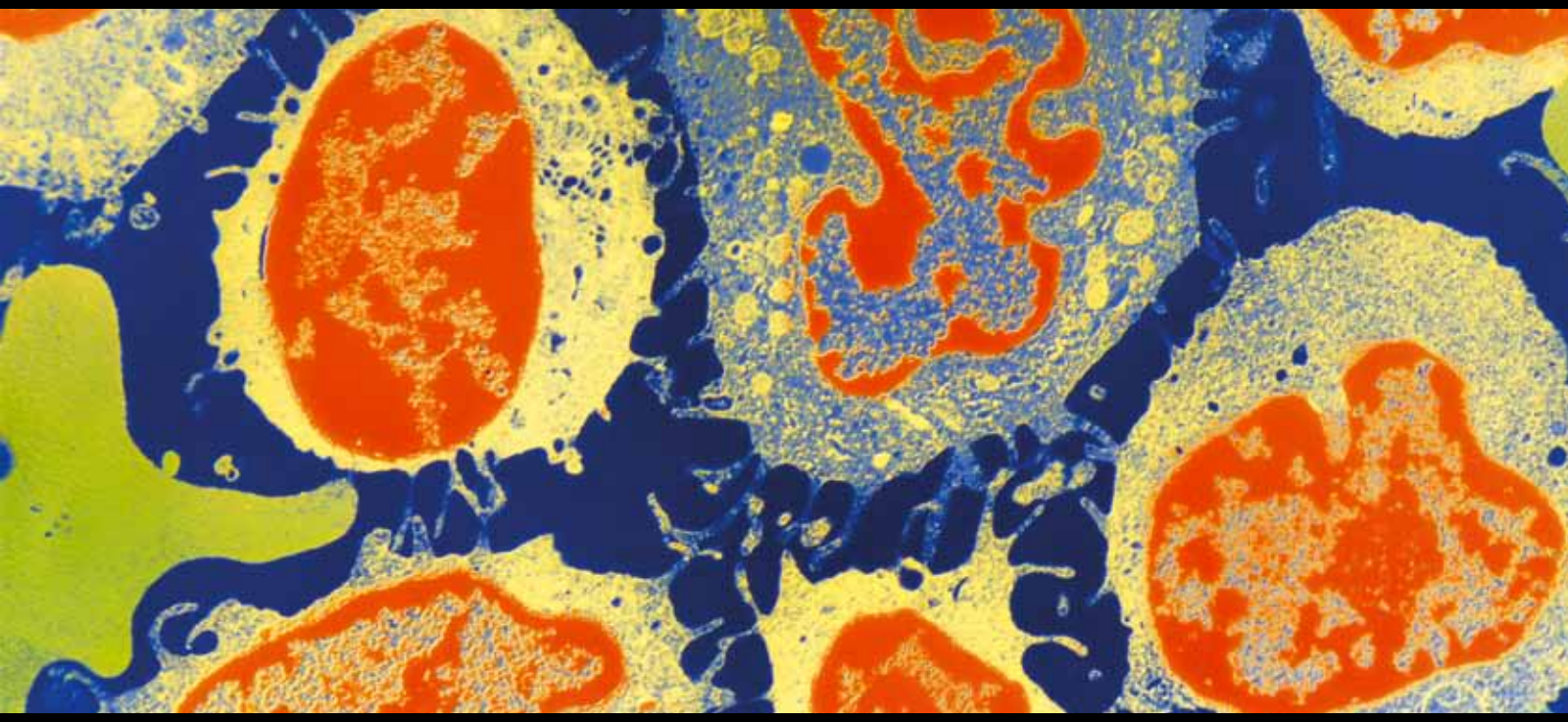
Journal of Oncology

Molecular Genetic Markers in Female Reproductive Cancers

Guest Editors: Tian-Li Wang, Ben Davidson, Phillip J. Buckhaults, Chiun-Sheng Huang, and Gudrun Pohl



Molecular Genetic Markers in Female Reproductive Cancers

Journal of Oncology

Molecular Genetic Markers in Female Reproductive Cancers

Guest Editors: Tian-Li Wang, Ben Davidson,
Phillip J. Buckhaults, Chiun-Sheng Huang, and Gudrun Pohl

Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 307460, 2 pages
doi:10.1155/2010/307460

Editorial

Molecular Genetic Markers in Female Reproductive Cancers

Tian-Li Wang,[1] Ben Davidson,[2] Phillip J. Buckhaults,[3] Chiun-Sheng Huang,[4] and Gudrun Pohl[5]

[1] *Departments of Gynecology and Obstetrics and Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA*
[2] *Department of Pathology, Norwegian Radium Hospital, Rikshospitalet University Hospital, Montebello, 0310 Oslo, Norway*
[3] *Departments of Pathology and Microbiology and Immunology, The University of South Carolina School of Medicine, Columbia, SC 29203, USA*
[4] *Department of Medicine, National Taiwan University, Taipei 10617, Taiwan*
[5] *Department of Internal Medicine I, Center for Hematology and Oncology, Wilhelminenspital, 1160 Vienna, Austria*

Correspondence should be addressed to Tian-Li Wang, tlw@jhmi.edu

Received 31 December 2010; Accepted 31 December 2010

Cancer is a complex genetic disease as a result of accumulated genomic alterations which serve as the driving force in initiating tumor development and propelling tumor progression. Recent advances in molecular genomic technology and the success of human genome project have empowered investigators with new tools in analyzing cancer genome and transcriptome in great details and have expedited the discovery of new cancer-associated genes. Decoding the genetic history present in tumor DNA, as well as identification and characterization of molecular changes involving cancer-associated genes and the pathways they controlled, has not only shed new light on the molecular etiology of cancer, but also has promises for the development of new diagnostic markers and novel therapeutics. In this special issue, the papers focus on recent findings of markers identified in female reproductive cancers including breast, ovarian, endometrial, and trophoblast tumors. The selected papers are not an exhaustive representation of the area of biomarkers in female reproductive cancers. Nonetheless, they represent the rich and many-faceted knowledge that we have the pleasure of sharing that with readers. We would like to thank the authors for their excellent contributions to this special issue and the reviewers' insightful input to ensure the quality of this special issue.

Of particularly interest are the two timely review articles by A. M. Karst et al. and A. L. Gross et al. focusing on the possible precursor lesions in the development of female reproductive cancer. The tubal origin of "ovarian" cancer has recently been in the research spot-light because of the identification of potential precursor lesions of ovarian cancer, termed "serous tubal intraepithelial carcinoma" in the fallopian tubes. Both articles critically review the published studies and provide unique perspectives of the impact and significance of this new hypothesis in the field of cancer research.

Review articles focusing on biomarkers including genetic (K. M. Hirshfield et al.), microRNA (H. M. Heneghan et al.), and cytokine (F. Barbieri et al.) summarize recent advances in biomarker discovery and the translational application. Those articles will serve as the source for the investigators who wish to pursue in evaluating new potential markers in the female reproductive cancer research. They also present the new challenges in biomarker discovery, validation, and verification.

In addition to the five review articles, there are several exciting original research articles describing discovery and validation of biomarkers in female reproductive cancers. Those papers include the studies of fatty acid synthase (S. M. Ueda et al.), miR200c (D. R. Cochrane et al.), gene expression (S. Konstantinovsky et al.), and cytogenetic markers (F. Micci et al.). The findings from those studies could serve as biological foundation for future exploration of how those markers contribute to disease progression. The biomarkers identified in these studies could also serve as a road map for development of novel diagnosis tools and new targeted therapies. This issue also reports a synergistic effect of Pten loss and oncogenic Kras mutation on the endometrial cancer development (I. H. Kim et al.) and the result provides

new insight into the pathogenesis of endometrial carcinoma. Finally, K. L. Yap et al. applied molecular genetic markers to demonstrate a lack of a Y-chromosomal complement in the majority of gestational trophoblastic neoplasms, suggesting that most trophoblastic tumors are derived from previous molar gestations. Because the molecular characters in gestational trophoblastic disease are understudied, this short report should provide new insight into the pathogenesis of this rare but highly interesting gynecological neoplasm.

In conclusion, this special issue is by no means an attempt to be comprehensive in covering the field of female reproductive cancer, but it provides excellent reviews and research articles that may be useful for readers in this field. We hope that this special issue will inspire investigators in launching new research endeavor in this field and are grateful for the opportunity to manage this special issue.

Tian-Li Wang
Ben Davidson
Phillip J. Buckhaults
Chiun-Sheng Huang
Gudrun Pohl

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 932371, 13 pages
doi:10.1155/2010/932371

Review Article

Ovarian Cancer Pathogenesis: A Model in Evolution

Alison M. Karst[1] and Ronny Drapkin[1,2]

[1] *Department of Medical Oncology, Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA*
[2] *Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA*

Correspondence should be addressed to Ronny Drapkin, ronny_drapkin@dfci.harvard.edu

Received 1 June 2009; Accepted 23 June 2009

Academic Editor: Tian-Li Wang

Ovarian cancer is a deadly disease for which there is no effective means of early detection. Ovarian carcinomas comprise a diverse group of neoplasms, exhibiting a wide range of morphological characteristics, clinical manifestations, genetic alterations, and tumor behaviors. This high degree of heterogeneity presents a major clinical challenge in both diagnosing and treating ovarian cancer. Furthermore, the early events leading to ovarian carcinoma development are poorly understood, thus complicating efforts to develop screening modalities for this disease. Here, we provide an overview of the current models of ovarian cancer pathogenesis, highlighting recent findings implicating the fallopian tube fimbria as a possible site of origin of ovarian carcinomas. The ovarian cancer model will continue to evolve as we learn more about the genetics and etiology of this disease.

1. Introduction

Ovarian cancer afflicts ~204 000 women worldwide each year, including ~21 650 Americans [1, 2]. Despite its relatively low incidence rate, ovarian cancer is an extremely lethal disease. Globally, it claims 125 000 lives per year, making it the seventh leading cause of cancer-related deaths among women [2]. In the United States, ovarian cancer mortality is even higher; it ranks as the fifth deadliest malignancy among women, with an estimated 15 520 deaths per year [1]. In fact, of the top ten cancer types afflicting American women in 2008, ovarian cancer had the highest death-to-incidence ratio, exceeding even that of lung cancer [1]. Its high mortality is primarily due to difficulties in diagnosing early stage disease. Although the 5 years survival rate for stage I ovarian cancer is >90%, stage I diagnoses are more often the exception than the rule. Most patients (~75%) present with advanced stage (III/IV) tumors, for which the 5 years survival rate is a dismal 30% [1]. This is not surprising when one considers the anatomical problem—the ovaries are a pair of tiny organs, only ~2–4 cm in diameter, suspended on either side of the uterus and not readily accessible by pelvic examination unless significantly enlarged. By definition, a stage I tumor is confined to the ovary [3] and is therefore unlikely to be noticed without the aid of a sensitive screening test. Unfortunately, there are currently no effective screening modalities for detecting ovarian cancer in asymptomatic individuals. Furthermore, there are no tell-tale physical signs of the disease. Typical symptoms—which include abdominal discomfort, bloating, gas, nausea, and urinary urgency—are vague and often mistaken for gastrointestinal problems [4, 5]. In many cases, symptoms may not even present until the tumor has reached an advanced stage. Consequently, ovarian cancer is frequently nicknamed the "silent killer" [5–7].

2. Early Detection

The best tools currently available for detecting early-stage ovarian cancer are transvaginal sonography (TVS) and serum biomarker testing. TVS is a noninvasive technique that provides detailed images of ovary size and shape, facilitating the detection of ovarian masses. Large-scale studies evaluating its ability to identify early-stage tumors have reported mixed results [8–13]. Overall, TVS has not demonstrated adequate sensitivity to warrant its use in screening general populations. Furthermore, even in the

most optimistic reports, it is clear that TVS can only detect tumors that cause a significant increase in ovarian volume [13]. This is especially worrisome in the case of serous-type tumors which may spread rapidly from the ovary to other pelvic sites prior to ovarian enlargement. For instance, in a large study by van Nagell et al., four women were diagnosed with late-stage ovarian cancer within 12 months of a negative TVS scan [9]. In addition, there is debate as to whether all ovarian tumors actually arise from the ovary and not from adjacent pelvic sites such as the fallopian tube or peritoneum [14]. Serous tumors involving the ovary but originating from extraovarian sites can never be classified as "stage I" according to current staging systems, since they are never confined to the ovary. It has therefore been proposed that the definition of "early stage" ovarian carcinoma should be based on minimal tumor volume rather than anatomical location [14]. TVS screening may also be prone to false-positive results because it cannot always distinguish malignant ovarian tumors from benign adnexal masses, such as cysts and fibromas, which are highly prevalent among postmenopausal women [15, 16]. While TVS alone is not a suitable front-line screening modality, it can serve as a useful secondary screening tool after serum biomarker testing [17, 18].

Serum biomarker testing is an ideal form of cancer detection because it is minimally invasive, cost effective, easily administered, nonsubjective, and is not contingent upon primary ovarian involvement. The most well-studied ovarian cancer biomarker is CA-125, a high molecular weight transmembrane glycoprotein expressed by coelomic- and Müllerian-derived epithelia, including that of the fallopian tube, endometrium, and endocervix [19]. It is not expressed by normal ovarian epithelium [20]. CA-125 is detected at low levels (<35 U/mL) in the serum of healthy individuals but is elevated in ~50% of stage I ovarian cancer patients and ~90% of advanced-stage patients [21–24]. CA-125 elevation is predominantly associated with serous tumors, the most common and most lethal subtype of ovarian carcinoma [25]. Serum levels directly correlate with the level of CA-125 protein production in tumor cells and appear to reflect a state of active tumor growth [25–27]. Following its discovery in 1981 [28], CA-125 was intensely studied to evaluate its potential for detecting early-stage ovarian cancer, and many encouraging results were reported. For example, a study of prediagnostic serum samples found that CA-125 was elevated in 25% of ovarian cancer patients 5 years prior to their diagnoses [29]. However, it was later discovered that serum CA-125 levels can be increased by a range of benign conditions (such as pelvic inflammatory disease, endometriosis, uterine fibroids, and ovarian cysts) making false positivity a problem [30]. So far, CA-125 has not demonstrated adequate sensitivity to support its use in screening asymptomatic women for early-stage ovarian cancer [31], but longer-term studies are still underway. In the meantime, CA-125 remains a valuable tool for monitoring response to chemotherapy and for detecting disease relapse following treatment [32, 33].

In an attempt to improve sensitivity for early disease detection, two approaches have been taken. (1) Obtaining longitudinal measurements of CA-125, and (2) using multiple tumor markers. The former approach assumes that CA-125 levels are likely to remain stable in patients with benign conditions but will increase over time if an ovarian cancer is in progress. Thus, by plotting serial CA-125 measurements over a period of years, one can calculate the "probability of ovarian cancer" for an individual patient using a Bayesian algorithm [34–36].

The latter approach aims to combine CA-125 with one or more additional tumor markers, most notably human epididymis protein 4 (HE4). HE4 is a glycoprotein secreted by Müllerian epithelia of the female reproductive tract as well as male epididymis [37]. Like CA-125, it is not expressed by normal ovarian epithelium but appears in some premalignant ovarian cysts (cortical inclusion cysts) and is strongly expressed by the most common ovarian tumor subtypes (serous and endometrioid) [37]. HE4 is both more sensitive and specific than CA-125 in detecting early-stage ovarian cancer and is not associated with benign conditions to the same degree, enabling HE4 to distinguish malignant ovarian tumors from benign cystic lesions [38–40]. When used in combination to detect early-stage disease, CA-125 and HE4 perform better than either marker alone and can be used to stratify patients into high- and low-risk groups [40, 41]. Although HE4 was recently approved by the US Food and Drug Administration for use in monitoring ovarian cancer patients following treatment, larger-scale studies are required to determine whether a dual CA-125/HE4 biomarker test is suitable for screening asymptomatic women in the general population.

In addition, some groups have assembled panels of biomarkers to create a so-called "composite marker." For example, Zhang et al. measured the serum levels of four tumor markers (CA-125II, CA72-4, CA15-3, and M-CSF) in sets of healthy women, women with benign ovarian conditions, and women with ovarian cancer [42]. From their measurements, they derived an artificial neural network (ANN) model that could distinguish healthy women from those with early-stage ovarian cancer. The sensitivity of their ANN-derived composite index was 25–28% higher than that of CA-125 alone. Similar reports of increased sensitivity upon employment of multiple tumor markers have been reported by other groups [43, 44]. However, serum tests for multiple biomarkers are not yet widely available.

3. Ovarian Cancer Is a Heterogeneous Disease

Some of the greatest challenges in detecting and treating ovarian cancer stem from its heterogeneous nature. The term "ovarian cancer" refers not to a single disease, but to a diverse group of malignancies affecting the ovary. In general, ovarian tumors may develop from one of three cell types: epithelial cells, sex cord-stromal cells (including granulosa, theca, and hilus cells), or germ cells (oocytes). Although ~40% of all ovarian tumors are nonepithelial in origin, such lesions rarely progress to a malignant state and account for only 10% of ovarian cancers [45]. This paper will therefore focus exclusively on epithelial-derived ovarian tumors, which

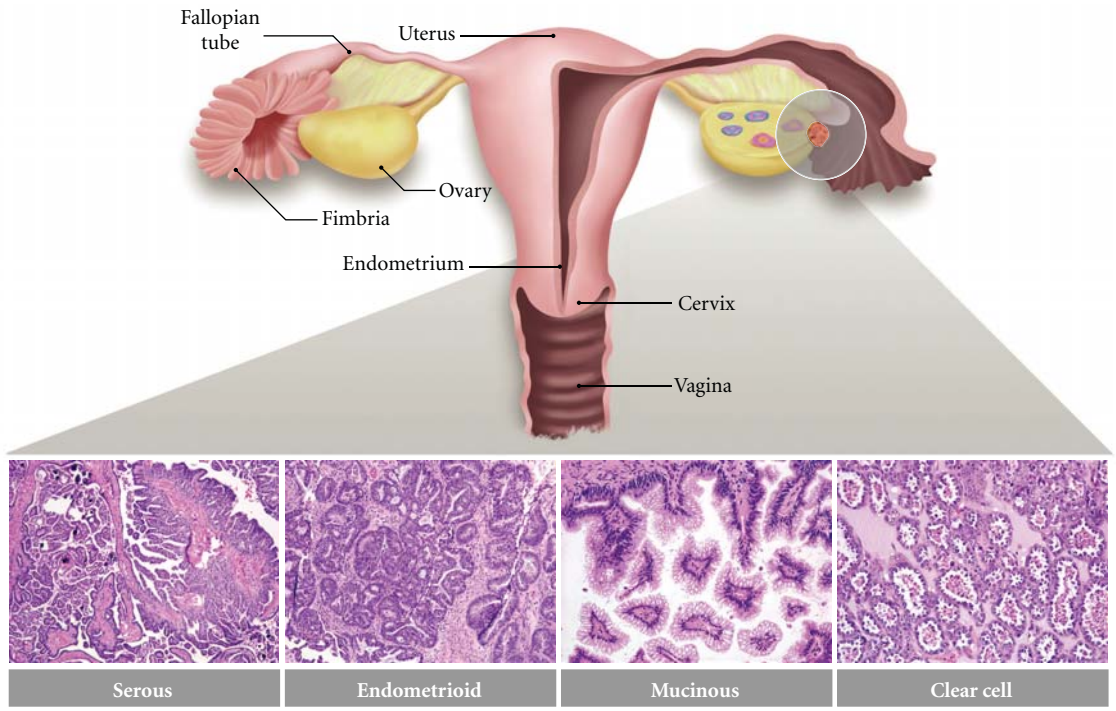


Figure 1: The major histologic subtypes of ovarian carcinoma. Serous carcinomas resemble fallopian tube epithelium, endometrioid carcinomas resemble endometrial glands, and mucinous carcinomas resemble endocervical epithelium. Photographs show representative tumor sections stained with hematoxylin and eosin. The shaded circle represents the general anatomical location from which ovarian carcinomas are thought to arise. The pink and blue entities within the cross-sected ovary represent maturing ovarian follicles.

constitute the predominant and most lethal forms of the disease. Epithelial ovarian carcinomas are themselves a heterogeneous group of neoplasms that exhibit a wide range of tumor morphologies, clinical manifestations, and underlying genetic alterations. Upon diagnosis of malignancy, ovarian tumors are surgically staged to determine how far they have extended beyond the ovary [3]. Stage I indicates confinement to the ovary. Stage II tumors extend beyond the ovary to adjacent pelvic structures such as the fallopian tube or uterus. Stage III indicates metastasis to the peritoneum and/or regional lymph nodes. Stage IV tumors have metastasized beyond the peritoneum to distant sites. Additionally, tumors are classified by subtype. Current clinical guidelines set forth by the World Health Organization (WHO) recognize eight histologic tumor subtypes: serous, endometrioid, mucinous, clear cell, transitional cell, squamous cell, mixed epithelial, and undifferentiated [46]. The three most common—serous, endometrioid, and mucinous—are characterized by their morphological resemblance to various mucosal tissues of the female reproductive tract, all of which exhibit Müllerian differentiation (Figure 1). More specifically, serous tumors resemble fallopian tube epithelium, endometrioid tumors resemble endometrial glands, and mucinous tumors resemble endocervical epithelium (though clinically, distinguishing mucinous ovarian tumors from those of the gastrointestinal tract is more relevant) [47]. This phenomenon is quite remarkable when one considers that all ovarian tumor subtypes are conventionally thought to develop from ovarian surface epithelium, a monolayer of nondescript, poorly differentiated mesothelial cells [48]. Within each subtype, tumors are further described as either benign, malignant, or borderline and, depending upon tumor subtype, classified as low- or high-grade. Borderline tumors are considered to have low malignant potential and/or indolent behavior.

There are major differences in incidence, tumor behavior (low versus high malignant potential), and clinical outcome between each histologic subtype. For example, it has been estimated that ~50% of malignant ovarian tumors are serous carcinomas, while ~25% are endometrioid carcinomas, ~10% are mucinous carcinomas, and only ~5% are clear cell carcinomas [45]. However, a study by Seidman et al. reported incidences of 70%, 7%, 10%, and <3% for serous, endometrioid, clear cell, and mucinous carcinomas, respectively, in a series of 220 consecutive cases of invasive ovarian cancer, suggesting that traditional distribution figures may vary considerably [49]. In terms of behavior, serous carcinomas tend to be aggressive, high-grade neoplasms that spread rapidly throughout the pelvis, while endometrioid and mucinous carcinomas are typically low-grade lesions, confined to the ovary [50, 51]. Clear cell and endometrioid carcinomas, unlike other subtypes, are strongly linked to endometriosis [52–56], leading some to believe that endometriosis may be a precursor to these lesions [57, 58]. Tumor histology can also strongly impact clinical response. For instance, late-stage serous and clear cell carcinomas, both of which have similar 5 years survival rates (20–30%), differ

markedly in their response to chemotherapy; serous tumors are (initially) highly responsive, while clear cell tumors are notoriously resistant [59, 60].

Genetic and biomarker profiling studies of ovarian cancer have revealed that each tumor subtype is associated with a unique "molecular signature." For example, gene expression profiling can readily distinguish mucinous and clear cell ovarian tumors from other subtypes, regardless of tumor stage or grade [61, 62]. Similarly, microRNA (miRNA) profiling of ovarian carcinomas has identified certain miRNAs that exhibit histotype specificity [63]. Gene expression profiling has also demonstrated that ovarian clear cell tumors are distinctly different from other forms of ovarian cancer and have more in common with clear cell tumors of other organs, such as renal clear cell carcinomas [64]. Even within one histotype, differences in tumor behavior may be underscored by distinct expression profiles. For example, Bonome et al. found that serous borderline tumors (SBTs), which account for 10–15% of serous ovarian neoplasms and are associated with vastly improved survival, cluster separately from high-grade serous carcinomas in hierarchical clustering analyses [65]. Moreover, SBTs are genetically more similar to normal ovarian surface epithelium than to advanced serous tumors. A recent immunohistochemical biomarker study of 500 ovarian carcinomas found that 20 of 21 candidate tumor biomarkers had significantly different expression patterns in each tumor subtype and that two thirds of the biomarkers lost their prognostic value when survival analyses were made subtype specific [66]. These results clearly indicate that each ovarian tumor subtype constitutes a distinct disease and should be treated as such in the contexts of detection, treatment, and prognosis.

Perhaps the most important characteristic of any tumor is the combination of genetic alterations that underlie its development and drive its progression. In this arena, ovarian tumors again exhibit heterogeneity. Mucinous, endometrioid, and low-grade serous tumors typically acquire mutations in a variety of genes such as *KRAS, BRAF, PTEN*, *β-catenin*, and *TFG-βRII* [51], all of which belong to signaling pathways controlling cell growth and proliferation, among other processes. Conversely, high-grade serous tumors appear to arise following a mutation in *TP53* [67] or, in the case of familial ovarian carcinoma, *BRCA1, BRCA2, MLH1*, or *MSH2* [68, 69]. All five genes are tumor suppressors that function in DNA damage signaling and repair [69, 70], suggesting that DNA damage is an especially important factor in the etiology of serous ovarian carcinoma. Mutations in *TP53* can even prompt an immunological response, leading to *p53* autoantibody production in some patients with high-grade ovarian carcinomas [71].

Despite the high degree of phenotypic and genotypic variability that exists between different forms of ovarian carcinoma, virtually all patients are treated identically upon diagnosis: cytoreductive surgery, followed by platinum-based chemotherapy. Although many tumors are initially responsive to this treatment, most develop platinum resistance and ~70% recur at some point [72]. Ultimately, only 30% of advanced-stage patients survive five years beyond their diagnoses [1]. Over the past several decades, great advances have been made in the surgical techniques and chemotherapy regimens used to treat ovarian cancer. Yet, despite the best efforts of surgeons, oncologists, and researchers, the 5 years survival rate has improved by only 8% since 1975 [1]. It has thus become apparent that a "blanket approach" to ovarian cancer treatment does not suffice. We must now shift our focus towards the development of targeted therapies capable of exploiting the molecular and genetic characteristics of individual tumor subtypes. This task is made difficult by the fact that we are still very much in the dark when it comes to understanding ovarian cancer etiology. Unlike other malignancies such as cervical or colon cancer, whose pathogeneses are well characterized, the sequence of events leading to ovarian carcinoma development remains a subject of ongoing debate.

4. Evolution of the Ovarian Cancer Model

4.1. Ovarian Surface Epithelium and Cortical Inclusion Cysts. The traditional view of ovarian cancer asserts that all tumor subtypes share a common origin in ovarian surface epithelium (OSE). OSE is a flat-to-cuboidal layer of uncommitted mesothelial cells covering the exterior surface of the ovary. During ovulation, follicular rupture and oocyte release inflict physical trauma upon the ovarian surface, creating a breach in the OSE that must be repaired. Over the course of a woman's reproductive life, this process of damage and repair is repeated multiple times. Accordingly, OSE cells exhibit a high degree of plasticity that facilitates tissue remodeling; they express both epithelial and mesenchymal markers and can transition from an epithelial to mesenchymal phenotype [73–76]. In addition to physical trauma, OSE cells are subjected to ovulation-associated inflammatory cytokines and reactive oxygen species that are capable of damaging DNA [77]. Accrual of DNA damage by OSE cells may increase their susceptibility to transformation. Furthermore, as women age, the ovarian surface develops numerous invaginations into the cortical stroma. These invaginations frequently pinch off and become entrapped within the stroma, forming circular OSE-lined structures termed "cortical inclusion cysts" (CICs) [78]. Once inside the ovary, the epithelial cells lining CICs are exposed to a new hormone-rich milieu that is thought to induce a differentiation or "metaplasia" into more complex epithelium resembling that of Müllerian-derived organs [78, 79]. Alternatively, it is postulated that in women experiencing endometriosis or endosalpingiosis (i.e., abnormal shedding of endometrial or tubal mucosa, respectively, into the pelvis), remnants of Müllerian-derived epithelia may adhere to the ovarian surface and become incorporated into a CIC [78, 80]. Several hormones acting upon the ovary (e.g., gonadotropins, estrogens, and androgens) have growth-promoting properties that may induce proliferation of epithelial cells within CICs [81]. If the epithelial cells also happen to harbor unresolved DNA damage, they may be prime targets for neoplastic transformation, eventually giving rise to ovarian carcinomas. The OSE-CIC model can account for several

important features of ovarian tumorigenesis, including (1) acquisition of Müllerian characteristics by OSE-derived tumors, (2) the cystic nature of benign ovarian tumors and the retention of cystic features by their malignant counterparts, and (3) the presence of low-grade and borderline tumors within the cortical stroma of the ovary. This model is also consistent with well-established epidemiologic data indicating that a decrease in ovulatory cycles (most commonly due to parity or oral contraceptive use) is the greatest risk-reducing factor for ovarian cancer in female populations [82]. However, this model has its limitations. For example, it does not explain why invasive endometrioid and mucinous carcinomas are frequently associated with borderline tumors in the ovary, whereas invasive serous carcinomas are not. Nor does this model address the clear differences in genetic alterations that exist between tumor subtypes. If all ovarian tumors develop from CICs within the ovary, then why do they have such different outcomes and such divergent genotypes? Perhaps the most curious phenomenon, not accounted for by this model, is the existence of extraovarian peritoneal carcinomas. Such tumors are histologically identical to serous ovarian carcinomas but do not involve the ovary and thus, are considered to arise de novo in the peritoneum.

4.2. Two-Pathway Model. The two-pathway model was proposed by Shih and Kurman in 2004 in an attempt to integrate most of the clinical, histopathological, and molecular genetic findings concerning ovarian cancer. In particular, they sought to account for the differences in *TP53* and *KRAS* mutational frequencies observed between serous borderline tumors (SBTs) and serous carcinomas [83–85]. SBTs comprise a subset of serous ovarian tumors (including those referred to as atypical proliferative serous tumors and micropapillary serous carcinomas) that are noninvasive, appear to develop from benign serous cystadenomas, and progress very slowly towards low-grade serous carcinoma [86, 87]. The indolent behavior of SBTs contrasts sharply with that of conventional high-grade serous tumors, which spread rapidly and metastasize early in their course. Furthermore, SBTs do not harbor the *TP53* mutations that are characteristic of high-grade serous carcinomas. These observations prompted the formulation of a new model that classifies all ovarian tumors as either Type I or Type II [51].

Type I tumors include all major histotypes (serous, endometrioid, mucinous, clear cell, and transitional) but exhibit low-grade nuclear and architectural features, slow growth, and can be linked to well-defined benign ovarian precursor lesions. The most common genetic alterations seen among Type I tumors are *KRAS* and *BRAF* mutations, both of which activate the oncogenic *MAPK* signaling pathway [88]. Mutually exclusive *KRAS* and *BRAF* mutations are observed in ~65% of SBTs but are rarely seen in high-grade serous carcinomas [58, 89]. *KRAS* mutations also occur in ~60% of mucinous, 5–16% of clear cell, and 4-5% of endometrioid Type I carcinomas [51]. *PTEN* mutations, which typically result in constitutive *PI3K* signaling, occur in ~20% of Type I endometrioid neoplasms [90]. The *MAPK* and *PI3K* pathways are related; they eventually converge upon a common downstream translation factor, eIF4B [91], which may represent an important signaling axis in Type I tumor development. *WNT* and *TGF-β* signaling pathways are also of potential importance for Type I tumor pathogenesis, based on the presence of *β-catenin* mutations in 16–54% of endometrioid tumors and *TGF-β* RII mutations in 66% of Type I clear cell tumors [51]. Interestingly, all of the genes altered in Type I ovarian tumors are components of pathways that become intimately related during the process of epithelial-to-mesenchymal transition [92, 93].

Type II ovarian tumors, on the other hand, are infrequently associated with benign or borderline ovarian precursor lesions. They are comprised almost exclusively of high-grade serous carcinomas but also include two less common subtypes—mixed epithelial and undifferentiated carcinomas. Type II ovarian tumors are overwhelmingly *TP53* mutated (50–80%) and may also exhibit gene amplification and overexpression of *HER2/neu* (10–20%) and *AKT2* (12–18%) oncogenes [94–99]. Shih and Kurman's two-pathway hypothesis reconciles most of the phenotypic and genotypic observations pertaining to ovarian tumors and it certainly improves upon the conventional OSE-CIC model. However, their model leaves one critical question unanswered—how do Type II tumors arise and does their pathogenesis include a well-defined precursor lesion?

4.3. Fallopian Tube as a Site of Origin. This question may have been answered by a series of studies investigating the prevalence of occult fallopian tube cancer in women with germline *BRCA* gene mutations. Inherited mutations in *BRCA*1 or *BRCA*2 are associated with familial ovarian and breast cancer syndromes and account for ~11–15% of ovarian carcinomas [100]. Mutations in either gene confer a 15–40% lifetime risk of developing ovarian cancer [101]. Many women with germline *BRCA* mutations (*BRCA*+) elect to undergo risk-reducing bilateral salpingo-oophorectomy (ovary and fallopian tube removal), after which their ovaries are thoroughly examined for evidence of occult cancer. Until recently, the fallopian tubes were not closely examined following such surgeries, and thus, early-stage tubal cancers were rarely detected and severely under reported in *BRCA*+ patients.

In 2001, Piek et al. drew attention to this issue when they reported that sectioning and examination of fallopian tubes from 12 *BRCA*+ patients revealed a high incidence (50%) of epithelial dysplasia [102]. The fallopian tube epithelium (FTE) is a columnar cell layer composed of two specialized cell types—secretory and ciliated cells. In their study, Piek et al. noticed that dysplastic regions of FTE exhibited a shift towards the secretory phenotype, with complete loss of ciliated cells and the acquisition of proliferative capacity (indicated by Ki67 immunoreactivity). Shortly thereafter, several other groups reported finding occult tubal cancers in the fallopian tubes of *BRCA*+ women, with incidence rates ranging from 0.9–17% [103–105].

However, not until a few years later did it become apparent that the distal end of the fallopian tube, the "fimbria", rather than the proximal region of the tube, was the most crucial site to look for early serous tumors. In 2006, Medeiros et al. conducted a study similar to that of Piek et al., examining the fallopian tubes of 13 *BRCA*+ women undergoing bilateral salpingo-oophorectomy [106]. Medeiros et al. employed a specific protocol for Sectioning and Extensively Examining the FIMbria (SEE-FIM). Their examination uncovered a high incidence (38%) of serous tubal intraepithelial carcinomas in the fallopian tube, but none in the ovaries. Furthermore, 80% of these carcinomas appeared exclusively in the fimbriated end of the fallopian tube, indicating that the fimbria is the preferred site of serous carcinogenesis in *BRCA*+ women. Similarly, Callahan et al. reported on a cohort of 122 *BRCA*+ women in which 7 early carcinomas were found, all originating in the fimbrial/ampullary region. A third study by Kindelberger et al. documented the occurrence of tubal intraepithelial carcinomas (TICs) in 55 consecutive cases of pelvic (i.e., ovarian, tubal, and peritoneal) serous cancer, not selected for *BRCA* status [107]. Of 42 cases designated "serous ovarian carcinoma," 71% involved the fallopian tube, and 48% of these contained a TIC. Again, TICs were located predominantly (93%) in the fimbrial region. These results suggest that many high-grade serous "ovarian" tumors may actually be of tubal origin, arising from the distal region of the fallopian tube, but then quickly spreading to the nearby ovary.

This concept, while at first provocative, is quite plausible upon consideration. The fimbria lies in extremely close proximity to ovarian surface epithelium and is therefore exposed to the same inflammatory (and potentially carcinogenic) microenvironment. At the fimbriated end of the tube the internal tubal mucosa (endosalpinx) meets the outer tubal serosa which, in turn, is continuous and indistinguishable from the peritoneum. It is therefore easy to imagine that transformed FTE cells, early in their progression, may slough off and migrate to the ovarian surface or directly to the peritoneum, with minimal ovarian involvement. This could explain the existence of some extraovarian peritoneal carcinomas.

During their initial study of tubal mucosa [106], Medeiros et al. noted stretches of secretory-type cells exhibiting strong *p53* immunoreactivity but appearing nonproliferative and histologically benign. The observed loss of ciliated cells was reminiscent of dysplastic lesions earlier described by Piek et al. [102]. They termed these regions of strong *p53* immunoreactivity "*p53* signatures" (Figure 2). In an attempt to further characterize these entities, Lee et al. examined the occurrence of *p53* signatures in the fimbria of both *BRCA*+ women and women undergoing hysterectomies for benign indications (such as fibroids, endometriosis, or prolapse) [108]. They found that *p53* signatures were equally common in the nonneoplastic fimbria of both *BRCA*+ and control subjects, suggesting that they are a "normal" phenomenon. They also discovered that *p53* signatures stain strongly for the DNA damage marker γ-H2AX (Figure 2). Gamma-H2AX is a phosphorylated form of the histone protein H2AX. Phosphorylation of H2AX by DNA damage-sensing kinases ATM and ATR occurs rapidly at sites of DNA double strand breakage [109]. The presence of so-called "*p53* signatures" in the fimbriae of normal women provides the first evidence that, under normal physiologic conditions, fimbrial epithelial cells experience genotoxic damage and trigger a DNA damage response. Lee et al.'s study also made several key observations about the relationship between *p53* signatures and TICs: 1 *p53* signatures occur more frequently in fimbriae where TICs are also present, 2 *p53* signatures are composed exclusively of secretory cells which, like TICs, exhibit a serous phenotype, 3 *p53* signatures and TICs, when concurrent, share evidence of DNA damage and exhibit identical *TP53* mutations, indicating a common origin, and 4 TICs can be distinguished from *p53* signatures by their increased proliferative capacity (i.e., MIB1 positivity and increased Cyclin E expression). Based on their observations, Lee et al. hypothesized that *p53* signatures could represent the elusive ovarian serous carcinoma precursor. To determine whether *p53* signatures also occur in ovarian epithelium, Folkins et al. examined the ovaries and fallopian tubes of 75 *BRCA*+ women. They detected a total of 29 signatures in tubal mucosa but only 1 in OSE and 0 in CICs [110], confirming that *p53* signatures preferentially arise in FTE rather than OSE.

In light of these developments, Lee et al. formulated a model of ovarian cancer which incorporates the fimbria as a major site of origin for serous carcinomas. Their model asserts that there are two distinct pathways leading to ovarian tumorigenesis. The first route is the traditional OSE-CIC pathway, in which OSE (or in some cases FTE, endometrium, or peritoneum) is entrapped within CICs and induced to undergo Müllerian metaplasia within the ovarian stroma, giving rise to mostly endometrioid, mucinous, and serous borderline tumors via a series of step-wise mutations (reviewed in [111]). This pathway leads to the formation of Shih and Kurman's "Type I" tumors. The second pathway involves the fallopian tube fimbria, where a combination of *TP53* mutation and genotoxic stress leads to the clonal expansion of secretory epithelial cells, forming a preneoplastic precursor lesion or "*p53* signature." Additional genetic "hits" in the absence of functional *TP53* enable these cells to acquire a proliferative capacity, facilitating progression to TIC (Figure 3). Identifying the genetic targets of such "hits" is currently a subject of intense research. Serous TICs have the ability to spread rapidly, moving from the fimbria to adjacent pelvic structures (e.g., the ovarian surface, uterine serosa, or peritoneal membranes) or exfoliating into the peritoneal cavity. This second pathway leads to the formation of Shih and Kurman's "Type II" tumors and, importantly, defines a precursor lesion for these tumors. The origin of genotoxic stress in the fimbrial microenvironment remains speculative at this point but is thought to include inflammatory cytokines and reactive oxygen species associated with ovulation. Accordingly, a recent paper has reported that *p53* signatures are associated with lower parity in *BRCA*+ women, suggesting that ovulation is indeed a risk factor for *p53* signature development in the fimbria [112].

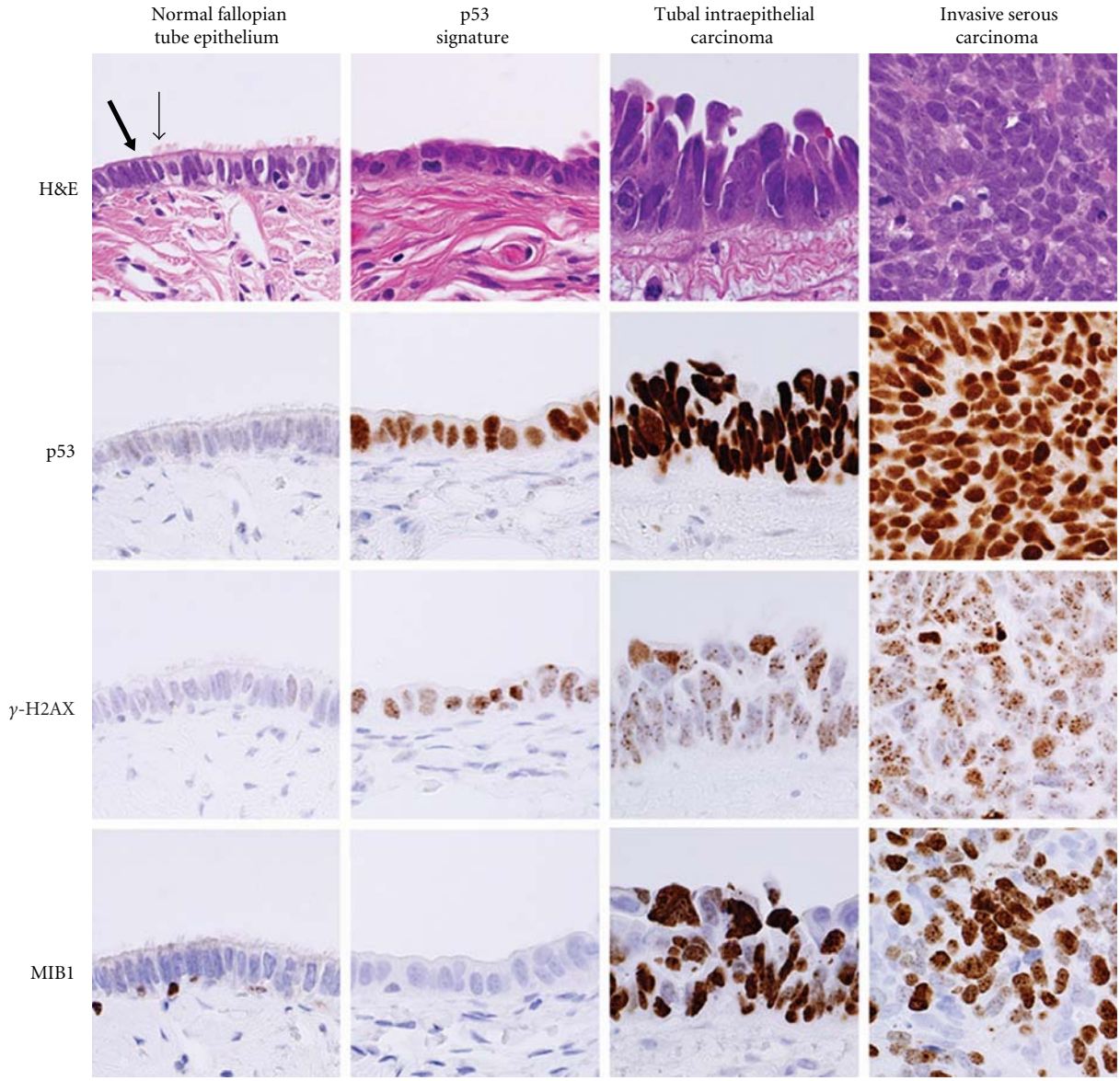


Figure 2: Pathologic features of the morphological continuum from normal fallopian tube epithelium to invasive serous carcinoma. Normal fallopian tube epithelium (FTE), containing both secretory (thick arrow) and ciliated (thin arrow) cells, is typically immunonegative for *p53*, γ-H2AX (a marker of DNA damage), and MIB1 (antibody against Ki67; a proliferation marker). The benign "*p53* signature" is composed of a stretch of secretory cells exhibiting strong *p53* expression and evidence of DNA damage (i.e., nuclear γ-H2AX foci), but showing no signs of proliferation. Upon progression to TIC, there is an acquisition of proliferative capacity, as evidenced by gain of MIB1 immunoreactivity. High levels of *p53*, γ-H2AX, and MIB1 typically persist after a TIC develops into invasive serous carcinoma.

This new model of ovarian cancer accounts for nearly all aspects of the disease and, for the first time, describes a step-by-step pathogenesis model for the deadliest and most enigmatic of all ovarian tumors—serous carcinoma. The clinical implications of this model were recently reviewed [111, 113] and suggest that a thorough examination of the fallopian tube fimbria should be conducted during routine pathologic evaluations of salpingo-oophorectomy specimens. It is important to note, however, that this new model is based largely upon descriptive pathological evidence and has not been experimentally validated.

5. Experimental Models of Ovarian Cancer

In order to elucidate the molecular mechanisms driving ovarian carcinoma development, we must first be able to model the disease using suitable in vitro and in vivo systems. Several sophisticated mouse models of ovarian carcinoma currently exist. Most are based on the traditional OSE-CIC model of ovarian tumorigenesis and therefore seek to transform murine ovarian surface epithelium (MOSE) in vivo. For example, Connolly et al. induced MOSE transformation in vivo by introducing SV40 T-Ag (large and small)

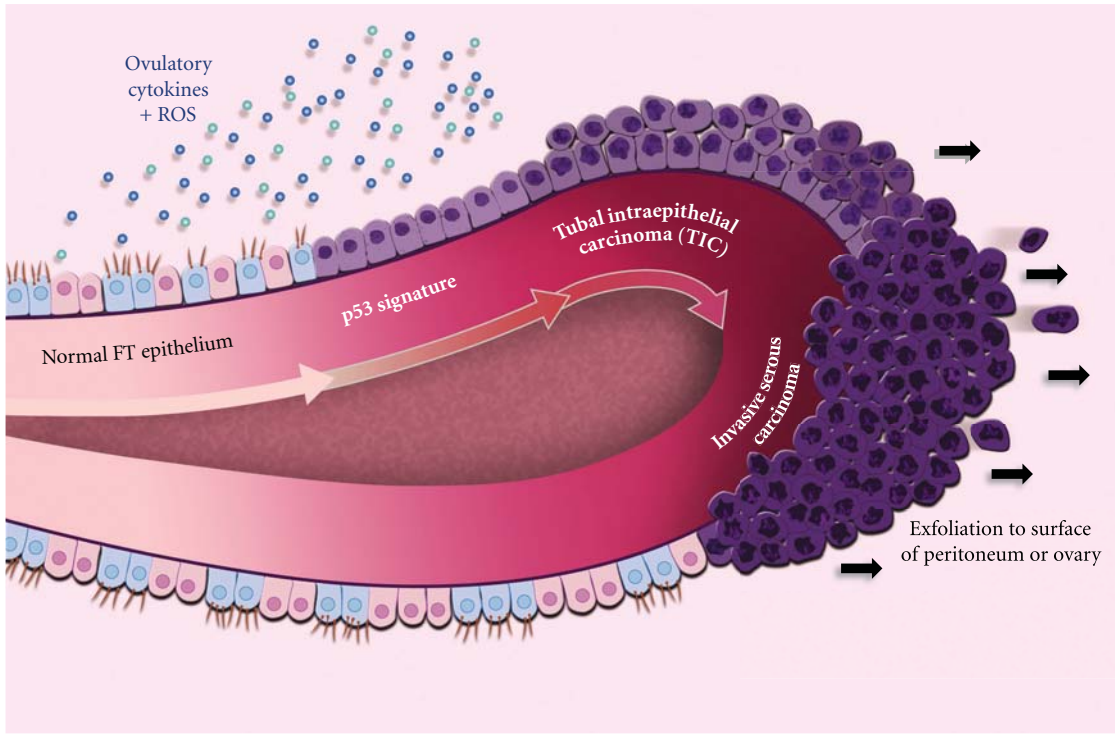


Figure 3: Diagram of a fimbrial plica, illustrating the stepwise progression of normal fallopian tube epithelium to invasive serous carcinoma. The fallopian tube epithelium (FTE) is composed of a single layer of ciliated and secretory cells that are exposed to ovulation-associated inflammatory cytokines and reactive oxygen species (ROS). Repetitive genotoxic stress causes DNA damage and induces *p53* mutation, leading to the clonal expansion of normal looking FTE cells of secretory phenotype. This stretch of damaged cells—termed a "*p53* signature"—stains strongly for *p53* and *γ*-H2AX. Further genetic "hits" enable cells to acquire a proliferative capacity, giving rise to tubal intraepithelial carcinoma (TIC). As a TIC progresses to invasive serous carcinoma, malignant cells are exfoliated from the fimbria, whereupon they may spread rapidly to the surface of the peritoneum and/or ovary. Exfoliation may also occur from TICs prior to fimbrial invasion.

into these cells under the control of a Müllerian inhibitory substance II receptor (MISIIR) promoter element [114]. Other groups have used the Cre-Lox system to conditionally knockout tumor suppressor genes *TP53* and *Rb* in murine ovaries [115]. Orsulic et al. developed a unique model in which a series of oncogenes was introduced directly into the MOSE cells of an adult mouse [116]. Their system employed an avian retroviral vector that requires retroviral receptor (TVA) expression by target cells. This is normally achieved by breeding transgenic animals that express TVA under the control of a tissue-specific gene promoter. However, since no MOSE-specific promoters exist, they removed the ovaries from a TVA expressing mouse, infected them in vitro, then implanted them orthotopically into a recipient mouse. Using this method, they were able to demonstrate the importance of *TP53* in suppressing ovarian tumorigenesis. Specifically, they showed that introduction of a trio of oncogenes (*c-MYC*, *KRAS*, and *AKT*) into $p53^{+/+}$ MOSE could not induce tumor formation, while introducing any two of the same oncogenes induced rapid tumor formation in $p53^{-/-}$ mice. All three of these models gave rise to poorly differentiated tumors resembling serous ovarian carcinomas and exhibited intraperitoneal dissemination.

There have also been attempts to create subtype-specific mouse models. Dinulescu et al. successfully generated tumors with endometrioid histology by using the Cre-Lox system to conditionally activate oncogenic *KRAS* and inactivate *PTEN* [117]. Using this system, they demonstrated that expression of activated *KRAS* or deletion of *PTEN* in MOSE led to an endometriosis-like condition, characterized by the presence of benign glandular lesions in the ovary. In contrast, the combination of both genetic mutations induced formation of invasive and metastatic adenocarcinomas resembling human ovarian endometrioid tumors. Their model nicely recapitulates the stepwise progression of Type I ovarian tumors. However, *KRAS* mutations are not commonly found in the endometrioid subtype. A second endometrioid tumor model developed by Wu et al. used the Cre-Lox system to delete *PTEN* and *APC* tumor suppressor genes, again giving rise to tumors with endometrioid histologies [118]. Although *APC* mutations are not characteristic of endometrioid ovarian carcinomas, they serve to deregulate the same pathway as *β-catenin* mutations—specifically, the *WNT* signaling pathway.

Mouse models that can faithfully reproduce both the genotypic and phenotypic characteristics of ovarian tumors are crucial for the development of targeted therapies. Current ovarian carcinoma models indicate that there are two divergent pathways to ovarian tumorigenesis, each giving rise to tumors with very different characteristics (Types I

and II). In attempting to model these tumor types, it is important to consider that many Type II tumors may arise from extraovarian sites—most notably, the fallopian tube epithelium. This is of critical importance, for example, in the context of orthotopic tumor cell implantation.

Recent revelations regarding the tubal origin of high-grade serous ovarian carcinomas present a challenge because, while human OSE cells have been routinely cultured for many years, FTE cells are a relatively scare commodity. There are no readily available human FTE cell lines and primary human FTE can be difficult to obtain. Nevertheless, models of FTE must be created in order to study the mechanisms of tubal/ovarian serous carcinoma development. Only a handful of labs thus far have attempted to culture or propagate FTE cells in vitro, the majority of them working within the field of reproductive biology [119–124]. As mentioned earlier, the FTE is a complex columnar epithelium composed of both secretory and ciliated cell types. However, ciliated FTE cells tend to either die or rapidly dedifferentiate when grown in vitro, resulting in a complete loss of the ciliated cell phenotype from FTE cocultures. Serous tubal/ovarian carcinomas are exclusively secretory in nature, suggesting that ciliated cells must be either less susceptible to malignant transformation or more apt to undergo cell death in response to genotoxic stressors in their microenvironment. To address such issues, a model system is required in which both ciliated and secretory cells remain viable, so that the behaviors of each cell type may be readily compared and contrasted.

To preserve the phenotypic integrity of FTE cocultures, they may be grown on collagen-coated porous filters, as described by Rajagopal et al. for the culture of monkey oviductal cells [125]. Similar techniques have been used to construct polarized cultures of respiratory epithelial cells [126]. Our lab has recently established an analogous "ex vivo" model of human FTE (unpublished data). Using this FTE coculture system we aim to answer several important questions, including the following: What are the molecular mechanisms driving FTE malignant transformation? Can transformed FTE cells progress to serous tubal intraepithelial carcinoma? Which molecules may be targeted in a therapeutic context to halt serous carcinoma progression? Are there FTE-specific genes that can be exploited for the development of a tubal serous carcinoma mouse model? Can we identify novel biomarkers relevant to serous tumor progression? Our studies must ultimately also consider the role of the tumor microenvironment, since it is apparent that multicellular interactions contribute to serous tumor metastasis and are important factors in a therapeutic context [127, 128]. Overall, these research efforts will offer new insights into the nature of the FTE and its propensity for malignant transformation, and thus, advance our understanding of ovarian cancer pathogenesis.

Acknowledgments

The authors wish to thank Drs. Kevin Elias and Michelle Hirsch for their insightful comments and thoughtful suggestions in reviewing this manuscript. Their research is supported by grants from the NIH-National Cancer Institute (K08 CA108748, R21 CA12468, P50 CA105009), the Ovarian Cancer Research Fund, the Fannie E. Ripple Foundation, the Robert and Deborah First Ovarian Cancer Research Fund, and the Randi and Joel Cutler Ovarian Cancer Research Fund.

References

[1] A. Jemal, R. Siegel, E. Ward, et al., "Cancer statistics, 2008," *CA: A Cancer Journal for Clinicians*, vol. 58, no. 2, pp. 71–96, 2008.

[2] P. Boyle and B. Levin, *International Agency for Research on Cancer, and World Health Organization, "World cancer report 2008"*, Distributed by WHO Press, Geneva, Switzerland, 2008.

[3] J. L. Benedet, H. Bender, H. Jones III, H. Y. Ngan, and S. Pecorelli, "FIGO staging classifications and clinical practice guidelines in the management of gynecologic cancers. FIGO Committee on Gynecologic Oncology," *International Journal of Gynecology & Obstetrics*, vol. 70, no. 2, pp. 209–262, 2000.

[4] L. H. Smith, C. R. Morris, S. Yasmeen, A. Parikh-Patel, R. D. Cress, and P. S. Romano, "Ovarian cancer: can we make the clinical diagnosis earlier?" *Cancer*, vol. 104, no. 7, pp. 1398–1407, 2005.

[5] B. A. Goff, L. Mandel, H. G. Muntz, and C. H. Melancon, "Ovarian carcinoma diagnosis: results of a national ovarian cancer survey," *Cancer*, vol. 89, no. 10, pp. 2068–2075, 2000.

[6] C. R. Bankhead, C. Collins, H. Stokes-Lampard, et al., "Identifying symptoms of ovarian cancer: a qualitative and quantitative study," *BJOG*, vol. 115, no. 8, pp. 1008–1014, 2008.

[7] R. Twombly, "Cancer killer may be "silent" no more," *Journal of the National Cancer Institute*, vol. 99, no. 18, pp. 1359–1361, 2007.

[8] T. H. Bourne, S. Campbell, K. M. Reynolds, et al., "Screening for early familial ovarian cancer with transvaginal ultrasonography and colour blood flow imaging," *British Medical Journal*, vol. 306, no. 6884, pp. 1025–1029, 1993.

[9] J. R. van Nagell Jr., P. D. DePriest, M. B. Reedy, et al., "The efficacy of transvaginal sonographic screening in asymptomatic women at risk for ovarian cancer," *Gynecologic Oncology*, vol. 77, no. 3, pp. 350–356, 2000.

[10] S. Sato, Y. Yokoyama, T. Sakamoto, M. Futagami, and Y. Saito, "Usefulness of mass screening for ovarian carcinoma using transvaginal ultrasonography," *Cancer*, vol. 89, no. 3, pp. 582–588, 2000.

[11] J. P. Lerner, I. E. Timor-Tritsch, A. Federman, and G. Abramovich, "Transvaginal ultrasonographic characterization of ovarian masses with an improved, weighted scoring system," *American Journal of Obstetrics & Gynecology*, vol. 170, no. 1, pp. 81–85, 1994.

[12] D. A. Fishman, L. Cohen, S. V. Blank, et al., "The role of ultrasound evaluation in the detection of early-stage epithelial ovarian cancer," *American Journal of Obstetrics & Gynecology*, vol. 192, no. 4, pp. 1214–1222, 2005.

[13] J. R. van Nagell Jr., P. D. DePriest, F. R. Ueland, et al., "Ovarian cancer screening with annual transvaginal sonography: findings of 25,000 women screened," *Cancer*, vol. 109, no. 9, pp. 1887–1896, 2007.

[14] R. J. Kurman, K. Visvanathan, R. Roden, T. C. Wu, and I.-M. Shih, "Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis," *American Journal of Obstetrics & Gynecology*, vol. 198, no. 4, pp. 351–356, 2008.

[15] L. Valentin, L. Skoog, and E. Epstein, "Frequency and type of adnexal lesions in autopsy material from postmenopausal women: ultrasound study with histological correlation," *Ultrasound in Obstetrics and Gynecology*, vol. 22, no. 3, pp. 284–289, 2003.

[16] E. F. C. Murta and R. S. Nomelini, "Early diagnosis and predictors of malignancy of adnexal masses," *Current Opinion in Obstetrics and Gynecology*, vol. 18, no. 1, pp. 14–19, 2006.

[17] U. Menon, A. Gentry-Maharaj, R. Hallett, et al., "Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS)," *The Lancet Oncology*, vol. 10, no. 4, pp. 327–340, 2009.

[18] I. J. Jacobs, S. J. Skates, N. MacDonald, et al., "Screening for ovarian cancer: a pilot randomised controlled trial," *The Lancet*, vol. 353, no. 9160, pp. 1207–1210, 1999.

[19] I. Jacobs and R. C. Bast Jr., "The CA 125 tumour-associated antigen: a review of the literature," *Human Reproduction*, vol. 4, no. 1, pp. 1–12, 1989.

[20] S. E. Kabawat, R. C. Bast Jr., A. K. Bhan, W. R. Welch, R. C. Knapp, and R. B. Colvin, "Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125," *International Journal of Gynecological Pathology*, vol. 2, no. 3, pp. 275–285, 1983.

[21] R. C. Bast Jr., T. L. Klug, E. St. John, et al., "A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer," *The New England Journal of Medicine*, vol. 309, no. 15, pp. 883–887, 1983.

[22] P. Kenemans, G. J. van Kamp, P. Oehr, and R. A. Verstraeten, "Heterologous double determinant immunoradiometric assay CA 125 II: reliable second-generation immunoassay for determining CA 125 in serum," *Clinical Chemistry*, vol. 39, no. 12, pp. 2509–2513, 1993.

[23] K. Nustad, R. C. Bast Jr., T. J. O'Brien, et al., "Specificity and affinity of 26 monoclonal antibodies against the CA 125 antigen: first report from the ISOBM TD-1 workshop," *Tumor Biology*, vol. 17, no. 4, pp. 196–219, 1996.

[24] R. C. Bast Jr., N. Urban, V. Shridhar, et al., "Early detection of ovarian cancer: promise and reality," *Cancer Treatment and Research*, vol. 107, pp. 61–97, 2002.

[25] E. V. S. Høgdall, L. Christensen, S. K. Kjaer, et al., "CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study," *Gynecologic Oncology*, vol. 104, no. 3, pp. 508–515, 2007.

[26] E. P. Beck, A. Moldenhauer, E. Merkle, et al., "CA 125 production and release by ovarian cancer cells in vitro," *International Journal of Biological Markers*, vol. 13, no. 4, pp. 200–206, 1998.

[27] C. Marth, A. G. Zeimet, G. Bock, and G. Daxenbichler, "Modulation of tumour marker CA-125 expression in cultured ovarian carcinoma cells," *European Journal of Cancer Part A*, vol. 28, no. 12, pp. 2002–2006, 1992.

[28] R. C. Bast Jr., M. Feeney, H. Lazarus, L. M. Nadler, R. B. Colvin, and R. C. Knapp, "Reactivity of a monoclonal antibody with human ovarian carcinoma," *Journal of Clinical Investigation*, vol. 68, no. 5, pp. 1331–1337, 1981.

[29] V. R. Zurawski Jr., H. Orjaseter, A. Andersen, and E. Jellum, "Elevated serum CA 125 levels prior to diagnosis of ovarian neoplasia: relevance for early detection of ovarian cancer," *International Journal of Cancer*, vol. 42, no. 5, pp. 677–680, 1988.

[30] H. Meden and A. Fattani-Meiodi, "CA 125 in benign gynecological conditions," *International Journal of Biological Markers*, vol. 13, no. 4, pp. 231–237, 1998.

[31] K. J. Helzlsouer, T. L. Bush, A. J. Alberg, K. M. Bass, H. Zacur, and G. W. Comstock, "Prospective study of serum CA-125 levels as markers of ovarian cancer," *Journal of the American Medical Association*, vol. 269, no. 9, pp. 1123–1126, 1993.

[32] R. E. Hawkins, K. Roberts, E. Wiltshaw, J. Mundy, I. J. Fryatt, and V. R. McCready, "The prognostic significance of the half-life of serum CA 125 in patients responding to chemotherapy for epithelial ovarian carcinoma," *British Journal of Obstetrics and Gynaecology*, vol. 96, no. 12, pp. 1395–1399, 1989.

[33] A. Gadducci, P. Zola, F. Landoni, et al., "Serum half-life of CA 125 during early chemotherapy as an independent prognostic variable for patients with advanced epithelial ovarian cancer: results of a Multicentric Italian Study," *Gynecologic Oncology*, vol. 58, no. 1, pp. 42–47, 1995.

[34] S. J. Skates, U. Menon, N. MacDonald, et al., "Calculation of the risk of ovarian cancer from serial CA-125 values for preclinical detection in postmenopausal women," *Journal of Clinical Oncology*, vol. 21, supplement 10, pp. 206s–210s, 2003.

[35] S. J. Skates, F.-J. Xu, Y.-H. Yu, et al., "Toward an optimal algorithm for ovarian cancer screening with longitudinal tumor markers," *Cancer*, vol. 76, supplement 10, pp. 2004–2010, 1995.

[36] S. J. Skates, D. K. Pauler, and I. J. Jacobs, "Screening based on the risk of cancer calculation from Bayesian hierarchical changepoint and mixture models of longitudinal markers," *Journal of the American Statistical Association*, vol. 96, pp. 429–439, 2001.

[37] R. Drapkin, H. H. von Horsten, Y. Lin, et al., "Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas," *Cancer Research*, vol. 65, no. 6, pp. 2162–2169, 2005.

[38] R. G. Moore, A. K. Brown, M. C. Miller, et al., "The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass," *Gynecologic Oncology*, vol. 108, no. 2, pp. 402–408, 2008.

[39] I. Hellström, J. Raycraft, M. Hayden-Ledbetter, et al., "The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma," *Cancer Research*, vol. 63, no. 13, pp. 3695–3700, 2003.

[40] K. Huhtinen, P. Suvitie, J. Hiissa, et al., "Serum HE4 concentration differentiates malignant ovarian tumours from ovarian endometriotic cysts," *British Journal of Cancer*, vol. 100, no. 8, pp. 1315–1319, 2009.

[41] R. G. Moore, D. S. McMeekin, A. K. Brown, et al., "A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass," *Gynecologic Oncology*, vol. 112, no. 1, pp. 40–46, 2009.

[42] Z. Zhang, Y. Yu, F. Xu, et al., "Combining multiple serum tumor markers improves detection of stage I epithelial ovarian cancer," *Gynecologic Oncology*, vol. 107, no. 3, pp. 526–531, 2007.

[43] I. Visintin, Z. Feng, G. Longton, et al., "Diagnostic markers for early detection of ovarian cancer," *Clinical Cancer Research*, vol. 14, no. 4, pp. 1065–1072, 2008.

[44] V. Nosov, F. Su, M. Amneus, et al., "Validation of serum biomarkers for detection of early-stage ovarian cancer," *American Journal of Obstetrics & Gynecology*, vol. 200, no. 6, pp. 639.e1–639.e5, 2009.

[45] V. W. Chen, B. Ruiz, J. L. Killeen, et al., "Pathology and classification of ovarian tumors," *Cancer*, vol. 97, supplement 10, pp. 2631–2642, 2003.

[46] F. A. Tavassoli and P. Devilee, *Pathology and Genetics of Tumours of the Breast and Female Genital Organs*, International Agency for Research on Cancer, and World Health Organization, IAPS Press, Lyon, France, 2003.

[47] L. Dubeau, "The cell of origin of ovarian epithelial tumors and the ovarian surface epithelium dogma: does the emperor have no clothes?" *Gynecologic Oncology*, vol. 72, no. 3, pp. 437–442, 1999.

[48] N. Auersperg, T. Ota, and G. W. E. Mitchell, "Early events in ovarian epithelial carcinogenesis: progress and problems in experimental approaches," *International Journal of Gynecological Cancer*, vol. 12, no. 6, pp. 691–703, 2002.

[49] J. D. Seidman, I. Horkayne-Szakaly, M. Haiba, C. R. Boice, R. J. Kurman, and B. M. Ronnett, "The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin," *International Journal of Gynecological Pathology*, vol. 23, no. 1, pp. 41–44, 2004.

[50] C. N. Landen Jr., M. J. Birrer, and A. K. Sood, "Early events in the pathogenesis of epithelial ovarian cancer," *Journal of Clinical Oncology*, vol. 26, no. 6, pp. 995–1005, 2008.

[51] I.-M. Shih and R. J. Kurman, "Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis," *American Journal of Pathology*, vol. 164, no. 5, pp. 1511–1518, 2004.

[52] D. S. McMeekin, R. A. Burger, A. Manetta, P. DiSaia, and M. L. Berman, "Endometrioid adenocarcinoma of the ovary and its relationship to endometriosis," *Gynecologic Oncology*, vol. 59, no. 1, pp. 81–86, 1995.

[53] H. Yoshikawa, H. Jimbo, S. Okada, et al., "Prevalence of endometriosis in ovarian cancer," *Gynecologic and Obstetric Investigation*, vol. 50, supplement 1, pp. 11–17, 2000.

[54] M. Erzen, S. Rakar, B. Klancnik, and K. Syrjanen, "Endometriosis-associated ovarian carcinoma (EAOC): an entity distinct from other ovarian carcinomas as suggested by a nested case-control study," *Gynecologic Oncology*, vol. 83, no. 1, pp. 100–108, 2001.

[55] S. C. Modesitt, G. Tortolero-Luna, J. B. Robinson, D. M. Gershenson, and J. K. Wolf, "Ovarian and extraovarian endometriosis-associated cancer," *Obstetrics and Gynecology*, vol. 100, no. 4, pp. 788–795, 2002.

[56] R. C. Stern, R. Dash, R. C. Bentley, M. J. Snyder, A. F. Haney, and S. J. Robboy, "Malignancy in endometriosis: frequency and comparison of ovarian and extraovarian types," *International Journal of Gynecological Pathology*, vol. 20, no. 2, pp. 133–139, 2001.

[57] R. Sainz de la Cuesta, J. H. Eichhorn, L. W. Rice, A. F. Fuller Jr., N. Nikrui, and B. A. Goff, "Histologic transformation of benign endometriosis to early epithelial ovarian cancer," *Gynecologic Oncology*, vol. 60, no. 2, pp. 238–244, 1996.

[58] E. J. Thomas and I. G. Campbell, "Molecular genetic defects in endometriosis," *Gynecologic and Obstetric Investigation*, vol. 50, supplement 1, pp. 44–50, 2000.

[59] H. Itamochi, J. Kigawa, T. Sugiyama, Y. Kikuchi, M. Suzuki, and N. Terakawa, "Low proliferation activity may be associated with chemoresistance in clear cell carcinoma of the ovary," *Obstetrics and Gynecology*, vol. 100, no. 2, pp. 281–287, 2002.

[60] T. Sugiyama, T. Kamura, J. Kigawa, et al., "Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy," *Cancer*, vol. 88, no. 11, pp. 2584–2589, 2000.

[61] D. R. Schwartz, S. L. R. Kardia, K. A. Shedden, et al., "Gene expression in ovarian cancer reflects both morphology and biological behavior, distinguishing clear cell from other poor-prognosis ovarian carcinomas," *Cancer Research*, vol. 62, no. 16, pp. 4722–4729, 2002.

[62] S. Marchini, P. Mariani, G. Chiorino, et al., "Analysis of gene expression in early-stage ovarian cancer," *Clinical Cancer Research*, vol. 14, no. 23, pp. 7850–7860, 2008.

[63] M. V. Iorio, R. Visone, G. Di Leva, et al., "MicroRNA signatures in human ovarian cancer," *Cancer Research*, vol. 67, no. 18, pp. 8699–8707, 2007.

[64] K. K. Zorn, T. Bonome, L. Gangi, et al., "Gene expression profiles of serous, endometrioid, and clear cell subtypes of ovarian and endometrial cancer," *Clinical Cancer Research*, vol. 11, no. 18, pp. 6422–6430, 2005.

[65] T. Bonome, J.-Y. Lee, D.-C. Park, et al., "Expression profiling of serous low malignant potential, low-grade, and high-grade tumors of the ovary," *Cancer Research*, vol. 65, no. 22, pp. 10602–10612, 2005.

[66] M. Kobel, S. E. Kalloger, N. Boyd, et al., "Ovarian carcinoma subtypes are different diseases: implications for biomarker studies," *PLoS Medicine*, vol. 5, no. 12, article e232, 2008.

[67] G. Singer, R. Stöhr, L. Cope, et al., "Patterns of p53 mutations separate ovarian serous borderline tumors and low- and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis: a mutational analysis with immunohistochemical correlation," *The American Journal of Surgical Pathology*, vol. 29, no. 2, pp. 218–224, 2005.

[68] S. A. Cannistra, "Cancer of the ovary," *The New England Journal of Medicine*, vol. 351, no. 24, pp. 2519–2529, 2004.

[69] S. A. South, H. Vance, C. Farrell, et al., "Consideration of hereditary nonpolyposis colorectal cancer in BRCA mutation-negative familial ovarian cancers," *Cancer*, vol. 115, no. 2, pp. 324–333, 2009.

[70] R. Scully and D. M. Livingston, "In search of the tumour-suppressor functions of BRCA1 and BRCA2," *Nature*, vol. 408, no. 6811, pp. 429–432, 2000.

[71] M. Tsai-Turton, A. Santillan, D. Lu, et al., "p53 autoantibodies, cytokine levels and ovarian carcinogenesis," *Gynecologic Oncology*, vol. 114, no. 1, pp. 12–17, 2009.

[72] T. J. Herzog and B. Pothuri, "Ovarian cancer: a focus on management of recurrent disease," *Nature Clinical Practice Oncology*, vol. 3, no. 11, pp. 604–611, 2006.

[73] N. Auersperg, S. L. Maines-Bandiera, H. G. Dyck, and P. A. Kruk, "Characterization of cultured human ovarian surface epithelial cells: phenotypic plasticity and premalignant changes," *Laboratory Investigation*, vol. 71, no. 4, pp. 510–518, 1994.

[74] B. Czernobilsky, R. Moll, R. Levy, and W. W. Franke, "Co-expression of cytokeratin and vimentin filaments in mesothelial, granulosa and rete ovarii cells of the human ovary," *European Journal of Cell Biology*, vol. 37, pp. 175–190, 1985.

[75] C. H. Siemens and N. Auersperg, "Serial propagation of human ovarian surface epithelium in tissue culture," *Journal of Cellular Physiology*, vol. 134, no. 3, pp. 347–356, 1988.

[76] P. A. Kruk, V.-J. Uitto, J. D. Firth, S. Dedhar, and N. Auersperg, "Reciprocal interactions between human ovarian surface epithelial cells and adjacent extracellular matrix," *Experimental Cell Research*, vol. 215, no. 1, pp. 97–108, 1994.

[77] W. J. Murdoch and J. F. Martinchick, "Oxidative damage to DNA of ovarian surface epithelial cells affected by ovulation: carcinogenic implication and chemoprevention," *Experimental Biology and Medicine*, vol. 229, no. 6, pp. 546–552, 2004.

[78] R. Drapkin and J. L. Hecht, "Pathogenesis of ovarian cancer," in *Diagnostic gynecologic and obstetric pathology*, Elsevier Saunders, Philadelphia, Pa, USA, 2006.

[79] R. Drapkin, C. P. Crum, and J. L. Hecht, "Expression of candidate tumor markers in ovarian carcinoma and benign ovary: evidence for a link between epithelial phenotype and neoplasia," *Human Pathology*, vol. 35, no. 8, pp. 1014–1021, 2004.

[80] L. Dubeau, "The cell of origin of ovarian epithelial tumours," *The Lancet Oncology*, vol. 9, no. 12, pp. 1191–1197, 2008.

[81] A. S. T. Wong and P. C. K. Leung, "Role of endocrine and growth factors on the ovarian surface epithelium," *Journal of Obstetrics and Gynaecology Research*, vol. 33, no. 1, pp. 3–16, 2007.

[82] J. Permuth-Wey and T. A. Sellers, "Epidemiology of ovarian cancer," *Methods in Molecular Biology*, vol. 472, pp. 413–437, 2009.

[83] B. H. Ortiz, M. Ailawadi, C. Colitti, et al., "Second primary or recurrence? Comparative patterns of p53 and K-ras mutations suggest that serous borderline ovarian tumors and subsequent serous carcinomas are unrelated tumors," *Cancer Research*, vol. 61, no. 19, pp. 7264–7267, 2001.

[84] R. F. Caduff, S. M. Svoboda-Newman, A. W. Ferguson, C. M. Johnston, and T. S. Frank, "Comparison of mutations of Ki-RAS and p53 immunoreactivity in borderline and malignant epithelial ovarian tumors," *The American Journal of Surgical Pathology*, vol. 23, no. 3, pp. 323–328, 1999.

[85] G. Singer, R. J. Kurman, H.-W. Chang, S. K. R. Cho, and I.-M. Shih, "Diverse tumorigenic pathways in ovarian serous carcinoma," *American Journal of Pathology*, vol. 160, no. 4, pp. 1223–1228, 2002.

[86] J. D. Seidman and R. J. Kurman, "Subclassification of serous borderline tumors of the ovary into benign and malignant types: a clinicopathologic study of 65 advanced stage cases," *The American Journal of Surgical Pathology*, vol. 20, no. 11, pp. 1331–1345, 1996.

[87] A. E. S. Sehdev, P. S. Sehdev, and R. J. Kurman, "Noninvasive and invasive micropapillary (low-grade) serous carcinoma of the ovary: a clinicopathologic analysis of 135 cases," *The American Journal of Surgical Pathology*, vol. 27, no. 6, pp. 725–736, 2003.

[88] C. Peyssonnaux and A. Eychene, "The Raf/MEK/ERK pathway: new concepts of activation," *Biology of the Cell*, vol. 93, no. 1-2, pp. 53–62, 2001.

[89] G. Singer, R. Oldt III, Y. Cohen, et al., "Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma," *Journal of the National Cancer Institute*, vol. 95, no. 6, pp. 484–486, 2003.

[90] K. Obata, S. J. Morland, R. H. Watson, et al., "Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors," *Cancer Research*, vol. 58, no. 10, pp. 2095–2097, 1998.

[91] D. Shahbazian, P. P. Roux, V. Mieulet, et al., "The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity," *The EMBO Journal*, vol. 25, no. 12, pp. 2781–2791, 2006.

[92] E. Janda, K. Lehmann, I. Killisch, et al., "Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways," *Journal of Cell Biology*, vol. 156, no. 2, pp. 299–313, 2002.

[93] M. Sabbah, S. Emami, G. Redeuilh, et al., "Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers," *Drug Resistance Updates*, vol. 11, no. 4-5, pp. 123–151, 2008.

[94] W.-Y. Chan, K.-K. Cheung, J. O. Schorge, et al., "Bcl-2 and p53 protein expression, apoptosis, and p53 mutation in human epithelial ovarian cancers," *American Journal of Pathology*, vol. 156, no. 2, pp. 409–417, 2000.

[95] M. F. Kohler, J. R. Marks, R. W. Wiseman, et al., "Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer," *Journal of the National Cancer Institute*, vol. 85, no. 18, pp. 1513–1519, 1993.

[96] J. Kupryjanczyk, A. D. Thor, R. Beauchamp, et al., "p53 gene mutations and protein accumulation in human ovarian cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 11, pp. 4961–4965, 1993.

[97] W.-H. Wen, A. Reles, I. B. Runnebaum, et al., "p53 mutations and expression in ovarian cancers: correlation with overall survival," *International Journal of Gynecological Pathology*, vol. 18, no. 1, pp. 29–41, 1999.

[98] J. S. Ross, F. Yang, B. V. S. Kallakury, C. E. Sheehan, R. A. Ambros, and P. J. Muraca, "HER-2/neu oncogene amplification by fluorescence in situ hybridization in epithelial tumors of the ovary," *American Journal of Clinical Pathology*, vol. 111, no. 3, pp. 311–316, 1999.

[99] J. Q. Cheng, A. K. Godwin, A. Bellacosa, et al., "AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 19, pp. 9267–9271, 1992.

[100] H. A. Risch, J. R. McLaughlin, D. E. C. Cole, et al., "Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada," *Journal of the National Cancer Institute*, vol. 98, no. 23, pp. 1694–1706, 2006.

[101] R. Wooster and B. L. Weber, "Breast and ovarian cancer," *The New England Journal of Medicine*, vol. 348, no. 23, pp. 2339–2347, 2003.

[102] J. M. J. Piek, P. J. van Diest, R. P. Zweemer, et al., "Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer," *Journal of Pathology*, vol. 195, no. 4, pp. 451–456, 2001.

[103] M. L. Carcangiu, P. Radice, S. Manoukian, et al., "Atypical epithelial proliferation in fallopian tubes in prophylactic salpingo-oophorectomy specimens from BRCA1 and BRCA2 germline mutation carrier," *International Journal of Gynecological Pathology*, vol. 23, no. 1, pp. 35–40, 2004.

[104] A. Finch, P. Shaw, B. Rosen, J. Murphy, S. A. Narod, and T. J. Colgan, "Clinical and pathologic findings of prophylactic salpingo-oophorectomies in 159 BRCA1 and BRCA2 carriers," *Gynecologic Oncology*, vol. 100, no. 1, pp. 58–64, 2006.

[105] K. Leeper, R. Garcia, E. Swisher, B. Goff, B. Greer, and P. Paley, "Pathologic findings in prophylactic oophorectomy specimens in high-risk women," *Gynecologic Oncology*, vol. 87, no. 1, pp. 52–56, 2002.

[106] F. Medeiros, M. G. Muto, Y. Lee, et al., "The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome," *The American Journal of Surgical Pathology*, vol. 30, no. 2, pp. 230–236, 2006.

[107] D. W. Kindelberger, Y. Lee, A. Miron, et al., "Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: evidence for a causal relationship," *The American Journal of Surgical Pathology*, vol. 31, no. 2, pp. 161–169, 2007.

[108] Y. Lee, A. Miron, R. Drapkin, et al., "A candidate precursor to serous carcinoma that originates in the distal fallopian tube," *Journal of Pathology*, vol. 211, no. 1, pp. 26–35, 2007.

[109] E. P. Rogakou, C. Boon, C. Redon, and W. M. Bonner, "Megabase chromatin domains involved in DNA double-strand breaks in vivo," *Journal of Cell Biology*, vol. 146, no. 5, pp. 905–915, 1999.

[110] A. K. Folkins, E. A. Jarboe, A. Saleemuddin, et al., "A candidate precursor to pelvic serous cancer (p53 signature) and its prevalence in ovaries and fallopian tubes from women with BRCA mutations," *Gynecologic Oncology*, vol. 109, no. 2, pp. 168–173, 2008.

[111] K. Levanon, C. Crum, and R. Drapkin, "New insights into the pathogenesis of serous ovarian cancer and its clinical impact," *Journal of Clinical Oncology*, vol. 26, no. 32, pp. 5284–5293, 2008.

[112] A. Saleemuddin, A. K. Folkins, L. Garrett, et al., "Risk factors for a serous cancer precursor ("p53 signature") in women with inherited BRCA mutations," *Gynecologic Oncology*, vol. 111, no. 2, pp. 226–232, 2008.

[113] A. K. Folkins, E. A. Jarboe, M. H. Roh, and C. P. Crum, "Precursors to pelvic serous carcinoma and their clinical implications," *Gynecologic Oncology*, vol. 113, no. 3, pp. 391–396, 2009.

[114] D. C. Connolly, R. Bao, A. Y. Nikitin, et al., "Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer," *Cancer Research*, vol. 63, no. 6, pp. 1389–1397, 2003.

[115] A. Flesken-Nikitin, K.-C. Choi, J. P. Eng, E. N. Shmidt, and A. Y. Nikitin, "Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium," *Cancer Research*, vol. 63, no. 13, pp. 3459–3463, 2003.

[116] S. Orsulic, Y. Li, R. A. Soslow, L. A. Vitale-Cross, J. S. Gutkind, and H. E. Varmus, "Induction of ovarian cancer by defined multiple genetic changes in a mouse model system," *Cancer Cell*, vol. 1, no. 1, pp. 53–62, 2002.

[117] D. M. Dinulescu, T. A. Ince, B. J. Quade, S. A. Shafer, D. Crowley, and T. Jacks, "Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer," *Nature Medicine*, vol. 11, no. 1, pp. 63–70, 2005.

[118] R. Wu, N. Hendrix-Lucas, R. Kuick, et al., "Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/β-catenin and PI3K/Pten signaling pathways," *Cancer Cell*, vol. 11, no. 4, pp. 321–333, 2007.

[119] T. Umezu, M. Hanazono, S. Aizawa, and Y. Tomooka, "Characterization of newly established clonal oviductal cell lines and differential hormonal regulation of gene expression," *In Vitro Cellular and Developmental Biology—Animal*, vol. 39, no. 3-4, pp. 146–156, 2003.

[120] Y.-L. Lee, K.-F. Lee, J.-S. Xu, Y.-L. Wang, S.-W. Tsao, and W. S. B. Yeung, "Establishment and characterization of an immortalized human oviductal cell line," *Molecular Reproduction and Development*, vol. 59, no. 4, pp. 400–409, 2001.

[121] M. T. Comer, H. J. Leese, and J. Southgate, "Induction of a differentiated ciliated cell phenotype in primary cultures of Fallopian tube epithelium," *Human Reproduction*, vol. 13, no. 11, pp. 3114–3120, 1998.

[122] H. Ando, M. Kobayashi, S. Toda, F. Kikkawa, T. Masahashi, and S. Mizutani, "Establishment of a ciliated epithelial cell line from human Fallopian tube," *Human Reproduction*, vol. 15, no. 7, pp. 1597–1603, 2000.

[123] T. Henriksen, T. Tanbo, T. Abyholm, B. R. Oppedal, O. P. Claussen, and T. Hovig, "Epithelial cells from human Fallopian tube in culture," *Human Reproduction*, vol. 5, no. 1, pp. 25–31, 1990.

[124] A. Bongso, S. C. Ng, H. Sathananthan, P. L. Ng, M. Rauff, and S. S. Ratnam, "Establishment of human ampullary cell cultures," *Human Reproduction*, vol. 4, no. 5, pp. 486–494, 1989.

[125] M. Rajagopal, T. L. Tollner, W. E. Finkbeiner, G. N. Cherr, and J. H. Widdicombe, "Differentiated structure and function of primary cultures of monkey oviductal epithelium," *In Vitro Cellular and Developmental Biology—Animal*, vol. 42, no. 8-9, pp. 248–254, 2006.

[126] P. D. Vermeer, L. A. Einwalter, T. O. Moninger, et al., "Segregation of receptor and ligand regulates activation of epithelial growth factor receptor," *Nature*, vol. 422, no. 6929, pp. 322–326, 2003.

[127] L. Zhang, J. R. Conejo-Garcia, D. Katsaros, et al., "Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer," *The New England Journal of Medicine*, vol. 348, no. 3, pp. 203–213, 2003.

[128] J. A. Joyce and J. W. Pollard, "Microenvironmental regulation of metastasis," *Nature Reviews Cancer*, vol. 9, no. 4, pp. 239–252, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 126295, 9 pages
doi:10.1155/2010/126295

Review Article

Precursor Lesions of High-Grade Serous Ovarian Carcinoma: Morphological and Molecular Characteristics

Amy L. Gross,[1] Robert J. Kurman,[2] Russell Vang,[2] Ie-Ming Shih,[2] and Kala Visvanathan[1,3]

[1] *Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA*
[2] *Division of Gynecologic Pathology, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA*
[3] *Department of Medical Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA*

Correspondence should be addressed to Kala Visvanathan, kvisvana@jhsph.edu

Received 18 December 2009; Accepted 14 February 2010

Academic Editor: Tian-Li Wang

The lack of proven screening tools for early detection and the high mortality of ovarian serous carcinoma (OSC), particularly high grade, have focused attention on identifying putative precursor lesions with distinct morphological and molecular characteristics. The finding of occult invasive and intraepithelial fallopian tube carcinomas in prophylactically removed specimens from asymptomatic high-risk BRCA 1/2-mutation carriers supports the notion of an origin for OSC in the fallopian tube. The intraepithelial carcinomas have been referred to as serous intraepithelial carcinomas (STICs) but our own findings (unpublished data) and recent reports have drawn attention to a spectrum of changes that fall short of STICs that we have designated serous tubal intraepithelial lesions (STILs).

1. Introduction

Ovarian cancer is the most lethal gynecologic cancer, responsible for over 13,000 deaths in the US in 2009 (http://www.cancer.org/). The majority of these cancers are detected at an advanced stage, after they have spread to the peritoneal surfaces; the 5-year survival rate for women diagnosed at stage III-IV is only 28% (SEER Cancer Statistics Review, 1975–2004, National Cancer Institute http://seer.cancer.gov/csr/1975_2006/). A lack of effective screening tools for early detection of ovarian cancer in high-risk and general populations has led to increased interest in the identification of precursor lesions defined by both morphological and molecular changes that could be the target of not only early detection but prevention efforts.

2. Molecular Characteristics of Ovarian Cancer

Ovarian cancer is heterogeneous, like other cancers, comprising a collection of subtypes with different histologic and molecular characteristics that in turn inform prognosis [1]. Accumulating evidence suggests that there are two general pathways in the molecular pathogenesis of what is known as ovarian cancer [2, 3]. The first (Type I) pathway leads to borderline tumors, which can develop into low-grade serous, mucinous, endometrioid, and clear cell carcinomas. These are for the most part low-grade tumors that are characterized by a high frequency of mutations of *KRAS*, *BRAF, ERBB2, CTNNB1* (the gene encoding beta catenin), and *PIK3CA*, low proliferation, and a 5-year survival of approximately 55% [2]. A stepwise model of progression from cystadenomas to low-grade carcinomas has been proposed for these neoplasms.

In contrast to the Type I tumors, the Type II tumors are high-grade and highly aggressive, spreading rapidly throughout the pelvis. Type II tumors include high-grade serous carcinoma, malignant mixed mesodermal tumors, and undifferentiated carcinomas. They are characterized by a high frequency of mutations in *TP53*, a tumor suppressor gene, and a high proliferative index. It is estimated that 60% of sporadic ovarian carcinomas and the majority of those diagnosed in BRCA1 mutation carriers are of the high-grade serous type [4, 5]. Preliminary data suggests that these TP53 mutations may develop early in the carcinogenic process. If

in fact this is confirmed, new approaches to early detection and prevention can be developed.

3. Identifying the Cell of Origin

It has conventionally been assumed that ovarian cancers arise from the ovarian surface epithelium (OSE), which is viewed as a modified type of mesothelium similar to that which lines the peritoneal cavity, by a process of invagination leading to the development of cortical inclusion cysts (CICs). It has been argued that changes in the microenvironment of the ovarian stroma surrounding the CICs leads to müllerian-type differentiation. This step, which is viewed as a metaplastic change, would be necessary to explain the morphologic appearance of ovarian epithelial tumors which have a müllerian-type phenotype. However, others believe it is unlikely that CICs are precursors and have instead proposed that ovarian epithelial tumors develop from müllerian-type epithelium lining paraovarian and paratubal cysts (the so-called secondary müllerian system) [6]. A tubal origin for high-grade serous ovarian cancer is supported by gene expression profiles of OSC that reveal that they are more similar to normal müllerian epithelium than the ovarian surface epithelium [7].

4. Prophylactic Salpingo-Oophorectomy in BRCA1 and BRCA2 Mutation Carriers

Prophylactic bilateral salpingo-oophorectomy (BPSO) specimens from high-risk women have proved to be an invaluable resource for research into the origins and precursors for high-grade serous pelvic carcinomas. Women found to have a deleterious germline mutation in the *BRCA1* or *BRCA2* gene are known to be at increased risk for ovarian cancer, with lifetime risk estimates ranging from 40% to 60% [8, 9]. Given the limitations of current options for ovarian surveillance, bilateral prophylactic salpingo-oophorectomy (BPSO) after the completion of child-bearing is the current standard recommendation for these women [10]. This procedure greatly reduces the risk of subsequent development of pelvic serous carcinoma, by 80%–90%, but surprisingly does not eliminate it entirely [11]. Remaining risk is mainly attributed to primary peritoneal serous carcinoma, which is similar to high-grade OSC in terms of presentation and response to treatment [12] and appears to originate from the same cell lineage [11]. The estimated cumulative incidence of peritoneal cancer at 20 years after oophorectomy is 4.3% [13], and so far a survival benefit has been shown in the short term but not long term [14].

5. Occult Carcinoma

It is estimated that between 2% and 17% of all BPSO specimens from BRCA1/2 mutation carriers will contain an occult cancer (see Table 1). The range of estimates to some extent is reflective of the comparison groups and the protocols used for sectioning specimens [11, 13, 15–24]. Despite these inconsistencies a majority of early serous cancers found in these specimens are localized to the fallopian tube.

6. Serous Tubal Intraepithelial Carcinomas

High-grade serous tubal intraepithelial carcinomas (STICs) are noninvasive carcinomas of the fallopian tube that have been found with varying frequency in BPSO specimens (see Table 2). STICs are characterized morphologically by nuclear hyperchromasia and atypia, mitotic figures, and nuclear stratification [26]. Immunohistochemically, they exhibit increased staining for p53 and MIB-1 (Ki-67) [26] (Figure 1). As noted above, many of the occult malignancies detected on thorough sectioning of BPSO specimens were microscopic and restricted to the fallopian tube. Subsequently, a careful and thorough examination of the fallopian tubes from 55 consecutive cases of "serous carcinoma" (pelvic, ovarian, or tubal) revealed that over 70% involved the endosalpinx and approximately half contained STICs [27]. These findings led to the hypothesis that the fallopian tube may be the source of a significant proportion of all pelvic high-grade serous carcinomas. To further confirm the shared origin of these OSC with their coexisting STICs, the authors analyzed p53 mutations in both sites from five cases. Identical mutations were detected in both sites for all cases. An analysis of chromosomal copy number changes by FISH demonstrated similar results in 3/5 cases comparing ovarian serous tumors with synchronous fallopian tube serous carcinoma, providing some additional potential support for a common, monoclonal origin [28]. Further, another study from the same group examined 45 cases of primary peritoneal serous carcinoma and found that 9 out of 26 cases with incomplete tubal sampling and 9 out of 19 cases that underwent complete examination of the tube had STIC [29]. In all of these studies, the majority of STICs were found in the fimbriated end of the tube, adjacent to the ovarian surface.

In an effort to verify the fimbria as a preferred site for STICs in BPSO specimens, Medeiros et al. [25] investigated 13 BRCA+ BPSOs and 13 controls, who were women who had bilateral salpingo-oophorectomy due to other gynecological diseases. Six of the cases had mutations in BRCA1 and 7 were BRCA2 positive. Cases and controls had similar age ranges and mean ages (50 and 58 years, resp.), and all specimens were entirely submitted for review, sectioned at 2-3 mm intervals. In addition, all fimbriae were extensively sectioned in one of two ways: serial sectioning (7 cases) or by SEE-FIM protocol—Sectioning and Extensively Examining the Fimbriae—in 19 cases. The SEE-FIM protocol was developed to ensure maximal examination of the fimbria [25]. Cases were compared to controls for the rate of detection of early neoplasms, their locations, and expression patterns for p53 and Ki-67. Five cancers were identified, all of which were tubal and from the case group. Four of the five tumors involved the fimbria; four of these five also stained positive for both p53 (>75% nuclei staining positive in a region of 12 cells in length) and Mib-1. The reason for the tendency of these early cancers to be found in the fimbria is not entirely clear, but the authors suggest that it may be due

Table 1: Prevalence of occult carcinoma in bilateral prophylactic salpingo-oophorectomy specimens.

Author	Study design	Study population	Sectioning protocol	Findings
Colgan et al. 2001 [15]	Cross-sectional	60 BPSO specimens from women (mean age 48.4) with high likelihood of being BRCA mutation carriers according to family history (early criteria) or tested positive for a BRCA1 or BRCA2 mutation (later criteria)	Ovaries: multiple sections through the short axis; fallopian tubes: 40/60 had representative sections only, the other 20 were completely submitted with transverse sections	5 (8.3%) cases showed occult carcinoma; 4/5 located in fallopian tube
Leeper et al. 2002 [17]	Cross-sectional	30 BPSO specimens from women (mean age 46, range 30–65) at high risk of ovarian cancer according to family or personal history	Ovaries and tubes: first 7 cases had representative sections only, remaining 23 cases were serially sectioned	5 (16.7%) cases showed occult carcinoma; 3 located in fallopian tube
Olivier et al. 2004 [18]	Cross-sectional	38 BPO specimens and 90 BPSO specimens from women (mean age 46, range 26–74) with known BRCA1 or BRCA2 mutations or personal history of breast cancer and family history suggestive of hereditary breast and ovarian cancer	Ovaries from all procedures and tubes from BPSO procedures: sectioned in their entirety	5 (5.6%) cases from BPSO group showed occult carcinoma (all in BRCA1 carriers); 2 restricted to the fallopian tube; no occult cancers in ovary-only specimens
Lamb et al. 2006 [22]	Cross-sectional	113 BPSO specimens from women (median age 47, range 30–70) at high risk for ovarian cancer based on GOG criteria	Ovaries and tubes: sectioned at 2- to 3-mm intervals	7 (6.2%) cases showed ovarian, fallopian tube or peritoneal neoplasia; 5 were early high-grade serous tubal neoplasia
Finch et al. 2006 [21]	Cross-sectional	159 BPSO specimens from BRCA1 or BRCA2 mutation carriers (mean age 47.7, range 34–71)	Ovaries: serially sectioned; fallopian tubes initially representative sections—partway through protocol amended to submit tubes in entirety	7 (4.4%) cases showed occult carcinoma; 6 involved fallopian tube
Medeiros et al. 2006 [25]	Case-control	13 BPSO specimens from BRCA1 or BRCA2 mutation carriers (mean age 50, range 39–76) and 13 controls (mean age 58, range 43–76) undergoing surgery for benign reasons	Ovaries and tubes: sectioned at 2- to 3-mm intervals; fimbriae in some cases serially sectioned, in others by SEE-FIM protocol	5 (38%) cases showed early cancers; all in the fallopian tube; 4/5 stained positive for both p53 and MIB-1, the 5th scored positive for MIB-1 only; no cancers were found in controls
Hermsen et al. 2006 [24]	Case-control	85 BPSO specimens from high-risk women according to family history or BRCA1 or BRCA2 mutation status (median age 48, range 33–64) and 72 controls undergoing surgery for benign reasons (median age 37, range 23–79)	Sectioning details not specified	1 case of tubal carcinoma + 2 cases of severe tubal dysplasia/in situ carcinoma (3.5%) were identified in the BPSO group; no cancers were found in controls

Table 1: Continued.

Author	Study design	Study population	Sectioning protocol	Findings
Callahan et al. 2007 [23]	Cross-sectional	122 BPSO specimens from women with BRCA1 or BRCA2 mutations or variants (median age 46.5, range 23–76)	Ovaries and tubes: sectioned at 2- to 3-mm intervals for all cases; SEE-FIM protocol performed in a subset	7 (5.7%) cases showed occult tubal carcinoma
Finch et al. 2006 [13]	Cross-sectional	490 BPSO specimens from women with BRCA1 or BRCA2 mutations (mean age 47.6, range 19–76)	All reported cancers confirmed by review of medical records and/or pathology reports. The pathology reports were reviewed in order to correctly assign the diagnosis of ovarian, fallopian tube, or primary peritoneal cancer.	11 (2.2%) specimens showed occult cancer; 7 were identified as ovarian; 3 were classified as tubal; 1 case had positive peritoneal washings with no source of cancer identified
Powell et al. 2005 [19]	Cross-sectional	67 BPSO specimens from women with BRCA1 or BRCA2 mutations (mean age 47, range 31–64)	Ovaries and tubes: serially sectioned at 2 mm intervals ("full" adherence to specified protocol) in 20 cases; partial adherence in 21 cases; standard procedures (nonadherence) in 26 cases	7 (17%) specimens that were processed by fully or partially adherent protocols ($n = 41$) showed occult cancer; 4 were tubal and 3 were ovarian

BPO: bilateral prophylactic oophorectomy; BPSO: bilateral prophylactic salpingo-oophorectomy; GOG: gynecologic oncology group; SEE-FIM: sectioning and extensively examining the fimbriae.

to increased surface area of this site, or potential differences in characteristics of the cells from this region versus more proximal sections of the tube. Their findings support a possible means of spread to the ovary by exfoliation or tubal-ovary adhesions.

7. Resemblance of STIC to Serous Endometrial Intraepithelial Carcinoma

Like high-grade OSC, uterine serous carcinomas are aggressive cancers with poor prognosis [30, 31]. Despite comprising only 10%–15% of all endometrial carcinomas, USC causes a disproportionate number of deaths and appears to have a different etiologic pathway than the usual type of endometrial carcinoma (endometrioid adenocarcinoma). In contrast to the endometrioid type, which is typically found to be associated with endometrial hyperplasia and other signs of hyperestrogenism, most uterine serous carcinomas are diagnosed in older, postmenopausal women with atrophic endometrium and no evidence of endometrial hyperplasia. It has therefore been hypothesized that uterine serous carcinoma and its presumptive precursor, serous endometrial intraepithelial carcinoma, may originate in an estrogen-independent manner [32] and are associated with other factors [33].

Serous endometrial intraepithelial carcinoma (SEIC) is characterized by "replacement of endometrial surface epithelium and glands by malignant cells that resemble invasive high-grade endometrial carcinoma" [34]. It has been identified in greater than 90% of uteri containing serous carcinoma [31]. Interestingly, immunostaining for p53 expression in pairs of uterine serous carcinoma with SEIC showed the majority to be p53 positive and all pairs were concordant [34]. Shared p53 mutations have also been described in SEIC and the associated uterine serous carcinoma [35]. Uterine serous carcinoma and OSC both exhibit a tendency to spread rapidly throughout the pelvis and multifocal disease is often found at diagnosis. These multiple foci are thought to represent monoclonal disease, unlike endometrioid carcinomas, which have been shown to have multiple sites of origin [36]. The question of site of origin for these multifocal uterine serous carcinoma parallels that of the origin of other pelvic serous carcinomas.

A recent study attempted to document the frequency of concurrent STIC and SEIC, conjecturing that uterine serous carcinoma with STIC might represent a distinct subset of pelvic serous carcinomas, with as-yet unclear origin. Of 22 uterine serous carcinoma cases examined, the presence of STIC was confirmed in 5 [36]. It was found that the endometrial tumor in all five of these cases was either noninvasive or superficially invasive, and in 2 of the cases identical p53 mutations were identified in both tubal and

Table 2: Serous tubal intraepithelial carcinoma in pelvic serous carcinoma cases.

Author	Study design	Study population	Sectioning Protocol	Findings
Salvador et al. 2008 [28]	Cross-sectional	16 cases of epithelial ovarian malignancy with tubes submitted in toto	fallopian tubes submitted in toto and serially sectioned every 3-4 mm	10 of the 12 cases of high-grade serous carcinoma showed either unilateral tubal mucosal involvement by TIC ($n = 7$) or tubal obliteration ipsilateral to the dominant ovarian mass ($n = 3$). In 3 of 5 selected high grade serous carcinoma cases with TICs, FISH analysis showed similar copy number changes in foci of the ovarian and fallopian tube mucosal carcinoma; one case was not synchronous and the 5th was indeterminate
Kindelberger et al. 2007 [27]	Cross-sectional	55 cases containing pelvic serous carcinoma (mean age 61.5, range 43–82)	SEE-FIM for all cases	41(75%) showed involvement of endosalpinx; 11 were classified as tubal or peritoneal primary; (9 of these had TICs); 20/30 cases classified as ovarian had TICs; 93% of TICs involved the fimbria. Of 5 ovarian cases with TICs, p53 DNA analysis showed identical mutations in at least one focus of TIC and ovarian cancer
Carlson et al. 2008 [29]	Cross-sectional	45 cases of primary peritoneal serous carcinoma in which there was either nonuniform sampling of the fallopian tube ($n = 26$) or SEE-FIM protocol ($n = 19$)	nonuniform sampling (portion of tube submitted), or SEE-FIM protocol	9 (35%) of first sampling group and 9 (47%) of second samping group showed STICs. 5/5 cases tested showed identical p53 mutations in the peritoneal and tubal lesions

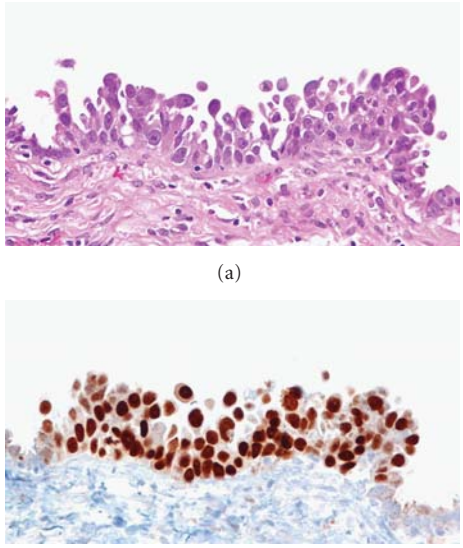
FISH: fluorescence in situ hybridization; SEE-FIM: sectioning and extensively examining the fimbriae; STIC: serous tubal intraepithelial carcinoma; TIC: tubal intraepithelial carcinoma.

endometrial lesions, suggesting a common origin, perhaps in the tube, for these cases. The proposal that these uterine tumors may also be of tubal origin is intriguing and certainly deserves further investigation.

8. Identification of Precursor Lesions

8.1. Serous Tubal Intraepithelial Lesions

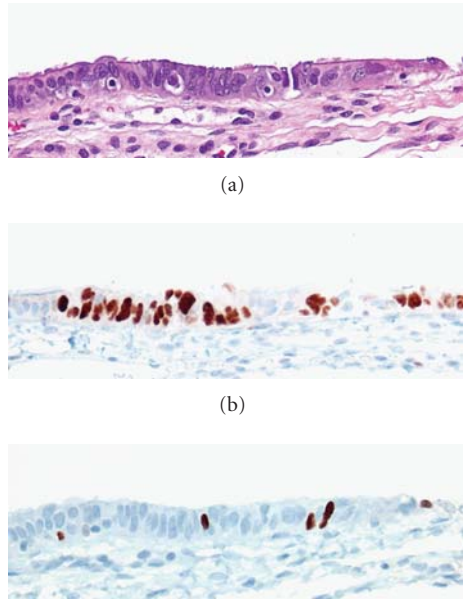
8.1.1. Molecular Changes. We use the term "serous tubal intraepithelial lesions" (STILs) to describe a spectrum of epithelial changes ranging from normal appearing tubal epithelium, expressing p53, to lesions with increasing degrees of cytologic atypia that fall short of an STIC (Figure 2). Others have reported these changes as tubal intraepithelial lesions in transition (TILT) [37]. Characterization of STILs has become a central focus of several groups, including ours, in order to determine the nature of these lesions and their relationship to STICs, specifically whether these are the earliest steps in the carcinogenic process. A 2007 study [38] sought to examine the relationship of what was designated "p53 signatures," characterized by high p53 immunostaining, defined as strong nuclear staining obscuring nuclear detail in at least 12 consecutive secretory nuclei in benign-appearing epithelium, and STIC. The fallopian tubes from three groups of women were included in this series: (1) 41 women with BRCA1 or BRCA2 mutations undergoing BPSO, (2) 58 consecutive women undergoing procedures for other gynecologic disease, and (3) 17 women already identified to have STICs, all of which were shown in a previous study to be associated with pelvic serous carcinoma [27]. All fimbriae were sectioned using the SEE-FIM protocol. Sections were examined for prevalence of p53 signatures. Similar percentages of p53 signatures were found in the benign BPSO specimens from BRCA+ women versus group 2 (24% and 33%, resp.) and were most often found in the fimbriated end of the tube. Nine of the 17 STICs (53%) contained at least one p53 signature and multiple p53 signatures were found in tubes containing STICs at twice the frequency found in nonneoplastic tubes. Staining



(a)

(b)

Figure 1: Serous tubal intraepithelial carcinoma (STIC). (a) The morphological features of a small STIC showing papillary architecture with intraepithelial carcinoma cells, many of which are detached and are able to freely disseminate onto ovaries, pelvic, or peritoneal wall. (b) The p53 immunohistochemistry demonstrates that all intraepithelial carcinoma cells are intensely positive for p53 nuclear immunoreactivity. In contrast, the normal-appearing tubal epithelial cells flanking the STIC are negative for p53.



(a)

(b)

(c)

Figure 2: Serous tubal intraepithelial lesion (STIL). (a) The morphological features of an STIL identified in a young cancer-free BRCA mutation carrier. The epithelium shows mild nuclear and architectural atypia but not to the level of a classical STIC. (b) An immunohistochemical stain for p53 shows overexpression in several consecutive secretory cells. (c) An immunohistochemical stain for Ki-67 shows a low proliferation index.

of a subset of tissues containing p53 signature, STIC, or serous carcinoma revealed the colocalization of γ-H2AX, an immunohistochemical marker of double-stranded DNA damage, with p53.

Another study from the same group compared the prevalence of p53 signatures in fallopian tubes versus ovarian cortical inclusion cysts in BRCA+ women [39]. Cases consisted of consecutive BPSOs performed in BRCA+ women. Controls, taken from a prior study, were a consecutive series of women free of ovarian cancer, having surgery for other gynecologic conditions, who had either tested negative for BRCA mutations or were untested. All tubes and ovaries were submitted entirely, and the SEE-FIM protocol was used for examining the fimbriae. The proportions of specimens that showed at least one p53 signature were similar between cases and controls (38% and 33%, resp.). No CICs were found to contain p53 signatures. Sixty-six percent of the p53 signatures in cases stained positive for γ-H2AX; staining for γ-H2AX in the p53 signatures of controls was not reported.

8.1.2. Hormonal Changes. One of the prevailing theories of ovarian epithelial carcinogenesis is that of "incessant ovulation" as a potential causative role in these cancers. Ovulation has been suggested to cause genotoxic damage in the ovarian surface epithelium where ovarian cancers have traditionally been thought to originate; the recent findings of colocalization of γ-H2AX with p53 staining in STILs and STICs in the fimbria, adjacent to the ovarian surface, is therefore highly intriguing and will be important to explore further.

A recent study exploiting genetic microarray technology compared the expression profiles of normal-appearing fallopian tube epithelium of BRCA+ mutation carriers and healthy controls to serous carcinomas [40]. Fallopian tube epithelium samples from the BRCA+ group (but not the normal controls) were found to cluster closely with serous carcinoma samples. In particular, BRCA+ fallopian tube epithelium taken during the luteal phase (versus follicular phase) expressed gene profiles most similar to those of the serous carcinoma tissues. Two differentially expressed genes, SKIL (ski-like), an oncogene, and DAB2, a tumor suppressor, emerged as candidates that have been implicated in the tumorigenic process [41, 42]. SKIL was found to be overexpressed, and DAB2 underexpressed, in serous carcinoma and BRCA+ luteal fallopian tube epithelium, relative to BRCA+ follicular fallopian tube epithelium samples, suggesting that the hormonal milieu during the luteal phase, particularly in BRCA+ carriers, may predispose to a carcinogenic process in the fallopian tube epithelium. The extent to which hormonal changes during the luteal phase represent a transitory phenomenon, and the manner in which the fallopian tube epithelium might retain a more permanent imprint of such cyclical fluctuations, is unclear.

Tubal-mucosal morphology is known to be influenced by hormones [15], although these changes have not been precisely quantified. Presumably, the endogenous and exogenous hormonal milieu plays a key role in the molecular

alterations of the fallopian tube epithelium, varying with age and menopausal status. Evidence from epidemiological studies provided further supports the role of hormonal exposures in affecting risk of ovarian cancer. It has been wellestablished in epidemiological studies that use of oral contraceptive pills (OCP) protects against the development of ovarian cancer; use for five or more years reduces risk by approximately 50% [43, 44]. Parity has also been shown in numerous studies to be protective, conferring a 30%–60% decreased risk compared to nulliparity [45]. Two theories have been postulated to explain these findings: that ovulation causes repetitive cell damage/repair which over time increases the risk of mutations or that increasing exposure to gonadotropin-releasing hormones (represented by lower OCP use and parity, or increased ovulatory cycles) may be the mechanism by which ovarian cancer risk is increased. But studies attempting to find a correlation between number of ovulatory cycles and accumulation of p53 mutations in ovarian cancers have been contradictory [46–48]. Schildkraut et al. (1997) examined the ovaries from 197 women with invasive ovarian cancer and found that cases overexpressing p53 (53%) were exposed to significantly more ovulatory cycles than those cases that did not overexpress p53. However, a study by Webb et al. examined 234 cases of invasive ovarian cancer and found no associations between p53 overexpression and other factors including years of ovulation, parity, family history of ovarian cancer, or age. One cross-sectional study examined the association between factors including parity, history of breast cancer, prior chemotherapy, smoking history, tamoxifen use, age at first birth, age at time of oophorectomy, BMI, age at menarche, and oral contraceptive use (never versus ever) obtained from medical record review and the presence of p53 signatures in the tubes of 75 BRCA+ mutation carriers [49]. Thirty eight percent of the tubes showed at least one p53 signature, and parity was significantly associated with the presence of p53 signatures. Due to the cross-sectional nature of the study temporality could not be assessed.

8.1.3. Cortical Inclusion Cysts. Several studies comparing the prevalence of CICs in "high-risk" prophylactically removed ovaries versus normal-risk ovaries have reported inconsistent findings [50]. One study comparing morphology of ovarian surface epithelium of 64 women with a family history of ovarian cancer with 30 women without a family history found significantly more CICs in the high-risk group [51], while others have found no significant difference in the frequency of CICs. CICs also appear to increase with age in both high-risk and normal-risk women, and this needs to be taken into consideration when evaluating its significance as a precursor lesion [50]. Barakat et al. [52] examined ovarian tissues from 18 BRCA1 mutation carriers and 20 age-matched controls and found no significant differences in frequency of inclusion cysts or other morphological alterations. Immunohistochemical staining for p53, ERBB-2, and Ki67 of the CICs also showed no difference in expression between cases and controls. In a cross-sectional study examining the relationship between demographic variables and the presence of CICs in 74 bilateral prophylactic salpingo-oophorectomies (BPSOs) from BRCA1/2 mutation carriers, Folkins et al. [53] found women with ≥ 7 versus <7 CICs per ovary pair had significantly older age at childbirth and menopause, older age at surgery, and higher body mass index (BMI). Limitations of this study include the small sample size, which prevented the analyses from being adjusted for confounders, such as age, and the retrospective collection of information on risk factors from medical records.

8.1.4. Future Directions. Large, well-powered studies with uniform sectioning methodology are needed to better define the prevalence of STICs and STILs in specimens from high-risk women and the general population, and to determine the incidence of subsequent peritoneal cancer. Further prospective data is needed to assess interactions between exposures and these lesions. Animal experiments could assist in validating the temporality of these precursor lesions to the development of ovarian serous carcinoma, as this type of study would not be feasible in clinical studies. The genetic changes accompanying and preceding these lesions may then be able to be targeted in prevention studies. In addition, a validated classification of STIL would help both internal validity and generalizability of studies.

References

[1] K. R. Cho and M. Shih Ie, "Ovarian cancer," *Annual Review of Pathology*, vol. 4, pp. 287–313, 2009.

[2] M. Shih Ie and R. J. Kurman, "Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis," *American Journal of Pathology*, vol. 164, no. 5, pp. 1511–1518, 2004.

[3] R. J. Kurman and M. Shih Ie, "Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications," *International Journal of Gynecological Pathology*, vol. 27, no. 2, pp. 151–160, 2008.

[4] H. T. Lynch, M. J. Casey, C. L. Snyder, et al., "Hereditary ovarian carcinoma: Heterogeneity, molecular genetics, pathology, and management," *Molecular Oncology*, vol. 3, no. 2, pp. 97–137, 2009.

[5] P. A. Shaw, J. R. McLaughlin, R. P. Zweemer, et al., "Histopathologic features of genetically determined ovarian cancer," *International Journal of Gynecological Pathology*, vol. 21, no. 4, pp. 407–411, 2002.

[6] L. Dubeau, "The cell of origin of ovarian epithelial tumours," *The Lancet Oncology*, vol. 9, no. 12, pp. 1191–1197, 2008.

[7] R. T. Marquez, K. A. Baggerly, A. P. Patterson, et al., "Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon," *Clinical Cancer Research*, vol. 11, no. 17, pp. 6116–6126, 2005.

[8] D. Ford, D. F. Easton, D. T. Bishop, S. A. Narod, and D. E. Goldgar, "Risks of cancer in *BRCA1*-mutation carriers," *The Lancet*, vol. 343, no. 8899, pp. 692–695, 1994.

[9] D. F. Easton, D. Ford, D. T. Bishop, et al., "Breast and ovarian cancer incidence in *BRCA1*-mutation carriers," *American Journal of Human Genetics*, vol. 56, no. 1, pp. 265–271, 1995.

[10] "The NCCN Clinical Practice Guidelines in Oncology Genetic/Familial High-Risk Assessment: Breast and Ovarian

(Version 1.2009)," National Comprehensive Cancer Network, June 2009, http://www.nccn.org.

[11] T. R. Rebbeck, H. T. Lynch, S. L. Neuhausen, et al., "Prophylactic oophorectomy in carriers of *BRCA1* or *BRCA2* mutations," *The New England Journal of Medicine*, vol. 346, no. 21, pp. 1616–1622, 2002.

[12] G. H. Eltabbakh and M. S. Piver, "Extraovarian primary peritoneal carcinoma," *Oncology*, vol. 12, no. 6, pp. 813–819, 1998.

[13] A. Finch, "Salpingo-oophorectomy and the risk of ovarian, fallopian tube, and peritoneal cancers in women with a *BRCA1* or *BRCA2* mutation," *Journal of the American Medical Association*, vol. 296, no. 2, pp. 185–192, 2006.

[14] S. M. Domchek, T. M. Friebel, S. L. Neuhausen, et al., "Mortality after bilateral salpingo-oophorectomy in *BRCA1* and *BRCA2* mutation carriers: a prospective cohort study," *The Lancet Oncology*, vol. 7, no. 3, pp. 223–229, 2006.

[15] T. J. Colgan, J. Murphy, D. E. C. Cole, S. Narod, and B. Rosen, "Occult carcinoma in prophylactic oophorectomy specimens: prevalence and association with BRCA germline mutation status," *American Journal of Surgical Pathology*, vol. 25, no. 10, pp. 1283–1289, 2001.

[16] N. D. Kauff, J. M. Satagopan, M. E. Robson, et al., "Risk-reducing salpingo-oophorectomy in women with a *BRCA1* or *BRCA2* mutation," *The New England Journal of Medicine*, vol. 346, no. 21, pp. 1609–1615, 2002.

[17] K. Leeper, R. Garcia, E. Swisher, B. Goff, B. Greer, and P. Paley, "Pathologic findings in prophylactic oophorectomy specimens in high-risk women," *Gynecologic Oncology*, vol. 87, no. 1, pp. 52–56, 2002.

[18] R. I. Olivier, M. Van Beurden, M. A. C. Lubsen, et al., "Clinical outcome of prophylactic oophorectomy in *BRCA1*/*BRCA2* mutation carriers and events during follow-up," *British Journal of Cancer*, vol. 90, no. 8, pp. 1492–1497, 2004.

[19] B. C. Powell, E. Kenley, L. M. Chen, et al., "Risk-reducing salpingo-oophorectomy in BRCA mutation carriers: role of serial sectioning in the detection of occult malignancy," *Journal of Clinical Oncology*, vol. 23, no. 1, pp. 127–132, 2005.

[20] P. A. M. Meeuwissen, C. Seynaeve, C. T. M. Brekelmans, H. J. Meijers-Heijboer, J. G. M. Klijn, and C. W. Burger, "Outcome of surveillance and prophylactic salpingo-oophorectomy in asymptomatic women at high risk for ovarian cancer," *Gynecologic Oncology*, vol. 97, no. 2, pp. 476–482, 2005.

[21] A. Finch, P. Shaw, B. Rosen, J. Murphy, S. A. Narod, and T. J. Colgan, "Clinical and pathologic findings of prophylactic salpingo-oophorectomies in 159 *BRCA1* and *BRCA2* carriers," *Gynecologic Oncology*, vol. 100, no. 1, pp. 58–64, 2006.

[22] J. D. Lamb, R. L. Garcia, B. A. Goff, P. J. Paley, and E. M. Swisher, "Predictors of occult neoplasia in women undergoing risk-reducing salpingo-oophorectomy," *American Journal of Obstetrics and Gynecology*, vol. 194, no. 6, pp. 1702–1709, 2006.

[23] M. J. Callahan, C. P. Crum, F. Medeiros, et al., "Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction," *Journal of Clinical Oncology*, vol. 25, no. 25, pp. 3985–3990, 2007.

[24] B. B. J. Hermsen, P. J. Van Diest, J. Berkhof, et al., "Low prevalence of (pre) malignant lesions in the breast and high prevalence in the ovary and fallopian tube in women at hereditary high risk of breast and ovarian cancer," *International Journal of Cancer*, vol. 119, no. 6, pp. 1412–1418, 2006.

[25] F. Medeiros, M. G. Muto, Y. Lee, et al., "The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome," *American Journal of Surgical Pathology*, vol. 30, no. 2, pp. 230–236, 2006.

[26] E. Jarboe, A. Folkins, M. R. Nucci, et al., "Serous carcinogenesis in the fallopian tube: a descriptive classification," *International Journal of Gynecological Pathology*, vol. 27, no. 1, pp. 1–9, 2008.

[27] D. W. Kindelberger, Y. Lee, A. Miron, et al., "Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: evidence for a causal relationship," *American Journal of Surgical Pathology*, vol. 31, no. 2, pp. 161–169, 2007.

[28] S. Salvador, A. Rempel, R. A. Soslow, B. Gilks, D. Huntsman, and D. Miller, "Chromosomal instability in fallopian tube precursor lesions of serous carcinoma and frequent monoclonality of synchronous ovarian and fallopian tube mucosal serous carcinoma," *Gynecologic Oncology*, vol. 110, no. 3, pp. 408–417, 2008.

[29] J. W. Carlson, A. Miron, E. A. Jarboe, et al., "Serous tubal intraepithelial carcinoma: its potential role in primary peritoneal serous carcinoma and serous cancer prevention," *Journal of Clinical Oncology*, vol. 26, no. 25, pp. 4160–4165, 2008.

[30] M. Hendrickson, J. Ross, A. Martinez, et al., "Uterine papillary serous carcinoma. A highly malignant form of endometrial adenocarcinoma," *American Journal of Surgical Pathology*, vol. 6, no. 2, pp. 93–108, 1982.

[31] M. E. Sherman, P. Bitterman, N. B. Rosenshein, G. Delgado, and R. J. Kurman, "Uterine serous carcinoma: a morphologically diverse neoplasm with unifying clinicopathologic features," *American Journal of Surgical Pathology*, vol. 16, no. 6, pp. 600–610, 1992.

[32] J. V. Bokhman, "Two pathogenetic types of endometrial carcinoma," *Gynecologic Oncology*, vol. 15, no. 1, pp. 10–17, 1983.

[33] M. E. Sherman, M. E. Bur, and R. J. Kurman, "p53 In endometrial cancer and its putative precursors: evidence for diverse pathways of tumorigenesis," *Human Pathology*, vol. 26, no. 11, pp. 1268–1274, 1995.

[34] R. A. Ambros, M. E. Sherman, C. M. Zahn, P. Bitterman, and R. J. Kurman, "Endometrial intraepithelial carcinoma: a distinctive lesion specifically associated with tumors displaying serous differentiation," *Human Pathology*, vol. 26, no. 11, pp. 1260–1267, 1995.

[35] H. Tashiro, C. Isacson, R. Levine, R. J. Kurman, K. R. Cho, and L. Hedrick, "p53 gene mutations are common in uterine serous carcinoma and occur early in their pathogenesis," *American Journal of Pathology*, vol. 150, no. 1, pp. 177–185, 1997.

[36] E. A. Jarboe, A. Miron, J. W. Carlson, et al., "Coexisting intraepithelial serous carcinomas of the endometrium and fallopian tube: frequency and potential significance," *International Journal of Gynecological Pathology*, vol. 28, no. 4, pp. 308–315, 2009.

[37] C. P. Crum, "Intercepting pelvic cancer in the distal fallopian tube: theories and realities," *Molecular Oncology*, vol. 3, no. 2, pp. 165–170, 2009.

[38] Y. Lee, A. Miron, R. Drapkin, et al., "A candidate precursor to serous carcinoma that originates in the distal fallopian tube," *Journal of Pathology*, vol. 211, no. 1, pp. 26–35, 2007.

[39] A. K. Folkins, E. A. Jarboe, A. Saleemuddin, et al., "A candidate precursor to pelvic serous cancer (p53 signature) and its prevalence in ovaries and fallopian tubes from women with BRCA mutations," *Gynecologic Oncology*, vol. 109, no. 2, pp. 168–173, 2008.

[40] A. A. Tone, H. Begley, M. Sharma, et al., "Gene expression profiles of luteal phase fallopian tube epithelium from BRCA mutation carriers resemble high-grade serous carcinoma," *Clinical Cancer Research*, vol. 14, no. 13, pp. 4067–4078, 2008.

[41] J. Deheuninck and K. Luo, "Ski and SnoN, potent negative regulators of TGF-β signaling," *Cell Research*, vol. 19, no. 1, pp. 47–57, 2009.

[42] D. H. Yang, Z. Fazili, E. R. Smith, et al., "Disabled-2 heterozygous mice are predisposed to endometrial and ovarian tumorigenesis and exhibit sex-biased embryonic lethality in a p53-null background," *American Journal of Pathology*, vol. 169, no. 1, pp. 258–267, 2006.

[43] H. A. Risch, N. S. Weiss, J. L. Lyon, et al., "Events of reproductive life and the incidence of epithelial ovarian cancer," *American Journal of Epidemiology*, vol. 117, no. 2, pp. 128–139, 1983.

[44] V. Beral, "Ovarian cancer and hormone replacement therapy in the Million Women Study," *The Lancet*, vol. 369, no. 9574, pp. 1703–1710, 2007.

[45] J. Permuth-Wey and T. A. Sellers, "Epidemiology of ovarian cancer," *Methods in Molecular Biology*, vol. 472, pp. 413–437, 2009.

[46] J. M. Schildkraut, E. Bastos, and A. Berchuck, "Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer," *Journal of the National Cancer Institute*, vol. 89, no. 13, pp. 932–938, 1997.

[47] J. M. Schildkraut, B. Calingaert, P. A. Marchbanks, P. G. Moorman, and G. C. Rodriguez, "Impact of progestin and estrogen potency in oral contraceptives on ovarian cancer risk," *Journal of the National Cancer Institute*, vol. 94, no. 1, pp. 32–38, 2002.

[48] P. M. Webb, A. Green, M. C. Cummings, D. M. Purdie, M. D. Walsh, and G. Chenevix-Trench, "Relationship between number of ovulatory cycles and accumulation of mutant p53 in epithelial ovarian cancer," *Journal of the National Cancer Institute*, vol. 90, no. 22, pp. 1729–1734, 1998.

[49] A. Saleemuddin, A. K. Folkins, L. Garrett, et al., "Risk factors for a serous cancer precursor ("p53 signature") in women with inherited BRCA mutations," *Gynecologic Oncology*, vol. 111, no. 2, pp. 226–232, 2008.

[50] K. Q. Cai, A. Klein-Szanto, D. Karthik, et al., "Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations," *Gynecologic Oncology*, vol. 103, no. 2, pp. 719–728, 2006.

[51] B. A. Werness, A. M. Afify, K. L. Bielat, G. H. Eltabbakh, M. S. Piver, and J. M. Paterson, "Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer," *Human Pathology*, vol. 30, no. 2, pp. 151–157, 1999.

[52] R. R. Barakat, M. G. Federici, P. E. Saigo, M. E. Robson, K. Offit, and J. Boyd, "Absence of premalignant histologic, molecular, or cell biologic alterations in prophylactic oophorectomy specimens from *BRCA1* heterozygotes," *Cancer*, vol. 89, no. 2, pp. 383–390, 2000.

[53] A. K. Folkins, A. Saleemuddin, L. A. Garrett, et al., "Epidemiologic correlates of ovarian cortical inclusion cysts (CICs) support a dual precursor pathway to pelvic epithelial cancer," *Gynecologic Oncology*, vol. 115, no. 1, pp. 108–111, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 297671, 11 pages
doi:10.1155/2010/297671

Research Article

Germline Mutations and Polymorphisms in the Origins of Cancers in Women

Kim M. Hirshfield,[1] Timothy R. Rebbeck,[2] and Arnold J. Levine[1,3]

[1] *The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, New Brunswick, NJ 08901, USA*
[2] *Department of Biostatistics and Epidemiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*
[3] *Institute for Advanced Study, Princeton, NJ 08540, USA*

Correspondence should be addressed to Arnold J. Levine, alevine@ias.edu

Received 23 June 2009; Accepted 14 November 2009

Academic Editor: Tian-Li Wang

Several female malignancies including breast, ovarian, and endometrial cancers can be characterized based on known somatic and germline mutations. Initiation and propagation of tumors reflect underlying genomic alterations such as mutations, polymorphisms, and copy number variations found in genes of multiple cellular pathways. The contributions of any single genetic variation or mutation in a population depend on its frequency and penetrance as well as tissue-specific functionality. Genome wide association studies, fluorescence in situ hybridization, comparative genomic hybridization, and candidate gene studies have enumerated genetic contributors to cancers in women. These include p53, BRCA1, BRCA2, STK11, PTEN, CHEK2, ATM, BRIP1, PALB2, FGFR2, TGFB1, MDM2, MDM4 as well as several other chromosomal loci. Based on the heterogeneity within a specific tumor type, a combination of genomic alterations defines the cancer subtype, biologic behavior, and in some cases, response to therapeutics. Consideration of tumor heterogeneity is therefore important in the critical analysis of gene associations in cancer.

1. Inherited Mutations that Predispose to Cancers in Women

There is strong evidence that inherited genetic factors (mutations plus single nucleotide polymorphisms) can play a major role in breast cancer susceptibility [1]. Inherited mutations in a small number of genes account for about five to ten percent of women's cancers. These inherited variations, identified in breast, ovarian, and endometrial cancer susceptibility, can be characterized in the general population by their frequency and the magnitude of their impact upon a patient (Table 1). Some inherited variants occur rarely in the general population, but confer large risks to the individual. Examples of these genes are BRCA1 and BRCA2 in breast and ovarian cancers. A second class of inherited variants confers a lower risk, and these variants are also rare in the general population. An example of this class of genes is a mutation in the CHEK2 gene in breast cancer. The third class, composed of high-risk variants that are also common in the population, has never been identified by the methods presently available and may in fact not exist because it may well be strongly selected against in populations. Finally, a fourth class of inherited variants includes those that confer low disease risk to the individual, but occur at higher frequencies in populations. These include some of the recent findings from genome-wide association studies (GWASs) mostly with breast cancers. A summary of the major findings to date for these genes is in Table 1 and is discussed in what follows.

Despite these advances made in identifying inherited breast cancer susceptibility genes, the vast majority of breast cancers are sporadic, that is, no identifiable mutation in one of the known breast cancer susceptibility genes. While this may reflect the fact that we have yet to identify the next BRCA gene, it may also reflect the polygenic nature of breast cancer susceptibility. Other contributors to genetic susceptibility, for example, polymorphisms, may have a higher relative contribution to risk, but their lower penetrance makes identification more difficult. Furthermore, modification of genetic susceptibility by environmental factors,

Table 1: Genetic loci implicated in hereditary, familial, and sporadic breast cancer susceptibility.

High penetrance, low frequency	Low penetrance, low frequency	Low penetrance, High frequency
BRCA1	CHEK2	FGFR2
BRCA2	ATM	LSP1
PTEN	PALB2	MAP3K1
p53	BRIP1	TGFB1
STK11		TOX3
		2q35
		8q

both endogenous and exogenous, may alter the degree of penetrance. Supporters of the polygenic nature of breast cancer suggest that the contributions from polymorphisms are very important because of their high frequency in the population.

1.1. High-Penetrance, Low-Frequency Inherited Variants. Although inherited mutations in a small number of genes account for only about five to ten percent of women's cancers, by far the BRCA1 and BRCA2 gene mutations are the most common examples of this observation (50–70% of familial breast cancers) [2]. In some populations BRCA1 and BRCA2 mutations can account for ten percent of all breast cancers (Ashkenazi Jewish populations) and ovarian cancers but in many ethnic groups and in all populations taken together these mutations are much rarer (reviewed in [3]). The BRCA1 and BRCA2 proteins appear to be scaffolding proteins that assemble DNA repair complexes of proteins at double-strand DNA breaks (mediating homologous DNA repair processes) (reviewed in [4]). Mutations in these genes result in a faulty repair process and a high mutation rate, especially during DNA replication, leading to cancers. The penetrance of these mutations for cancer occurrence and the age of onset of these cancers in women can be quite variable. There have been a number of other possible functions ascribed to the BRCA1 and BRCA2 proteins such as ubiquitin ligase activity and a modifier of transcription and it is certainly possible that these protein complexes act in several ways [5]. Breast cancers initiated in women who are heterozygous for BRCA1 or BRCA2 often have a reduction to homozygosity at the BRCA-locus eliminating its functions. This results in DNA damage in the tumor which should activate the p53 protein resulting in apoptosis, senescence, or cell cycle arrest. If this is the case, the p53 gene product would be a suppressor of this cancer phenotype and contribute to the variable penetrance of these breast cancer genes. Consistent with this is the observation that BRCA1/2-initiated breast cancers have very high rates (29–84%) of somatic p53 mutations compared to 14–35% in non-BRCA1/2-related breast cancer [6].

Inherited mutations in several other genes, such as PTEN and p53, can give rise to cancers in women. Cowden's Disease is a heterozygous deficiency in the PTEN gene that can result in breast, endometrial, and other cancers [3, 7]. The PTEN protein is a lipid (PIP-3) phosphatase that modulates a growth factor pathway, in turn regulating metabolic pathways in cells, angiogenesis, mitochondrial functions and apoptotic functions [8]. Genetic alterations in this pathway are among the most common somatic mutations observed in breast and endometrial cancers [9, 10]. Mutations in LKB1 also predispose to breast and ovarian cancers as one of the phenotypes in Peutz-Jeghers syndrome [3, 11]. Inherited defects in one allele of the p53 gene give rise to Li-Fraumeni syndrome, where a subset of the cancers observed at an early age are breast cancers [12].

1.2. Low-Penetrance, Low-Frequency Inherited Variants. This class of inherited variants is difficult to detect with existing methods because the rarity of these variants and coupled with small effect sizes this means that most association studies will not be able to detect them due to limitations in population sizes under study. In the extreme, these variants may represent "private" mutations that confer a small degree of risk to very few individuals in this population, such that nearly every person would have a unique set of predisposing alleles. While it has been difficult to detect inherited variants of this type there are several examples of this type of variant which were uncovered by examining candidate genes that an investigator suspected played a role in a cancer. Inherited alterations in the CHEK2 gene which normally produces a protein kinase found in signal transduction pathways (p53 pathway and others), alerts the cell that there is DNA damage and its loss can have an impact upon several types of cancer [13]. Similarly the ATM protein kinase harbors genetic variants that detect single- and double-strand breaks in the DNA and signals to the p53 pathway and other DNA repair processes. Variants in this gene could lower or raise the sensitivity of this DNA damage detector and impact upon the efficiency of p53 and its tumor suppressor pathway and can predispose women to breast cancers [14]. The BRIP1 gene (BRCA1 interacting protein-1) encodes a protein that is a DNA/RNA helicase of the REC Q family that binds to the carboxy-terminus of BRCA1 protein conferring an activity involved in DNA repair and variants of this gene can predispose to breast cancers [15]. Interestingly this gene product is also a component of the Fanconi anemia gene pathway for DNA repair processes. Finally the PALB2 gene product (partner and localizer of BRCA2) is part of the BRCA2 protein complex and plays a role in DNA repair. It has recently been shown to be a genetic determinant of familial breast and other cancers primarily in the certain populations, but found at even lower frequency in other populations [16].

1.3. Low-Penetrance, High-Frequency Inherited Variants. Fewer than 10% of breast cancers are attributable to known mutations in breast cancer susceptibility genes BRCA1 and BRCA2. The multigenic susceptibility due to common, low-penetrance risk markers is yet to be defined [1, 17–20]. Both candidate gene [21] and genome-wide association studies have identified novel markers for susceptibility [22–25] and prognosis [26]. Genome-wide association studies

have become widely used to identify commonly occurring alleles at disease susceptibility loci. These studies use a large number of high-density markers to identify associations with disease that rely upon patterns of linkage disequilibrium in the human genome. GWASs have been successful in identifying genes for breast cancer, and GWASs for ovarian and endometrial cancers are underway although several investigators have validated findings from GWAS studies designed originally for breast cancer studies but employed for ovarian cancer [27]. Some of the more reproducible genes that GWASs studies have indicated can play a role in the risk for developing breast cancers include FGFR2, LSP1, MAP3K1, TGFB1, TOX3, 2q35, and 8q [17, 22, 24].

2. Somatic Mutations That Are Commonly Observed in Women's Cancers

Both gene amplifications and deletions can lead to common somatic mutations in women's cancers. Among the amplifications are the following. (1) HER-2/Neu, amplified in about 15% of the breast cancers, is a growth receptor that activates the Ras-MEK and the PI3K pathways in cancer cells [28]. (2) Cyclin D, amplified in about 10–12% of the breast cancers, is a subunit of the cyclin dependent kinase −4/6 that acts upon the Rb protein freeing the E2F transcription factor for entry into the cell cycle [28, 29]. (3) WIP1, amplified in about 13% of breast cancers, is a serine/threonine phosphatase that inactivates the ATM kinase and the p53 protein [30]. The GASC1 gene, which produces a histone demethylase activity, is amplified in about 5–10% of breast cancers but 20–25% of the basal breast cancers. This enzyme removes dimethyl and trimethyl groups from histone H-3 lysine-9 and 36 residues which results in altered transcriptional patterns in these cells. Inactivation of gene functions by deletion or other mechanisms commonly occurs in (1) PTEN in breast, ovarian, and endometrial cancers, and (2) p53 in HER2/neu positive breast cancers, triple negative breast cancers, and BRCA-associated breast and ovarian cancers. PI3K amplifications and activating mutations are common in breast and endometrial cancers [31, 32] and Ras activating mutations are common in endometrial cancers. Several genes such as AKT and STAT3 are often expressed at high activities in all of these cancers but without detectable amplifications of those genes. Epigenetic alterations, such as methylation of cytosine residues in CpG dinucleotides, can bring about the inactivation of genes (p16 gene in breast cancers) while mismatch repair defects have been observed to enhance the mutation rate of many genes in endometrial cancers. In addition to those somatic mutations discussed here, a large number of mutations in many oncogenes and tumor suppressor genes have been observed at lower rates in women's cancers.

Large copy number variations in genetic loci from tumor tissues have been observed using fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH), and a reduced heterozygosity of single nucleotide polymorphisms over large regions of a chromosome. This type of genomic instability has been observed at many loci in all chromosomes in some breast tumors. Other breast tumors demonstrate little or no genomic instability (below the level of detection). As a generalization those individuals who have tumors that demonstrate very high levels of genomic instability have a poorer prognosis [33]. While some loci are repeatedly amplified, as occurs in Her2 overexpressing breast cancers, or deleted, as with PTEN in endometrial cancers, the heterogeneity of mutations in women's cancers is striking. There are many mutational paths to initiate and propagate a tumor.

Notably however, somatic mutations often occur in genes where germline mutations in those same genes are the etiologic factors in cancer susceptibility syndromes. Alternatively, somatic mutations occur in other genes involved in regulatory aspects of those vital pathways. Despite the number of mutational pathways to initiate and propagate tumors, several specific genomic alterations are associated with particular breast cancer phenotypes. These phenotypes are manifested in their molecular profile, biology, and prognosis. Patterns of transcriptional profiles obtained from breast tumors have permitted a fairly reproducible classification of breast cancers that are derived from different cell types or have evolved under the influence of different gene expression patterns [34–38]. These different transcriptional patterns correlate well with critical diagnostic criteria (ER+, PR+, HER-2/neu+, triple negative, BRCA1) that guide both diagnosis and treatment protocols for these types of breast cancers. The classification also correlates well with some mutations such as p53, but other causal mutations such as cyclin D and WIP1 amplifications, PI3K and STAT3 activations need to be explored. Classification based upon transcriptional profiles also associates well with several clinical parameters. For example, luminal A cancers are hormone receptor positive, are diagnosed primarily in older women, are low grade with low proliferative index, and have mainly wildtype p53 [35, 38]. Luminal B cancers also tend to retain wildtype p53 but have reduced or absent expression of progesterone receptor and are more likely to recur than luminal A cancers [35, 36, 38]. In contrast to luminal tumors, basal cancers are hormone receptor negative and Her2 negative, are more likely to be diagnosed in young, premenopausal women, are high grade with high proliferative index, and are associated with higher risk of recurrence [35–38]. Her2-amplified breast cancers, regardless of hormone receptor status, are of higher grade and proliferative index, have worse prognosis with higher recurrences in first five years after diagnosis, and commonly have p53 mutations [35, 36]. Like basal tumors, BRCA1-associated breast cancers predominantly occur in young, premenopausal women, are primarily hormone receptor negative, and the most likely to carry p53 mutations [34]. Unfortunately this type of detail and analysis does not yet exist for ovarian and endometrial cancers.

Thus, it is now clear that there are at least five types of breast cancer with characteristic transcriptional profiles that can harbor some subset of mutations that drive these cancers [39]. Importantly, each type of breast cancer calls for different treatment protocols and often results in different

outcomes. We have only partially established the critical mutational patterns in each type of breast cancer and we have only begun to extend this type of analysis to other women's cancers. However, it is apparent that breast cancer heterogeneity reflects underlying genomic alterations leading to different biology and phenotypes.

3. Single Nucleotide Polymorphisms (SNPs) and Their Phenotypes

Inherited mutations in genes involved in DNA repair processes (BRCA1, BRCA2), cell cycle checkpoints and apoptosis (p53 , Rb), and gene products that regulate critical pathways (PTEN) clearly play a central role in predetermining the initiation of cancers, often with an incomplete penetrance. Polymorphic alleles in many additional genes, often in these same signal transduction pathways, can also contribute, albeit in a smaller quantitative fashion, to the origins of a cancer, the propagation of a cancer, and the treatment responses of a cancer. By definition a mutation in a gene occurs rarely in a population (below 1% of the population under study) while a polymorphism occurs more commonly. Because these polymorphic alleles can act cooperatively and many genes in the same signal transduction pathway can show epistatic relationships, single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) can have observable impact upon the incidence of a cancer in a defined population, the age of onset of a cancer, the response to treatment, the frequency of relapse, and the overall survival of a patient population. Thus in addition to inherited mutations, SNPs and CNVs in a population provide a genetic background that can influence the cancer cells harboring the inherited and somatic mutations that arise and cause a tumor. The phenotypes observed in people with inherited mutations in cancer causing genes are an increased incidence of cancers in a family or population and an earlier age of onset of a cancer than observed in the total population. Mutations in the p53 gene show this pattern and in addition multiple independent cancers in the same individual can be observed [3, 12]. Inherited mutations also often produce a limited set or tissue type of cancer such as BRCA1 or BRCA2 with breast and ovarian tumors [3]. It is thought that the BRCA1 and BRCA2 proteins function in many tissues to repair DNA damage, so the limited cancer causation to breast and ovary remains a mystery. Indeed all of the tumor suppressor genes demonstrate a tissue preference in the tumors they cause when they function as inherited alleles but somatic mutations in those same genes are often found in a much wider group of cancers of different tissues [40]. SNPs and CNVs will likely also have limited tissue impact upon cancers and like inherited mutations, functioning throughout development and life, can have cumulative impact over a lifetime.

The possible role of a SNP or an CNV in cancer is usually demonstrated by an association study correlating the presence of an "at-risk" allele with the incidence of a cancer or a related phenotype. This is fundamentally a statistical argument that provides correlation, not causality. The situation gets better if the at-risk allele can be shown, in vitro or in vivo, to have a different level or activity that could lead to the population wide phenotype. Examples of these correlations are now being demonstrated for results from GWAS and are taking into account tumor subtypes [41]. Thus molecular and cellular studies can provide an important rational for the population study results. In some cases it may also be possible to model the at-risk allele in another genetic system, such as a mouse carrying the alternate human alleles in the orthologous gene of the mouse. One would then explore the phenotypes observed in humans using such a mouse model. In this way it may be possible to move from correlation to causality. In the process of these studies one may learn about the details of the properties of an SNP or CNV that enlightens the population studies. A very good example of this is an SNP, SNP309, in the first intron of the MDM2 gene in humans. The MDM2 protein is a ubiquitin ligase which negatively regulates p53 levels in a cell by polyubiquitination of the p53 protein followed by its degradation. Thus MDM2 levels and activity in a cell regulate the p53 protein levels in a cell. SNP309 in the MDM2 gene comes in two forms, a G-allele and a T-allele. The first intron of the MDM2 gene contains sites for transcription factors that regulate the levels of the MDM2 mRNA. The G-allele binds a transcription factor, Sp-1, better than the T-allele [42]. Ten base pairs away from this Sp-1 site is an ER binding site and the Sp-1 and ER transcription factors can interact so that the highest levels of MDM2 mRNA and protein are produced in cells that are G/G homozygotes and ER+ and exposed to estrogen as shown in breast cancer cells in culture. Indeed the association of the G-allele of SNP 309 with an early age of onset of a cancer is most commonly observed in premenopausal women with ER+ tumors [43]. Thus if one analyzes all women with breast cancers for an association with the presence of the "at-risk" allele of this SNP the statistical test for an association commonly fails. Only when the association is tested with premenopausal females with ER+ tumors can a clear association be found. This is a good example of understanding the biology and genetics before one undertakes large association studies.

The human genome of any individual contains about three million SNPs that distinguish that person from another. In the population of humans there are an estimated fifty million SNPs. Most of these differences have no detectable phenotype. Because of this, large genome wide scans (GWAS) of SNPs now employ a million SNPs to test for an association with a disease [44]. This is clearly an exercise in multiple hypothesis testing and so one requires very large populations of cases (and controls) and a statistical significance (a type 1 error rate) that provides a $P = 10^{-7}$ value. Even then the number of false positives can be large and so repeated independent studies are required to refine the truly significant associations. This is in part why many SNP associations with cancers have been so poorly reproducible. Small study populations will often give lots of false positives not observed in independent repeat studies. In case-control studies where one is examining the different allele frequencies in a case and a control

group there is presently no mathematical test to prove that these two populations are equivalent. Allele frequencies can differ in racial groups or other populations and while it is easy to control for some parameters we do not know all of the variables. In spite of these difficulties several recent publications employing GWAS approaches with large populations have been reported for associations with breast cancers, colorectal cancers, lung cancers, melanomas and prostate cancers [17, 22, 24, 25, 45–51].

A different approach to uncovering active SNPs with associations to cancers is to examine a small number of candidate genes for the presence of SNPs that impact upon a phenotype. The criticism of this approach is that novel genes and SNPs that impact upon a cancer will not be discovered by this approach. Rather it is a chance to delve deeper into the diversity and properties of a gene, its protein and its phenotypes in a population. The most likely candidate genes that have functional SNPs are the ones that have mutations in some cancers or provide an inherited basis for cancer when they are mutated. Because SNPs are expected to be less deleterious than a mutation which inactivates or fully activates a function, it is helpful to look for candidate SNPs in genes that demonstrate haploinsufficiency. This encompasses those genes that have a cancer-related phenotype when an individual has only one wild type allele, and presumably half the activity and level of a protein. Interestingly the p53 gene, MDM2 gene, and the MDM4 gene (a second negative regulator of p53 that acts upon MDM2) are all haploinsufficient genes in mice and p53 is haploinsufficient in humans (there is presently no test for MDM2 or MDM4 haploinsufficiency in humans) [52, 53]. These three proteins make up the core of p53 regulatory activities in a cell (see Figure 1). There is a great deal of evidence demonstrating that the levels and/or activities of these proteins in a cell are tightly controlled by extensive feedback loops and that small changes in these proteins have phenotypes that are readily observed.

Another way to look for SNPs that have biological activity is to examine whether a mutant allele or a polymorphic allele is under negative or positive selection in a population. If that is the case then that allele must have a biological activity that impacts upon the organism. There are now a growing number of methods to look for regions of a genetic locus under positive or negative selection. Selection pressures in humans commonly result from genes that contribute to resistance to infectious diseases (about 20% of human cancers are caused by or associated with viruses), optimal use of nutritional opportunities (the IGF-PI3K-PTEN-mTOR pathways help to regulate this), or the highest levels of fecundity, leaving more offspring in a population (the p53 pathway can participate in this and is discussed below). Employing information theory-entropy based methods Atwal et al. have suggested that some alleles of MDM2 and MDM4 are under positive selective pressures in Caucasian populations [54, 55]. Based on identification of selected loci in MDM4, studies have now demonstrated associations between MDM4 SNP loci with risk of breast and ovarian cancers as well as age of onset of ovarian cancers and hormone receptor negative breast cancers [55, 56].

There is also evidence that an allele in the coding region of the p53 protein may also be under positive selective pressure in Caucasian populations. This SNP at codon 72 in the p53 protein (out of 393 amino acids) either encodes an arginine (Arg) or a proline (Pro) residue. The Pro-allele is the ancestral form and Africans near the equator have very high levels of the Pro-allele. As populations move to northern latitudes in Europe (Caucasians) and in Asia (Asians) there is an increasing frequency of the Arg-allele reaching 75–85% in Scandinavia. One explanation for this distribution comes from the observation that p53 induces the synthesis of pro-opiomelanocortin which regulates the tanning response. This could be thought of as a protective mechanism for light-skinned populations or helping to protect individuals of lighter skin color which was developed to enhance the production of vitamin D in northern climates. In a recent study in China, a correlation was found that implicates both temperature and ultra-violet light sources as the driving forces upon selection of the p53 Arg/Pro and SNP309 polymorphisms [57].

There is a growing body of evidence that the newly formed and selected Arg-allele in Caucasian and Asian populations has quite different properties then the Pro-allele. Cells in culture with the Arg-allele transcribe several pro-apoptotic genes at higher rates than the same cell lines with Pro-alleles. Several studies have demonstrated that cells with the Arg-allele undergo higher frequencies of apoptosis than the same cells with Pro-alleles. A deletion of the p53 protein proline rich domain, in which the Arg/Pro polymorphism resides, reduces the efficiency of apoptosis by that mutant p53 protein. These studies demonstrate at the cellular and molecular level that functional differences exist between these two alleles of the p53 gene [58–61].

Perhaps the best explanation for the selection of the p53 Arg allele in Caucasian populations is the observation that the cytokine leukemia inhibitory factor (LIF) is regulated at the transcriptional level by p53 and two times more LIF is produced in cells by the Arg-allele than the Pro-allele of p53 [62]. p53-mediated production of LIF in the uterus is required for implantation of mouse embryos after fertilization (LIF is also produced in humans for implantation) and so both p53 and LIF are required for high levels of fecundity [63]. Interestingly, the frequency of the p53 Pro-allele is quite enriched in women who are at an in vitro fertilization clinic and demonstrate lower levels of implantation of fertilized eggs [62]. This observation may explain the selective pressures on these alleles in Caucasians. Obviously there are not similar fertility difficulties in Africans with the Pro-allele, suggesting that the genetic background (other alleles in genes in the p53 pathway) is an important factor for this phenotype. Poor fertility was observed in p53 knockout mice and varied in different genetic backgrounds [63]. Some of the compensating MDM2 and MDM4 alleles are also under selection pressures in Caucasian populations. It has been these types of studies that have identified functional SNPs that can now be tested for their activities and associations with specific cancers.

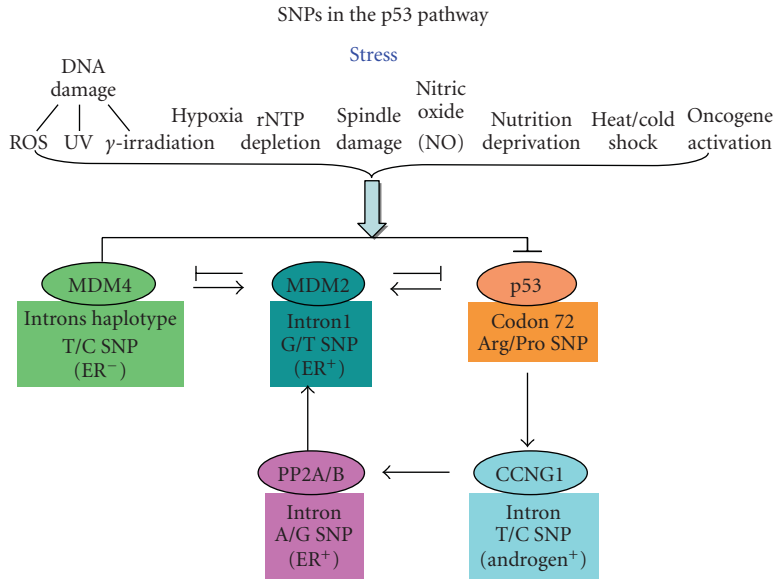


Figure 1

4. The p53 Pathway and Cancer Prevention

The p53 protein and its signal transduction pathway respond to a wide variety of stresses and act as a fidelity check point preventing mistakes leading to high mutation rates. Cellular stresses such as DNA damage, telomere shortening, hypoxia, nutrient deprivation, an interruption of ribosome biogenesis, errors in proper mitotic spindle functions, or even the mutational activation of selected oncogenes (myc, Ras) can activate the p53 protein so that it becomes an efficient transcription factor for selected genes. A wide variety of protein kinases, histone methylases, ubiquitin ligases, and so forth participate in detecting these stress signals and modifying the p53, MDM2, or MDM4 proteins. This results in shutting down the MDM2/4 negative regulation of p53, an increased half-life, and an increased concentration of the p53 protein in the cell (Figure 1). Higher levels of a modified p53 protein then give rise to a transcriptional response of p53 regulated genes. The most common outcomes of this signal transduction pathway are apoptosis, cellular senescence, or cell cycle arrest. Because these stresses upon a cell can cause a very high error rate in both DNA replication and cell division, p53 blocks progression through the cell cycle or eliminates clones that contain mutational events. In this way p53 acts as a tumor suppressor gene over a lifetime of stressful events. The presence of only one wild type p53 allele in mice or humans (Li-Fraumeni syndrome) leads to an early onset of tumors compared to the wild type population and often leads to multiple independent tumors in an individual with almost a one hundred percent penetrance [64, 65]. Four independent studies have now shown that the G-allele (at-risk allele) of SNP309 in the MDM2 gene, which raises the levels of this mRNA and protein inhibiting p53 activity, lowers the age of onset of tumors and increases the number of independent tumors observed in individuals with Li-Fraumeni syndrome [42, 66–68]. Those individuals with Li-Fraumeni syndrome who do not have a p53 mutation (patients with a high frequency of tumors due to an unrelated mutation) are not affected by the SNP309 G-allele demonstrating the specificity and epistatic relationship between MDM2 and p53. Li-Fraumeni patients with a p53 mutation also have more rapid telomere erosion, demonstrating the role of p53 and SNP309 in this process [67]. It is well known that p53 senses the loss of telemetric DNA and will stop cell division or cause cellular apoptosis. One study examining both the p53 Arg/Pro and SNP309 polymorphisms suggested that the p53 Pro-allele can have an impact upon the age of onset of cancers (earlier) and survival (poorer) in patients with Li-Fraumeni syndrome and SNP309 can make the situation worse, but the number of patients with both genotypes was too small to obtain a statistically confident result [68].

5. SNPs in the p53 Pathway Associated with Breast and Ovarian Cancers

The literature examining the association of SNPs in genes in the p53 pathway is fraught with contradictions. Undoubtedly, this comes about for several reasons. First, studies involving small population sizes do not necessarily provide adequate statistical power. Second, studies may fail to stratify populations into groups that reflect the biology and clinical impacts of a cancer. For example, MDM2 SNP309, which acts preferentially in premenopausal females with ER+ tumors, is a smaller group in the total cohort. Third, there is a failure to understand that SNPs in different genes may have very different phenotypes in the context of a cancer (tissue specificity, the age of onset phenotype, etc.). Finally, there is a tendency to make comparisons of cases and controls that are not biologically or genetically equivalent.

At present, the structure of many association studies leads to false positives and negatives as well as uncovering an occasional functional SNP. For example, a very large population of women with breast cancer was analyzed in a GWAS but the patients were not stratified by ER status or for any of the clear differences between different types of breast cancers [22]. Clearly, this will dilute any signal that comes from just one of these cancer types. In addition, when SNPs in tumor suppressor genes or oncogenes are under study it may be the case that a mutation in that gene will eliminate the SNP from being identified or all SNPs in genes epistatic to the mutated gene may no longer score in the association study. Because of these difficulties we must rely upon the independent replication of results as well as a functional explanation for how an SNP is acting to uncover an association. In a formal large meta-analysis of published results from the literaturevan Heemst et al. [69] studied the impact of p53 Pro/Pro and Arg/Arg polymorphisms upon the frequency of developing cancers and upon the longevity of the population under study. They found that individuals with a Pro/Pro genotype had an increased risk of developing a cancer over their lifetimes when compared to individuals with an Arg/Arg genotype. In a prospective study of individuals 85 years and older, carried out with 1226 people over a ten-year period, they found that people with the Pro/Pro genotype had a 2.45 increased proportional mortality from cancer ($P = .007$). But this group also showed a longer longevity (a 41% increased survival in the population, $P = .032$). One interpretation of this result is that the Arg/Arg genotype has a higher apoptotic rate in response to stress and so protects against cancer better, but also kills stem cells more efficiently over a lifetime, reducing longevity [69]. This suggests that studying older patients may reveal a phenotype in this p53 SNP because it acts over a lifetime to protect the host from stresses. By the same token, older mice show declines in p53 activities with age and this lower level of p53 responses could uncover a phenotype at older ages that is too robust to measure in younger groups [70]. If this interpretation is correct one can see why the p53 Arg/Pro SNP has given rise to such contradictory responses when the ages of the case and control groups are not taken into account.

Similarly, studies with MDM2 SNP309 have produced contradictory associations with cancers. Some of this has to do with mixing both males and females into the cohort under study (SNP309 is regulated by the ER), some of this has to do with a failure to separate ER+ and ER− tumors in the analysis, and some studies just choose the wrong phenotype or cohort to measure. For example, the observation that the G-allele (the at-risk allele) of SNP309 is associated with an earlier age of onset in a variety of cancers has now been reproduced by many independent groups employing soft tissue sarcomas [43], lymphomas [43], leukemia [71], melanomas [72], head neck [73] and oral squamous cell carcinomas [74], gastric cancer [75], colon cancers [76, 77], lung cancers [78–80], endometrial cancer [81, 82], bladder cancers [83, 84], glioblastoma [85], neuroblastoma [86], and both breast cancers [43] and ovarian [87] cancers. In a study of lung cancers, Lind and her colleagues [78] found that G/G homozygotes had a 1.62 odds ratio of developing cancer compared with T/T homozygotes. When only females in the study were considered the G/G to T/T odds ratio was 4.06. This is the same result observed in an independent study with large diffuse B-cell lymphomas where the G-allele of SNP309 was associated with cancer only in premenopausal females and not in postmenopausal females nor in men [43].

In a third recent study of melanomas, a similar association was found only in premenopausal females [72]. This pattern suggests that active estrogen receptors are present in a large number of tumors and can affect the outcome of the disease. This set of genetic observations opens up possible new routes for therapy with these tumors. In another large study with lung cancer patients carried out in China by Zhang et al. [80] they demonstrated an odds ratio of 1.83 for the G/G over T/T alleles and a 1.47 fold odds ratio for the p53 Pro/Pro over Arg/Arg individuals. Those patients who were G/G and Pro/Pro had an odds ratio of 4.36; whereas those smokers (a mutagenic stress that activates p53) who were Pro/Pro, G/G had an odds ratio of 10.41. Understanding the biology of the signal transduction pathway can permit one to study the relevant variables. Often combinations of SNPs that have an epistatic relationship can provide much more significant results.

Recently, biologically functional SNPs were detected in the MDM4 gene that appear to be under evolutionary selection pressures and have an impact upon fecundity in females at an IVF clinic [62, 63]. Association studies employing five different patient populations have indicated that selected alleles of these SNPs confer an increased risk for or early onset of breast cancers and ovarian cancers [55, 56]. The ethnic backgrounds of the cohorts under study made a difference in the ability to detect these associations so that once again a genetic background of other SNPs that reside in other genes in the same pathway could play an important role. The minor alleles of these same MDM4 SNPs demonstrated a clear enrichment in their frequencies in women at an IVF clinic who had difficulties with implantation of embryos. These diverse phenotypes suggest a functional consequence of these SNPs that can be selected for or against over recent (Caucasian and Asian) times of human evolution. It will be important to observe replications of these data in a wide variety of cancers.

Because many of the cancer treatments result in DNA damage and other stresses to a cell, the response (as determined by a combination of SNPs) to treatment and long-term survival could depend upon the combination of alleles in the p53 pathway SNPs. To test this notion association studies will have to assemble large groups of individuals who have experienced a defined cancer and treatment and record the outcomes over many years. Such cohorts are more difficult to assemble but are an important part of this effort. Characterizing the patient and the tumor genome prior to the selection of treatments is a growing concern and would be aided by the use of validated SNPs and mutations.

6. Limitations with SNP Studies

The success of these studies has been in part due to the use of large study samples (usually based around multicenter consortia) and replication data sets that have been designed into the gene discovery algorithm. Although this approach maximizes the identification of possible genes involved in contributing to breast cancers, these studies often give rise to a number of false positive findings due to multiple hypothesis testing with a very large number of SNPs in the GWAS scans. In addition, the GWAS approach tends to optimize the discovery of genes with statistically significant marginal effects. Therefore, it may miss significant genes. First, genes may not be detected whose effects are not significant on the margin but are significant in conjunction with other genes or exposures. Second, genes may not be identified if there is substantial genetic heterogeneity among cases, such that the proportion of individuals in the population whose disease is caused by a gene is so small that its effect is "washed out" in the total sample. Third, GWASs tend to favor large numbers over epidemiologically rigorous study designs. Although large samples are clearly required to detect small effects, and replication/validation of initial results maximizes the chances that reported associations are true positives, it is possible that unmeasured biases due to study design limitations may have resulted in high false negatives.

Likewise, meta-analyses, used to critically evaluate and statistically combine studies, have been performed for MDM2 SNP309. However, a caveat to such an analysis is that the studies are comparable. Population-specific effects and SNP functionality in independent racial genetic backgrounds may exist and limit the ability to combine heterogeneous study groups. To emphasize this, Shi et al. describe a causative selection of MDM2 SNP309 and p53 Arg72 associated with environmental stresses, that is, cold winter temperatures and UV intensity, wherein the two SNPs are not coselected [57]. This is further supported by two publications describing population-specific differences between African-Americans, Caucasians, and Caucasians of Ashkenazi Jewish descent for both MDM2 SNP309 and MDM4 haplotypes [54, 55]. A combined analysis for SNP309 was presented in Wilkening et al. [88]. Data from eleven breast cancer studies, five colorectal cancer studies, or seven lung cancer studies were each combined for a fixed meta-analysis. Based on their analysis, they concluded that the SNP309 variant did not have an impact on risk or colorectal cancers, but did exhibit increased risk in the homozygous state for lung cancer. They concluded that SNP309 alone has little effect on the risk of common cancers. In reviewing criteria of studies within the analysis, there is significant heterogeneity between study groups. Other studies have also previously concluded that the effects of SNP309 are evident in women but not in men and in hormone receptor-positive breast cancers [43]. Most studies do not differentiate between gender or hormone receptor positive diseases, both of which may dilute any effects. In contrast to the conclusions made by Wilkening et al. [88], those of Hu et al. [89] are that the homozygous variant is associated with increased risk of all types of tumors where tumor type and ethnicity contributed to substantial heterogeneity. The latter publication by Hu et al. [89] describes in detail methods for identifying appropriate studies, data extraction and analysis. The majority of publications fail to reduce heterogeneity in their populations based on molecular markers, gender, and degree of disease heterogeneity. Therefore, a meta-analysis would be limited by these factors and should be interpreted with caution.

The majority of useful and reproducible reports involving SNP associations with breast, ovarian, and endometrial cancers have occurred in the context of candidate gene studies. These studies typically identify loci that are hypothesized to contain genetic variants that may be associated with disease risk. Using this approach, a number of putative susceptibility genes have been identified for these tumor sites that have been validated. Furthermore, candidate gene studies have tended to have used more rigorous study designs, collected useful epidemiological and confounder data, and have detailed information that may define etiologically heterogeneous groups of individuals that may provide a setting in which genes that are not easily detectable in the usual GWAS setting may be found. This comes about because we have a great deal of information about those genes already known to play a role in cancer causation that modifies the questions we ask and the associations we look for in a study. It appears that both the GWAS algorithm and the candidate gene algorithm may have value in identifying susceptibility genes, and these genes may be different because of the strengths and weaknesses of each approach.

References

[1] A. C. Antoniou and D. F. Easton, "Models of genetic susceptibility to breast cancer," *Oncogene*, vol. 25, no. 43, pp. 5898–5905, 2006.

[2] D. Ford, D. F. Easton, M. Stratton, et al., "Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families," *American Journal of Human Genetics*, vol. 62, no. 3, pp. 676–689, 1998.

[3] J. E. Garber and K. Offit, "Hereditary cancer predisposition syndromes," *Journal of Clinical Oncology*, vol. 23, no. 2, pp. 276–292, 2005.

[4] A. R. Venkitaraman, "Functions of BRCA1 and BRCA2 in the biological response to DNA damage," *Journal of Cell Science*, vol. 114, no. 20, pp. 3591–3598, 2001.

[5] L. M. Starita, A. A. Horwitz, M.-C. Keogh, C. Ishioka, J. D. Parvin, and N. Chiba, "BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II," *Journal of Biological Chemistry*, vol. 280, no. 26, pp. 24498–24505, 2005.

[6] M. S. Greenblatt, P. O. Chappuis, J. P. Bond, N. Hamel, and W. D. Foulkes, "TP53 mutations in breast cancer associated with BRCA1 or BRCA2 germ-line mutations: distinctive spectrum and structural distribution," *Cancer Research*, vol. 61, no. 10, pp. 4092–4097, 2001.

[7] R. Pilarski, "Cowden syndrome: a critical review of the clinical literature," *Journal of Genetic Counseling*, vol. 18, no. 1, pp. 13–27, 2009.

[8] C. Eng, "Role of PTEN, a lipid phosphatase upstream effector of protein kinase B, in epithelial thyroid carcinogenesis," *Annals of the New York Academy of Sciences*, vol. 968, pp. 213–221, 2002.

[9] J. I. Risinger, A. K. Hayes, A. Berchuck, and J. C. Barrett, "PTEN/MMAC1 mutations in endometrial cancers," *Cancer Research*, vol. 57, no. 21, pp. 4736–4738, 1997.

[10] I. U. Ali, "Gatekeeper for endometrium: the PTEN tumor suppressor gene," *Journal of the National Cancer Institute*, vol. 92, no. 11, pp. 861–863, 2000.

[11] W. Lim, N. Hearle, B. Shah, et al., "Further observations on LKB1/STK11 status and cancer risk in Peutz-Jeghers syndrome," *British Journal of Cancer*, vol. 89, no. 2, pp. 308–313, 2003.

[12] D. Malkin, F. P. Li, L. C. Strong, et al., "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms," *Science*, vol. 250, no. 4985, pp. 1233–1238, 1990.

[13] H. Nevanlinna and J. Bartek, "The CHEK2 gene and inherited breast cancer susceptibility," *Oncogene*, vol. 25, no. 43, pp. 5912–5919, 2006.

[14] D. L. Stredrick, M. Garcia-Closas, M. A. Pineda, et al., "The ATM missense mutation p.Ser49Cys (c.146C>G) and the risk of breast cancer," *Human Mutation*, vol. 27, no. 6, pp. 538–544, 2006.

[15] S. Seal, D. Thompson, A. Renwick, et al., "Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles," *Nature Genetics*, vol. 38, no. 11, pp. 1239–1241, 2006.

[16] N. Rahman, S. Seal, D. Thompson, et al., "PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene," *Nature Genetics*, vol. 39, no. 2, pp. 165–167, 2006.

[17] D. F. Easton and R. A. Eeles, "Genome-wide association studies in cancer," *Human Molecular Genetics*, vol. 17, no. R2, pp. R109–R115, 2008.

[18] K. Hemminki, A. Försti, and J. L. Bermejo, "New cancer susceptibility loci: population and familial risks," *International Journal of Cancer*, vol. 123, no. 7, pp. 1726–1729, 2008.

[19] K. Hemminki, J. Sundquist, and J. L. Bermejo, "How common is familial cancer?" *Annals of Oncology*, vol. 19, no. 1, pp. 163–167, 2008.

[20] P. D. P. Pharoah, A. C. Antoniou, D. F. Easton, and B. A. J. Ponder, "Polygenes, risk prediction, and targeted prevention of breast cancer," *The New England Journal of Medicine*, vol. 358, no. 26, pp. 2796–2803, 2008.

[21] P. D. Pharoah, J. Tyrer, A. M. Dunning, D. F. Easton, and B. A. Ponder, "Association between common variation in 120 candidate genes and breast cancer risk," *PLoS Genetics*, vol. 3, no. 3, article e42, 2007.

[22] D. J. Hunter, P. Kraft, K. B. Jacobs, et al., "A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer," *Nature Genetics*, vol. 39, no. 7, pp. 870–874, 2007.

[23] S. N. Stacey, A. Manolescu, P. Sulem, et al., "Common variants on chromosome 5p12 confer susceptibility to estrogen receptor-positive breast cancer," *Nature Genetics*, vol. 40, no. 6, pp. 703–706, 2008.

[24] S. N. Stacey, A. Manolescu, P. Sulem, et al., "Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer," *Nature Genetics*, vol. 39, no. 7, pp. 865–869, 2007.

[25] D. F. Easton, K. A. Pooley, A. M. Dunning, et al., "Genome-wide association study identifies novel breast cancer susceptibility loci," *Nature*, vol. 447, no. 7148, pp. 1087–1093, 2007.

[26] M. Udler and P. D. P. Pharoah, "Germline genetic variation and breast cancer survival: prognostic and therapeutic implications," *Future Oncology*, vol. 3, no. 5, pp. 491–495, 2007.

[27] H. Song, S. J. Ramus, S. K. Kjaer, et al., "Association between invasive ovarian cancer susceptibility and 11 best candidate SNPs from breast cancer genome-wide association study," *Human Molecular Genetics*, vol. 18, no. 12, pp. 2297–2304, 2009.

[28] E. Y. Cho, Y.-L. Choi, J. J. Han, K.-M. Kim, and Y. L. Oh, "Expression and amplification of Her2, EGFR and cyclin D1 in breast cancer: immunohistochemistry and chromogenic in situ hybridization," *Pathology International*, vol. 58, no. 1, pp. 17–25, 2008.

[29] S. Elsheikh, A. R. Green, M. A. Aleskandarany, et al., "CCND1 amplification and cyclin D1 expression in breast cancer and their relation with proteomic subgroups and patient outcome," *Breast Cancer Research and Treatment*, vol. 109, no. 2, pp. 325–335, 2008.

[30] E. Yu, Y. S. Ahn, S. J. Jang, et al., "Overexpression of the wip1 gene abrogates the p38 MAPK/p53/Wip1 pathway and silences p16 expression in human breast cancers," *Breast Cancer Research and Treatment*, vol. 101, no. 3, pp. 269–278, 2007.

[31] K. Stemke-Hale, A.M. Gonzalez-Angulo, A. Lluch, et al., "An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer," *Cancer Research*, vol. 68, no. 15, pp. 6084–6091, 2008.

[32] M. Ollikainen, A. Gylling, M. Puputti, et al., "Patterns of PIK3CA alterations in familial colorectal and endometrial carcinoma," *International Journal of Cancer*, vol. 121, no. 4, pp. 915–920, 2007.

[33] J. K. Habermann, J. Doering, S. Hautaniemi, et al., "The gene expression signature of genomic instability in breast cancer is an independent predictor of clinical outcome," *International Journal of Cancer*, vol. 124, no. 7, pp. 1552–1564, 2009.

[34] J. Palacios, M. J. Robles-Frias, M. A. Castilla, M. A. Lopez-Garcia, and J. Benitez, "The molecular pathology of hereditary breast cancer," *Pathobiology*, vol. 75, no. 2, pp. 85–94, 2008.

[35] R. Bhargava, J. Striebel, S. Beriwal, et al., "Prevalence, morphologic features and proliferation indices of breast carcinoma molecular classes using immunohistochemical surrogate markers," *International Journal of Clinical and Experimental Pathology*, vol. 2, no. 5, pp. 444–455, 2009.

[36] A. Spitale, P. Mazzola, D. Soldini, L. Mazzucchelli, and A. Bordoni, "Breast cancer classification according to immunohistochemical markers: clinicopathologic features and short-term survival analysis in a population-based study from the South of Switzerland," *Annals of Oncology*, vol. 20, no. 4, pp. 628–635, 2009.

[37] M. C. U. Cheang, D. Voduc, C. Bajdik, et al., "Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype," *Clinical Cancer Research*, vol. 14, no. 5, pp. 1368–1376, 2008.

[38] F. Bertucci, P. Finetti, N. Cervera, et al., "How different are luminal A and basal breast cancers?" *International Journal of Cancer*, vol. 124, no. 6, pp. 1338–1348, 2009.

[39] T. Sorlie, C. M. Perou, R. Tibshirani, et al., "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 19, pp. 10869–10874, 2001.

[40] L. D. Wood, D. W. Parsons, S. Jones, et al., "The genomic landscapes of human breast and colorectal cancers," *Science*, vol. 318, no. 5853, pp. 1108–1113, 2007.

[41] S. H. Nordgard, F. E. Johansen, G. I. Alnaes, and B. Naume, "Genes harbouring susceptibility SNPs are differentially expressed in the breast cancer subtypes," *Breast Cancer Research*, vol. 9, no. 6, p. 113, 2007.

[42] G. L. Bond, W. Hu, E. E. Bond, et al., "A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans," *Cell*, vol. 119, no. 5, pp. 591–602, 2004.

[43] G. L. Bond, K. M. Hirshfield, T. Kirchhoff, et al., "MDM2 SNP309 accelerates tumor formation in a gender-specific and hormone-dependent manner," *Cancer Research*, vol. 66, no. 10, pp. 5104–5110, 2006.

[44] J. P. A. Ioannidis, G. Thomas, and M. J. Daly, "Validating, augmenting and refining genome-wide association signals," *Nature Reviews Genetics*, vol. 10, no. 5, pp. 318–329, 2009.

[45] G. Thomas, K. B. Jacobs, M. Yeager, et al., "Multiple loci identified in a genome-wide association study of prostate cancer," *Nature Genetics*, vol. 40, no. 3, pp. 310–315, 2008.

[46] C. I. Amos, X. Wu, P. Broderick, et al., "Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1," *Nature Genetics*, vol. 40, no. 5, pp. 616–622, 2008.

[47] R. J. Hung, J. D. McKay, V. Gaborieau, et al., "A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25," *Nature*, vol. 452, no. 7187, pp. 633–637, 2008.

[48] K. M. Brown, S. MacGregor, G. W. Montgomery, et al., "Common sequence variants on 20q11.22 confer melanoma susceptibility," *Nature Genetics*, vol. 40, no. 7, pp. 838–840, 2008.

[49] P. Broderick, L. Carvajal-Carmona, A. M. Pittman, et al., "A genome-wide association study shows that common alleles of SMAD7 influence colorectal cancer risk," *Nature Genetics*, vol. 39, no. 11, pp. 1315–1317, 2007.

[50] I. P. M. Tomlinson, E. Webb, L. Carvajal-Carmona, et al., "A genome-wide association study identifies colorectal cancer susceptibility loci on chromosomes 10p14 and 8q23.3," *Nature Genetics*, vol. 40, no. 5, pp. 623–630, 2008.

[51] A. Tenesa, S. M. Farrington, J. G. D. Prendergast, et al., "Genome-wide association scan identifies a colorectal cancer susceptibility locus on 11q23 and replicates risk loci at 8q24 and 18q21," *Nature Genetics*, vol. 40, no. 5, pp. 631–637, 2008.

[52] T. Terzian, Y. Wang, C. S. Van Pelt, N. F. Box, E. L. Travis, and G. Lozano, "Haploinsufficiency of Mdm2 and Mdm4 in tumorigenesis and development," *Molecular and Cellular Biology*, vol. 27, no. 15, pp. 5479–5485, 2007.

[53] J. M. Parant and G. Lozano, "Disrupting TP53 in mouse models of human cancers," *Human Mutation*, vol. 21, no. 3, pp. 321–326, 2003.

[54] G. S. Atwal, G. L. Bond, S. Metsuyanim, et al., "Haplotype structure and selection of the MDM2 oncogene in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 11, pp. 4524–4529, 2007.

[55] G. S. Atwal, T. Kirchhoff, E. E. Bond, et al., "Altered tumor formation and evolutionary selection of genetic variants in the human MDM4 oncogene," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 25, pp. 10236–10241, 2009.

[56] D. Kulkarni, A. Vazquez, B. G. Haffty, et al., "A polymorphic variant in human MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer," *Carcinogenesis*, vol. 30, no. 11, pp. 1910–1915, 2009.

[57] H. Shi, S.-j. Tan, H. Zhong, et al., "Winter temperature and UV are tightly linked to genetic changes in the p53 tumor suppressor pathway in Eastern Asia," *American Journal of Human Genetics*, vol. 84, no. 4, pp. 534–541, 2009.

[58] P. Dumont, J. I.-J. Leu, A. C. Della Pietra III, D. L. George, and M. Murphy, "The codon 72 polymorphic variants of p53 have markedly different apoptotic potential," *Nature Genetics*, vol. 33, no. 3, pp. 357–365, 2003.

[59] P. Sansone, G. Storci, S. Pandolfi, L. Montanaro, P. Chieco, and M. Bonafe, "The p53 codon 72 proline allele is endowed with enhanced cell-death inducing potential in cancer cells exposed to hypoxia," *British Journal of Cancer*, vol. 96, no. 8, pp. 1302–1308, 2007.

[60] M. Siddique and K. Sabapathy, "Trp53-dependent DNA-repair is affected by the codon 72 polymorphism," *Oncogene*, vol. 25, no. 25, pp. 3489–3500, 2006.

[61] M. Thomas, A. Kalita, S. Labrecque, D. Pim, L. Banks, and G. Matlashewski, "Two polymorphic variants of wild-type p53 differ biochemically and biologically," *Molecular and Cellular Biology*, vol. 19, no. 2, pp. 1092–1100, 1999.

[62] H.-J. Kang, Z. Feng, Y. Sun, et al., "Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 24, pp. 9761–9766, 2009.

[63] W. Hu, Z. Feng, A. K. Teresky, and A. J. Levine, "p53 regulates maternal reproduction through LIF," *Nature*, vol. 450, no. 7170, pp. 721–724, 2007.

[64] K. E. Nichols, D. Malkin, J. E. Garber, J. F. Fraumeni Jr., and F. P. Li, "Germ-line p53 mutations predispose to a wide spectrum of early-onset cancers," *Cancer Epidemiology Biomarkers and Prevention*, vol. 10, no. 2, pp. 83–87, 2001.

[65] A. Chompret, L. Brugieres, M. Ronsin, et al., "P53 germline mutations in childhood cancers and cancer risk for carrier individuals," *British Journal of Cancer*, vol. 82, no. 12, pp. 1932–1937, 2000.

[66] M. W. G. Ruijs, M. K. Schmidt, H. Nevanlinna, et al., "The single-nucleotide polymorphism 309 in the MDM2 gene contributes to the Li-Fraumeni syndrome and related phenotypes," *European Journal of Human Genetics*, vol. 15, no. 1, pp. 110–114, 2007.

[67] U. Tabori, S. Nanda, H. Druker, J. Lees, and D. Malkin, "Younger age of cancer initiation is associated with shorter telomere length in Li-Fraumeni syndrome," *Cancer Research*, vol. 67, no. 4, pp. 1415–1418, 2007.

[68] G. Bougeard, S. Baert-Desurmont, I. Tournier, et al., "Impact of the MDM2 SNP309 and p53 Arg72Pro polymorphism on age of tumour onset in Li-Fraumeni syndrome," *Journal of Medical Genetics*, vol. 43, no. 6, pp. 531–533, 2006.

[69] D. van Heemst, S. P. Mooijaart, M. Beekman, et al., "Variation in the human *TP53* gene affects old age survival and cancer mortality," *Experimental Gerontology*, vol. 40, no. 1-2, pp. 11–15, 2005.

[70] Z. Feng, W. Hu, A. K. Teresky, E. Hernando, C. Cordon-Cardo, and A. J. Levine, "Declining p53 function in the aging process: a possible mechanism for the increased tumor incidence in older populations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 42, pp. 16633–16638, 2007.

[71] X. Xiong, M. Wang, L. Wang, et al., "Risk of MDM2 SNP309 alone or in combination with the p53 codon 72 polymorphism in acute myeloid leukemia," *Leukemia Research*, vol. 33, no. 11, pp. 1454–1458, 2009.

[72] E. F. Firoz, M. Warycha, J. Zakrzewski, et al., "Association of MDM2 SNP309, age of onset, and gender in cutaneous melanoma," *Clinical Cancer Research*, vol. 15, no. 7, pp. 2573–2580, 2009.

[73] M. Nakashima, S. Kondo, Y. Shimizu, et al., "Impact of MDM2 single nucleotide polymorphism on tumor onset in head and neck squamous cell carcinoma," *Acta Oto-Laryngologica*, vol. 128, no. 7, pp. 808–813, 2008.

[74] S. Hamid, Y.-H. Yang, K. N. L. Peng, et al., "MDM2 SNP309 does not confer an increased risk to oral squamous cell carcinoma but may modulate the age of disease onset," *Oral Oncology*, vol. 45, no. 6, pp. 496–500, 2009.

[75] N. Ohmiya, A. Taguchi, N. Mabuchi, et al., "MDM2 promoter polymorphism is associated with both an increased susceptibility to gastric carcinoma and poor prognosis," *Journal of Clinical Oncology*, vol. 24, no. 27, pp. 4434–4440, 2006.

[76] C. Menin, M. C. Scaini, G. L. De Salvo, et al., "Association between MDM2-SNP309 and age at colorectal cancer diagnosis according to p53 mutation status," *Journal of the National Cancer Institute*, vol. 98, no. 4, pp. 285–288, 2006.

[77] G. L. Bond, C. Menin, R. Bertorelle, P. Alhopuro, L. A. Aaltonen, and A. J. Levine, "MDM2 SNP309 accelerates colorectal tumour formation in women," *Journal of Medical Genetics*, vol. 43, no. 12, pp. 950–952, 2006.

[78] H. Lind, S. Zienolddiny, P. O. Ekstrom, V. Skaug, and A. Haugen, "Association of a functional polymorphism in the promoter of the MDM2 gene with risk of nonsmall cell lung cancer," *International Journal of Cancer*, vol. 119, no. 3, pp. 718–721, 2006.

[79] G. Liu, P. Wheatley-Price, W. Zhou, et al., "Genetic polymorphisms of MDM2, cumulative cigarette smoking and nonsmall cell lung cancer risk," *International Journal of Cancer*, vol. 122, no. 4, pp. 915–918, 2008.

[80] X. Zhang, X. Miao, Y. Guo, et al., "Genetic polymorphisms in cell cycle regulatory genes MDM2 and TP53 are associated with susceptibility to lung cancer," *Human Mutation*, vol. 27, no. 1, pp. 110–117, 2006.

[81] K. Terry, M. McGrath, I.-M. Lee, J. Buring, and I. De Vivo, "MDM2 SNP309 is associated with endometrial cancer risk," *Cancer Epidemiology Biomarkers and Prevention*, vol. 17, no. 4, pp. 983–986, 2008.

[82] M. Ueda, M. Yamamoto, O. Nunobiki, et al., "Murine double-minute 2 homolog single nucleotide polymorphism 309 and the risk of gynecologic cancer," *Human Cell*, vol. 22, no. 2, pp. 49–54, 2009.

[83] M. Sanchez-Carbayo, N. D. Socci, T. Kirchoff, et al., "A polymorphismin HDM2 (SNP309) associates with early onset in superficial tumors, TP53 mutations, and poor outcome in invasive bladder cancer," *Clinical Cancer Research*, vol. 13, no. 11, pp. 3215–3220, 2007.

[84] Y. Horikawa, J. Nadaoka, M. Saito, et al., "Clinical implications of the MDM2 SNP309 and p53 Arg72Pro polymorphisms in transitional cell carcinoma of the bladder," *Oncology Reports*, vol. 20, no. 1, pp. 49–55, 2008.

[85] R. G. Khatri, K. Navaratne, and R. J. Weil, "The role of a single nucleotide polymorphism of MDM2 in glioblastoma multiforme: laboratory investigation," *Journal of Neurosurgery*, vol. 109, no. 5, pp. 842–848, 2008.

[86] S. Cattelani, R. Defferrari, S. Marsilio, et al., "Impact of a single nucleotide polymorphism in the MDM2 gene on neuroblastoma development and aggressiveness: results of a pilot study on 239 patients," *Clinical Cancer Research*, vol. 14, no. 11, pp. 3248–3253, 2008.

[87] F. Bartel, J. Jung, A. Bohnke, et al., "Both germ line and somatic genetics of the p53 pathway affect ovarian cancer incidence and survival," *Clinical Cancer Research*, vol. 14, no. 1, pp. 89–96, 2008.

[88] S. Wilkening, J. L. Bermejo, and K. Hemminki, "MDM2 SNP309 and cancer risk: a combined analysis," *Carcinogenesis*, vol. 28, no. 11, pp. 2262–2267, 2007.

[89] Z. Hu, G. Jin, L. Wang, F. Chen, X. Wang, and H. Shen, "MDM2 promoter polymorphism SNP309 contributes to tumor susceptibility: evidence from 21 case-control studies," *Cancer Epidemiology Biomarkers and Prevention*, vol. 16, no. 12, pp. 2717–2723, 2007.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 950201, 7 pages
doi:10.1155/2010/950201

Review Article

MicroRNAs as Novel Biomarkers for Breast Cancer

H. M. Heneghan, N. Miller, A. J. Lowery, K. J. Sweeney, and M. J. Kerin

Department of Surgery, Clinical Science Institute, National University of Ireland, Galway, Ireland

Correspondence should be addressed to H. M. Heneghan, helenheneghan@hotmail.com

Received 15 March 2009; Accepted 8 May 2009

Academic Editor: Ben Davidson

Breast cancer is a complex phenotypically diverse genetic disease, involving a variety of changes in gene expression and structure. Recent advances in molecular profiling technology have made great progress in unravelling the molecular taxonomy of breast cancer, which has shed new light on the aetiology of the disease and also heralded great potential for the development of novel biomarkers and therapeutic targets. Mi(cro)RNAs are a contemporary class of small noncoding endogenous RNA molecules, generating great excitement in the clinical and scientific communities. The recent discovery that miRNA expression is frequently dysregulated in cancer has uncovered an entirely new repertoire of molecular factors upstream of gene expression, which warrants extensive investigation to further elucidate their precise role in malignancy. We present a comprehensive and timely review of the role of miRNAs in cancer: addressing miRNA function, their putative role as oncogenes or tumor suppressors, with a particular emphasis on breast cancer throughout. We discuss the recent discovery of quantifiable circulating cancer-associated miRNAs, which heralds immense potential for their use as novel minimally invasive biomarkers for breast and other cancers. Finally, we comment on the potential role of miRNAs in breast cancer management, particularly in improving current prognostic tools and achieving the goal of individualized cancer treatment.

1. Introduction

The molecular biology of malignancy is diverse, complex, and remains poorly understood. The incidence of malignancies such as breast cancer is increasing consistently, and breast cancer has now become the commonest form of female malignancy among women in almost all of Europe and North America. Each year more than 1.3 million women will be diagnosed with breast cancer worldwide and approximately 4652 000 will die from the disease [1] despite the fact that breast cancer is highly curable if diagnosed and treated appropriately at an early stage. In Ireland alone, the annual incidence is currently over 2300 and rising [2]. The value of current histological prognostic indicators in predicting the course of the disease is weak and many of the molecular mechanisms underlying breast cancer progression remain poorly understood. This deficit has led to significant interest in the quest for novel predictive markers for breast cancer.

Mi(cro)RNAs are a contemporary class of tiny noncoding endogenous RNA molecules, only 18–25 nucleotides long. Since their discovery in 1993, these small molecules have been shown to play critical regulatory roles in a wide range of biological and pathological processes. Elucidating their mechanisms of action is still in its infancy. Nonetheless, work in this area to date has demonstrated that miRNAs may regulate cellular gene expression at the transcriptional or posttranscriptional level; by suppressing translation of protein coding genes, or cleaving target mRNAs to induce their degradation, through imperfect pairing with target mRNAs of protein coding genes [3]. MiRNA biogenesis in the human cell is a multistep complex process. A simplified representation is shown in Figure 1 [4]. The specific region of miRNA importance for mRNA target recognition is located at the 5′ end of the mature miRNA sequence, from bases 2 to 8. This is often referred to as the "seed sequence" [5]. Computational target prediction algorithms have been developed to identify putative mRNA targets, and these place considerable importance on this seed sequence, using it to search for complementary sequences in the 3′-UTRs of known genes that exhibit conservation across species. These algorithms predict that each miRNA may potentially bind to as many as 200 targets and estimate that miRNAs control the

expression of at least one third of human mRNAs, further highlighting their crucial role as regulators of gene expression [6].

At the time of writing, 8 273 mature miRNA sequences have been described in primates, rodents, birds, fish, worms, flies, plants, and viruses [7]. This represents a growth of over 200 microRNAs in the last 2 years. In the human genome, over 600 mature miRNAs have been reported to date; however, computational prediction estimates that this could increase to >1000 [8]. It is obvious that the microRNA story is just beginning.

2. Experimental Techniques for miRNA Analysis

The explosion of interest in miRNAs over the past two years necessitates effective tools for detecting their presence, quantification, and functional analysis. High-throughput profiling techniques such as miRNA microarrays and bead-based miRNA profiling have facilitated miRNA expression profiling, that is, far superior to existing low through-put techniques such as Northern blotting and cloning, and is essential for validation of microarray data. Castoldi et al. [9] described a novel miRNA microarray platform using locked nucleic acid-modified capture probes. Locked nucleic acid modification improved probe thermostability and increased specificity, thus enabling miRNAs with single nucleotide differences to be discriminated—an important consideration as sequence-related family members may be involved in different physiologic functions [10]. An alternative high-throughput miRNA profiling technique is the bead-based flow cytometric approach developed by Lu et al. [11]; a method which offers high specificity for closely related miRNAs because hybridization occurs in solution. Quantitative real-time PCR methodologies have been widely applied to miRNA research. To date, the most successful approach in terms of specificity and sensitivity is a two-step approach using looped miRNA-specific reverse transcription primers and TaqMan probes from Applied Biosystems [12].

To complement these miRNA profiling assays and to address functional questions necessitated the development of methods to manipulate miRNA expression. 2-*O*-Methyl antisense single-strand oligonucleotides and locked nucleic acid-modified oligonucleotides have been developed as miRNA inhibitors, making the suppression of endogenous miRNA activity and its downstream effect on mRNA expression achievable both in vitro and in vivo [13–16]. The effects of target miRNA knockdown on cell morphology and function can be determined using standard assays for processes such as cell proliferation, migration, invasion, and angiogenesis. MiRNA inhibition can be studied in animal models via transfection with tumor cells treated with miRNA inhibitors [17] or by the intravenous injection of "antagomirs" (2-*O*-methyl-modified nucleotides with a cholesterol moiety at the 3′-end [18]. The most recent development in the field of miRNA inhibition, led by Naldini and colleagues, describes techniques to manipulate miRNA expression in vivo by expressing decoy miRNA targets via lentiviral vectors [19]. This new approach to examine loss-of-function in vivo complements the results obtained by classic knockout technology as described above. It allows inhibition of specific miRNAs by building in multiple different decoys in the same miRNA inhibitor. This exciting new development should lead to answers for interesting functional questions with clinical or therapeutic relevance. For example, one could now potentially knock down the oncogenic proprieties of the miR-17-92-1 cluster which is well documented to be involved in human cancer [20]. This technique could also help one examine the let-7 microRNA family—a large, well-known tumor suppressor miRNA family [21] thereby providing insights into the functional consequence of knocking down all let-7 miRNAs [22].

MiRNA mimicry, a complementary technique to the aforementioned miRNA inhibition, has recently been used in vitro to identify the cellular processes and phenotypic changes associated with specific miRNAs transfected into cell lines [23]. Functional assays (e.g., proliferation, migration, invasion, and angiogenesis) then allow us to determine the effect of miRNA upregulation on tumorigenic or nontumorigenic cell populations. These revolutionary technologies will undoubtedly help us shed light on the functional roles of miRNAs and hold immense potential for application to the clinical arena as novel therapeutic targets.

2.1. MiRNA and Human Cancer. Early experimental work into the regulatory role of miRNAs uncovered their important role in various cellular processes such as differentiation, cell growth, and cell death. These processes are commonly dysregulated in cancer, implicating miRNAs in carcinogenesis. The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia [24]. Loss of miR-15a and miR-16-1 from this locus results in increased expression of the anti-apoptotic gene BCL2. Intensifying research in this field, using a range of techniques including miRNA cloning, quantitative PCR, microarrays and bead-based flow cytometric miRNA expression profiling has resulted in the identification and confirmation of abnormal miRNA expression in a number of human malignancies including breast cancer (Table 1). MiRNA expression has been observed to be upregulated or downregulated in tumours compared with normal tissue, supporting their dual role in carcinogenesis as either "Oncomirs" or tumour suppressors respectively [11].

The ability to obtain miRNA expression profiles from human tumors has led to remarkable insight and knowledge regarding the developmental lineage and differentiation states of tumours. Even within a single developmental lineage it has been shown that distinct patterns of miRNA expression are observed, that reflect mechanisms of transformation, and further support the idea that miRNA expression patterns encode the developmental history of human cancers. In contrast to messenger RNA (mRNA) profiles it is possible also to successfully classify poorly differentiated tumours using these new miRNA expression profiles [24, 25]. This has exciting implications clinically, in that miRNA expression may accurately diagnose poorly differentiated tissue samples which proved to be of uncertain histological origin thus facilitating treatment planning. Again in contrast to mRNA,

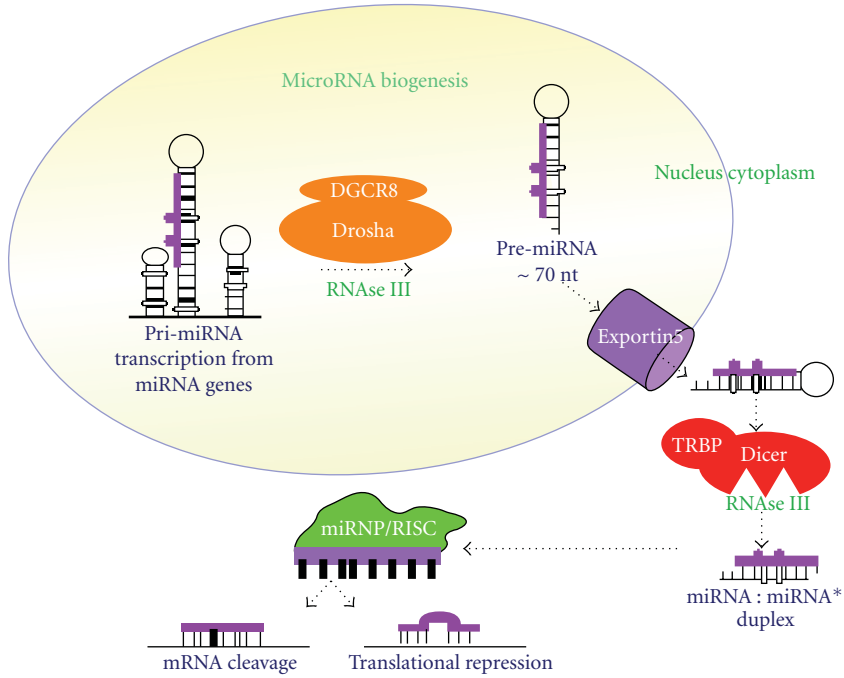


Figure 1: MiRNA biogenesis and processing in human cells: the multistep process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves nascent miRNA transcripts (pri-miRNA) into ~70 nucleotide precursors (pre-miRNA). These pre-miRNAs consist of an imperfect stem-loop structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner the transactivator RNA-binding protein TRBP into a small, imperfect dsRNA duplex (miRNA : miRNA*) that contains both the mature miRNA strand and its complementary strand. The miRNA strand is incorporated into the miRNP complex and targets complementary mRNA sequences, exerting its functionality via mRNA cleavage or translational repression.

Lu et al. showed that even a modest number of miRNAs are sufficient to classify human tumours and miRNAs remain largely intact in routinely collected, formalin-fixed, and paraffin-embedded clinical tissues [11]. Such information would eliminate the diagnostic uncertainty that previously existed in this setting and will be particularly useful for metastatic lesions of uncertain primary origin.

2.2. Breast Cancer and Genomic Signatures. Recent advances in phenotyping and molecular profiling of human cancers have greatly enhanced the diagnosis and biological classification of several tumors, in particular breast cancers where this technology has enhanced disease classification beyond single-gene markers. Prior to this a very limited armamentarium of prognostic markers beyond those offered by histopathological analysis was available in the clinical arena. Pioneering work by Sorlie et al. [33–35] identified microarray-generated gene expression signatures which stratified breast cancers into intrinsic subtypes largely based on their ER, progesterone (PR), and HER2/*neu* receptor status. Subtypes were designated *Luminal A*, which strongly expressed ER and/or PR, but were HER2/*neu* negative; *Luminal B*, which were ER, PR, and HER2/*neu* (triple) positive; *Basal* tumours which were ER, PR, and HER2/*neu* triple negative; and an *HER2* subset which was ER negative ER but had high expression of several genes in the HER2/*neu* amplicon, including *HER2* and *GRB7*. Survival analyses showed significantly different outcome for patients depending on their tumour subtype, emphasising the clinical relevance of stratification by such molecular profiling. This novel method of disease stratification based on the molecular taxonomy of the breast tumour heralds the promise of improving and individualizing patients' treatment regimens [36]. Great scientific endeavours in this field of microarray-based gene expression profiling are ongoing and intensifying, with the aim of translating such technical advances to the clinical arena, in providing us with a new tool for accurate molecular diagnosis of breast cancer [37]. One such application recently has been the development by Paik et al. of a multigene assay predictive of recurrence of tamoxifen-treated, node-negative breast cancer (*Oncotype DX*) [38]. This, and other similarly novel genomic tests (e.g., *MammaPrint, Theros, MapQuant Dx*) prove the feasibility of accelerating the transition between empirical and molecular medicine. Analogous to the derivation of intrinsic subtypes of breast cancer from gene expression signatures, it is predicted that in the very near future miRNA signatures, which are currently showing capability of accurately classifying tumours according to currently available prognostic variables, will serve as novel biomarkers and prognostic indicators thus providing strong rationale for individualised treatment. Additionally it is thought that miRNAs have the potential to improve greatly the precision of the recently derived genomic signatures, given that miRNA profiles have superior accuracy to mRNA

Table 1: MiRNAs with altered expression in malignancy.

Tissue/tumor type	Increased expression	Decreased expression
Breast [26, 27]	miR-21, miR-29b-2	miR-125b, miR-145 miR-10b, miR-155, miR-17-5p, miR-27b
Ovarian [28, 29]	miR-141, miR-200(a-c), miR-221	let-7f, miR-140, miR-145, miR199a, miR-424
Endometrial [30–32]	miR-103, miR-107, miR-185, miR-205, miR-210, miR-449	miR-99b, miR-152, miR-193, miR-204, miR-221, let-7i
Glioblastoma [4, 25]	miR-221, miR-21	miR-181a, miR-181b, miR-181c
Chronic lymphocytic leukaemia [24]		miR-15, miR-16
Lymphoma [4, 11]	miR-155, miR-17-92cluster	miR-15a
Colorectal [4, 11, 25]	miR-10a, miR-17-92 cluster, miR-20a, miR-24-1, miR-29b-2, miR-31	miR-143, miR-145, let-7
Thyroid [4, 25]	miR-221, miR-222, miR-146, miR-181b, miR-197, miR-346	
Hepatocellular [4, 25]	miR-18, miR-224	miR-199a, miR-195, miR-200a, miR-125a
Testicular [11]	miR-372, miR-373	
Pancreatic [4, 11, 25]	miR-221, miR-376a, miR301, miR-21, miR-24-2, miR-100, miR-103-1,2, miR-107, miR-125b-1	miR-375
Cholangiocarcinoma [25]	miR-21, miR-141, miR-200b	
Prostate [11]	let-7d, miR-195, miR-203	miR-128a
Gastric [4, 11, 25]	miR-223, miR-21, miR-103-2	miR-218-2
Lung [4, 11, 25]	mir-17-92 cluster, miR-17-5p	let-7 family

profiling in this regard [11]. A comprehensive interrogation of the breast cancer subclasses via miRNA expression profiling could further characterize the molecular basis underlying these subtypes, perhaps define more precise subsets of breast cancer, and provide opportunities for the identification of novel targets that can be exploited for targeted therapy.

2.3. MiRNA and Breast Cancer. Elucidation of the molecular mechanisms involved in breast cancer has been the subject of extensive research in recent years, yet several dilemmas and major challenges still prevail in the management of breast cancer patients including unpredictable response and development of resistance to adjuvant therapies. The emergence of miRNAs as regulators of gene expression identifies them as obvious novel candidate diagnostic and prognostic indicators, and potential therapeutic targets. Calin et al. [24] showed that half of the known mature human miRNAs are located in cancer-associated genomic regions, or fragile sites, thus potentiating their role in cancer. A specific example of this is the polycistron cluster miR-17-92 at the c13orf25 locus on chromosome 13q31. This locus is known to undergo loss of heterozygosity in a number of different cancer types, including breast cancer [39]. A number of other miRNAs (miR-196 and miR-10a) are located in homeobox clusters, which are known to be involved in the development of breast cancer and associated with the malignant capacity of cancer cells [40].

MiRNA expression studies in breast cancer indicate their importance and potential use as disease classifiers and prognostic tools in this field. In their analysis of 76 breast tumour and 34 normal specimens, Iorio et al. [26] identified 29 miRNAs that were differentially expressed in breast cancer tissue compared to normal, and a further set of 15 miRNAs that could correctly discriminate between tumour and normal. In addition, miRNA expression correlated with biopathological features such as ER and PR expression (*miR-30*) and tumour stage (*miR-213* and *miR-203*). The differential expression of several *let-7* isoforms was associated with biopathologic features including PR status (*let-7c*), lymph node metastasis (*let-7f-1, let-7a-3, let-7a-2*), or high proliferation index (*let-7c, let-7d*) in tumour samples. Mattie et al. identified unique sets of miRNAs associated with breast cancers currently defined by their HER2/*neu* or ER/PR status [27]. Significantly, there was overlap between the miRNAs identified in both studies. In initial studies in our own Department, we have shown that the expression levels of miR-195 and mir-154 are negatively correlated with ER positivity in a cohort of early breast cancers [41]. In another recent publication we were the first to identify reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue [42], subsequent to our validation of a two-gene normaliser (MRPL19 and PPIA) for analysis of gene expression in primary breast tissue [43].

3. Circulating microRNAs: Novel Minimally Invasive Biomarkers for Breast Cancer?

Current challenges in the management of breast cancer include a continuing search for sensitive minimally invasive markers that can be exploited to detect early neoplastic changes thus facilitating the detection of breast cancer at an early stage, as well as for monitoring the progress of patients with breast cancer and their response to treatments. Existing biomarkers for breast cancer have many inherent deficiencies. Mammography is currently the gold standard diagnostic tool however it is not without limitations, including its use of ionizing radiation and a false positive rate of 8–10% [44]. To date, only two markers have been established so far in the routine assessment of breast cancer: ER (for predicting response to endocrine therapies) and HER2 (for predicting response to Trastuzumab) [45]. Although these markers are currently available, ER and HER2 assessment is far from perfect [46]. A number of circulating tumour markers (e.g., carcinoembryonic antigen [CEA] and carbohydrate antigen 15-3 [CA 15-3]) are used clinically in the management of breast cancer, but the sensitivity of these markers is low, so that they are not useful as screening tools [47] though they have long been in clinical use as prognostic markers and to monitor for disease progression or recurrence. Despite their frequent use, CEA and Ca 15.3 remain poor markers for early stage disease with a documented preoperative sensitivity of only 9.11 and 5.36, respectively, as documented by Uehara et al. [48, 49].

The ideal biomarker should be easily accessible such that it can be sampled relatively noninvasively, sensitive enough to detect early presence of tumours in almost all patients and absent or minimal in healthy tumour-free individuals.

There is also great need for the identification of sensitive, reliable and acceptable markers of response to neoadjuvant and adjuvant therapies. MiRNAs have enormous potential to serve as an idea class of cancer biomarkers for the following reasons.

(1) MiRNA expression is known to be aberrant in cancer [11, 24].

(2) MiRNA expression profiles are pathognomonic, or tissue-specific [11].

(3) MiRNAs are remarkably stable molecules that have been shown to be well preserved in formalin fixed, paraffin embedded tissues as well as fresh snap frozen specimens [50, 51].

Acknowledging the exceptional stability of miRNAs in visceral tissue very recently instigated efforts to establish if miRNAs were also preserved, detectable, and quantifiable in the circulation and other bodily fluids (urine, saliva, etc.). This area of miRNA research is only now emerging, and is generating much excitement in clinical and scientific communities, such as its potential. MiRNA presence in serum was described for the first time in March 2008, in patients with diffuse large B-cell lymphoma [52]. Subsequent to this, a small number of studies have reported similarly, on the presence of miRNA in circulation and their potential for use as novel biomarkers for diseases and physiological states including malignancy, diabetes mellitus and pregnancy [53–55]. However these studies have been limited by small numbers and inconsistencies in methodologies [56]. This concept needs extensive investigation to validate the theory. To date no work has been published on the role of circulating miRNAs in breast cancer—an area where, if feasible, their use as novel minimally invasive biomarkers would be an incredible breakthrough in our management of this disease.

4. Therapeutic Potential

The association of aberrant miRNA expression with tumorigenesis and the functional analysis of specific miRNAs illustrate the feasibility of using miRNAs as targets of therapeutic intervention. Anti-miRNA 2-*O*-methyl or locked nucleic acid oligonucleotides used to inactivate oncomirs such as miR-21 in breast tumors may taper tumor growth [17]. Anti-miR-21-induced reduction in tumor growth, interestingly, was also shown by Si et al. to be potentiated by the addition of the chemotherapeutic agent topotecan, an inhibitor of DNA topoisomerase I. This suggests that suppression of the oncogenic miR-21 could sensitize tumor cells to anticancer therapy, which is an exciting prospect for patients exhibiting a poor response to primary chemotherapy. Conversely, the induction of tumor suppressor miRNA expression using viral or liposomal delivery of tissue-specific tumor suppressors to affected tissue may result in the prevention of progression, or even shrinking, of breast tumors. Tumor suppressor miRNA induction has also been shown to be subject to epigenetic control. Using chromatin remodelling drugs to simultaneously inhibit DNA methylation and histone deacetylation, epigenetic alterations in cancer and normal cells were manipulated by Saito et al. [57], who showed that certain miRNAs were upregulated in tumor cells but not in normal cells. MiR-127, which exhibited reduced expression in 75% of human cancer cells tested, was significantly upregulated after treatment. The induction of this miRNA was associated with downregulation of the proto-oncogene BCL6, suggesting a cancer-protective effect for miR-127 and a novel therapeutic strategy for the prevention and treatment of malignancy. This concept of inducing tumour suppressor miRNA expression has been termed "miRNA Replacement Therapy"; in anticipation of the promising clinical potential it holds.

5. Conclusion

The involvement of miRNAs in the initiation and progression of human malignancy holds great potential for new developments in current diagnostic and therapeutic strategies in the management of patients with breast cancer. Much of the work on microRNAs is still in its infancy and requires further exploration so that we may better understand their role in tumorigenesis. This scientific endeavour will undoubtedly lead to exciting developments in the future management of breast cancer. As the functional roles of miRNAs in cancer biology are further uncovered we predict

that; circulating miRNAs will serve as novel minimally invasive biomarkers for breast and other cancers, that improved methods of stratifying and subclassifying breast cancers will lead to tailored and individualized therapeutic regimens, thus sparing many patients from toxic effects of treatments from which they would derive no benefit. There is obviously great demand now for further intensive research into the identification of novel miRNAs, the elucidation of their mRNA targets, and an understanding of their functional effects, so as to improve our knowledge of the roles of these novel biomarkers in carcinogenesis and to expose their true potential as therapeutic agents.

References

[1] M. Garcia, et al., *Global Cancer Facts & Figures 2007*, American Cancer Society, Atlanta, Ga, USA, 2007.

[2] National Cancer Registry Ireland, Cancer projections 2005–2035, 2008.

[3] R. J. Jackson and N. Standart, "How do microRNA's regulate gene expression?" *Science's STKE*, vol. 367, no. 1, 2007.

[4] A. J. Lowery, N. Miller, R. E. McNeill, and M. J. Kerin, "MicroRNAs as prognostic indicators and therapeutic targets: potential effect on breast cancer management," *Clinical Cancer Research*, vol. 14, no. 2, pp. 360–365, 2008.

[5] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[6] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[7] S. Griffiths-Jones, H. K. Saini, S. van Dongen, and A. J. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acids Research*, vol. 36, supplement 1, pp. D154–D158, 2008.

[8] E. Berezikov, V. Guryev, and J. van de Belt, "Phylogenetic shadowing and computational identification of human microRNA genes," *Cell*, vol. 120, no. 1, pp. 21–24, 2005.

[9] M. Castoldi, S. Schmidt, V. Benes, et al., "A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA)," *RNA*, vol. 12, no. 5, pp. 913–920, 2006.

[10] A. L. Abbott, E. Alvarez-Saavedra, E. A. Miska, et al., "The *let-7* MicroRNA family members *mir-48*, *mir-84*, and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*," *Developmental Cell*, vol. 9, no. 3, pp. 403–414, 2005.

[11] J. Lu, G. Getz, E. A. Miska, et al., "MicroRNA expression profiles classify human cancers," *Nature*, vol. 435, no. 7043, pp. 834–838, 2005.

[12] K. Lao, N. L. Xu, V. Yeung, C. Chen, K. J. Livak, and N. A. Straus, "Multiplexing RT-PCR for the detection of multiple miRNA species in small samples," *Biochemical and Biophysical Research Communications*, vol. 343, no. 1, pp. 85–89, 2006.

[13] G. Hutvágner, M. J. Simard, C. C. Mello, and P. D. Zamore, "Sequence-specific inhibition of small RNA function," *PLoS Biology*, vol. 2, no. 4, article e98, 2004.

[14] G. Meister, M. Landthaler, Y. Dorsett, and T. Tuschl, "Sequence-specific inhibition of microRNA-and siRNA-induced RNA silencing," *RNA*, vol. 10, no. 3, pp. 544–550, 2004.

[15] A. Boutla, C. Delidakis, and M. Tabler, "Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in *Drosophila* and the identification of putative target genes," *Nucleic Acids Research*, vol. 31, no. 17, pp. 4973–4980, 2003.

[16] U. A. Ørom, S. Kauppinen, and A. H. Lund, "LNA-modified oligonucleotides mediate specific inhibition of microRNA function," *Gene*, vol. 372, no. 1-2, pp. 137–141, 2006.

[17] M.-L. Si, S. Zhu, H. Wu, Z. Lu, F. Wu, and Y.-Y. Mo, "miR-21-mediated tumor growth," *Oncogene*, vol. 26, no. 19, pp. 2799–2803, 2007.

[18] J. Krutzfeldt, N. Rajewsky, R. Braich, et al., "Silencing of microRNAs in vivo with 'antagomirs'," *Nature*, vol. 438, no. 7068, pp. 685–689, 2005.

[19] B. Gentner, G. Schira, A. Giustacchini, et al., "Stable knockdown of microRNA in vivo by lentiviral vectors," *Nature Methods*, vol. 6, no. 1, pp. 63–66, 2009.

[20] J. T. Mendell, "miRiad roles for the miR-17-92 cluster in development and disease," *Cell*, vol. 133, no. 2, pp. 217–222, 2008.

[21] C. D. Johnson, A. Esquela-Kerscher, G. Stefani, et al., "The let-7 microRNA represses cell proliferation pathways in human cells," *Cancer Research*, vol. 67, no. 16, pp. 7713–7722, 2007.

[22] P. P. Medina and F. J. Slack, "Inhibiting microRNA function in vivo," *Nature Methods*, vol. 6, no. 1, pp. 37–38, 2009.

[23] J. M. Franco-Zorrilla, A. Valli, M. Todesco, et al., "Target mimicry provides a new mechanism for regulation of microRNA activity," *Nature Genetics*, vol. 39, no. 8, pp. 1033–1037, 2007.

[24] G. A. Calin, C. D. Dumitru, M. Shimizu, et al., "Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15524–15529, 2002.

[25] S. Volinia, G. A. Calin, C.-G. Liu, et al., "A microRNA expression signature of human solid tumors defines cancer gene targets," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2257–2261, 2006.

[26] M. V. Iorio, M. Ferracin, C.-G. Liu, et al., "MicroRNA gene expression deregulation in human breast cancer," *Cancer Research*, vol. 65, no. 16, pp. 7065–7070, 2005.

[27] M. D. Mattie, C. C. Benz, J. Bowers, et al., "Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies," *Molecular Cancer*, vol. 5, article 24, 2006.

[28] M. V. Iorio, R. Visone, G. Di Leva, et al., "MicroRNA signatures in human ovarian cancer," *Cancer Research*, vol. 67, no. 18, pp. 8699–8707, 2007.

[29] N. Dahiya, C. A. Sherman-Baust, T.-L. Wang, et al., "MicroRNA expression and identification of putative miRNA targets in ovarian cancer," *PLoS One*, vol. 3, no. 6, article e2436, 2008.

[30] W. Wu, Z. Lin, Z. Zhuang, and X. Liang, "Expression profile of mammalian MicroRNAS in endometrioid adenocarcinoma," *European Journal of Cancer Prevention*, vol. 18, no. 1, pp. 50–55, 2009.

[31] T. K. H. Chung, T.-H. Cheung, N.-Y. Huen, et al., "Dysregulated microRNAs and their predicted targets associated with endometrioid endometrial adenocarcinoma in Hong Kong women," *International Journal of Cancer*, vol. 124, no. 6, pp. 1358–1365, 2009.

[32] T. Boren, Y. Xiong, A. Hakam, et al., "MicroRNAs and their target messenger RNAs associated with endometrial carcinogenesis," *Gynecologic Oncology*, vol. 110, no. 2, pp. 206–215, 2008.

[33] C. M. Perou, T. Sørile, M. B. Eisen, et al., "Molecular portraits of human breast tumours," *Nature*, vol. 406, no. 6797, pp. 747–752, 2000.

[34] T. Sørlie, C. M. Perou, R. Tibshirani, et al., "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 19, pp. 10869–10874, 2001.

[35] T. Sørlie and Y. Wang, "Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms," *BMC Genomics*, vol. 7, article 127, 2006.

[36] L. J. Van't Veer, H. Dai, M. J. van de Vijver, et al., "Gene expression profiling predicts clinical outcome of breast cancer," *Nature*, vol. 415, no. 6871, pp. 530–536, 2002.

[37] C. Sotiriou and L. Pusztai, "Gene-expression signatures in breast cancer," *The New England Journal of Medicine*, vol. 360, no. 8, pp. 790–800, 2009.

[38] S. Paik, S. Shak, G. Tang, et al., "A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer," *The New England Journal of Medicine*, vol. 351, no. 27, pp. 2817–2826, 2004.

[39] M. Negrini, D. Rasio, G. M. Hampton, et al., "Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: identification of a new region at 11q23.3," *Cancer Research*, vol. 55, no. 14, pp. 3003–3007, 1995.

[40] K. Makiyama, J. Hamada, M. Takada, et al., "Aberrant expression of HOX genes in human invasive breast carcinoma," *Oncology Reports*, vol. 13, no. 4, pp. 673–679, 2005.

[41] A. J. Lowery, et al., "Micro-RNA expression profiling in primary breast tumours," *European Journal of Cancer*, vol. 5, supplement 3, 2007.

[42] P. A. Davoren, R. E. McNeill, A. J. Lowery, M. J. Kerin, and N. Miller, "Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer," *BMC Molecular Biology*, vol. 9, article 76, 2008.

[43] R. E. McNeill, N. Miller, and M. J. Kerin, "Evaluation and validation of candidate endogenous control genes for real-time quantitative PCR studies of breast cancer," *BMC Molecular Biology*, vol. 8, article 107, 2007.

[44] S. Taplin, L. Abraham, W. E. Barlow, et al., "Mammography facility characteristics associated with interpretive accuracy of screening mammography," *Journal of the National Cancer Institute*, vol. 100, no. 12, pp. 876–887, 2008.

[45] A. Thompson, K. Brennan, A. Cox, et al., "Evaluation of the current knowledge limitations in breast cancer research: a gap analysis," *Breast Cancer Research*, vol. 10, no. 2, article R26, 2008.

[46] M. J. Piccart-Gebhart, M. Procter, B. Leyland-Jones, et al., "Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer," *The New England Journal of Medicine*, vol. 353, no. 16, pp. 1659–1672, 2005.

[47] L. Harris, H. Fritsche, R. Mennel, et al., "American society of clinical oncology 2007 update of recommendations for the use of tumor markers in breast cancer," *Journal of Clinical Oncology*, vol. 25, no. 33, pp. 5287–5312, 2007.

[48] D. M. O'Hanlon, M. J. Kerin, P. Kent, D. Maher, H. Grimes, and H. F. Given, "An evaluation of preoperative CA 15-3 measurement in primary breast carcinoma," *British Journal of Cancer*, vol. 71, no. 6, pp. 1288–1291, 1995.

[49] M. Uehara, T. Kinoshita, T. Hojo, S. Akashi-Tanaka, E. Iwamoto, and T. Fukutomi, "Long-term prognostic study of carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3) in breast cancer," *International Journal of Clinical Oncology*, vol. 13, no. 5, pp. 447–451, 2008.

[50] Y. Xi, G. Nakajima, E. Gavin, et al., "Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples," *RNA*, vol. 13, no. 10, pp. 1668–1674, 2007.

[51] J. Li, P. Smyth, R. Flavin, et al., "Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells," *BMC Biotechnology*, vol. 7, article 36, 2007.

[52] C. H. Lawrie, S. Gal, H. M. Dunlop, et al., "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma," *British Journal of Haematology*, vol. 141, no. 5, pp. 672–675, 2008.

[53] P. S. Mitchell, R. K. Parkin, E. M. Kroh, et al., "Circulating microRNAs as stable blood-based markers for cancer detection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 30, pp. 10513–10518, 2008.

[54] X. Chen, Y. Ba, L. Ma, et al., "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," *Cell Research*, vol. 18, no. 10, pp. 997–1006, 2008.

[55] S. Gilad, E. Meiri, Y. Yogev, et al., "Serum microRNAs are promising novel biomarkers," *PLoS One*, vol. 3, no. 9, article e3148, 2008.

[56] L. J. Chin and F. J. Slack, "A truth serum for cancer—microRNAs have major potential as cancer biomarkers," *Cell Research*, vol. 18, no. 10, pp. 983–984, 2008.

[57] Y. Saito, G. Liang, G. Egger, et al., "Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells," *Cancer Cell*, vol. 9, no. 6, pp. 435–443, 2006.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 426956, 15 pages
doi:10.1155/2010/426956

Review Article

Role of Chemokine Network in the Development and Progression of Ovarian Cancer: A Potential Novel Pharmacological Target

Federica Barbieri, Adriana Bajetto, and Tullio Florio

Laboratory of Pharmacology, Department of Oncology, Biology and Genetics, University of Genova, 16132 Genova, Italy

Correspondence should be addressed to Tullio Florio, tullio.florio@unige.it

Received 11 June 2009; Accepted 28 September 2009

Academic Editor: Tian-Li Wang

Ovarian cancer is the most common type of gynecologic malignancy. Despite advances in surgery and chemotherapy, the survival rate is still low since most ovarian cancers relapse and become drug-resistant. Chemokines are small chemoattractant peptides mainly involved in the immune responses. More recently, chemokines were also demonstrated to regulate extra-immunological functions. It was shown that the chemokine network plays crucial functions in the tumorigenesis in several tissues. In particular the imbalanced or aberrant expression of CXCL12 and its receptor CXCR4 strongly affects cancer cell proliferation, recruitment of immunosuppressive cells, neovascularization, and metastasization. In the last years, several molecules able to target CXCR4 or CXCL12 have been developed to interfere with tumor growth, including pharmacological inhibitors, antagonists, and specific antibodies. This chemokine ligand/receptor pair was also proposed to represent an innovative therapeutic target for the treatment of ovarian cancer. Thus, a thorough understanding of ovarian cancer biology, and how chemokines may control these different biological activities might lead to the development of more effective therapies. This paper will focus on the current biology of CXCL12/CXCR4 axis in the context of understanding their potential role in ovarian cancer development.

1. Introduction

Chemokines are small secreted cytokines, primarily involved in the regulation of the motility of hematopoietic cells (cells of the immune system) in their specific homing to lymphoid organs in normal hematopoiesis and during inflammation [1], through the activation of specific G-protein coupled receptors [2].

To date 53 human chemokines and 23 receptors have been cloned and characterized.

Chemokines display high structural homology and overlapping functions and often bind more than one receptor. In general, ligand binding causes chemokine receptor activation, hallmarked by the phosphorylation of C-terminal serine/threonine residues that, in turn, drives dissociation of heterotrimeric G-proteins into α and $\beta\gamma$ subunits, inhibition of adenylyl cyclase activity, increased generation of inositol trisphosphate, intracellular calcium release, and the activation of phosphatidyl inositol 3 kinase (PI3K)/Akt cascade and Ras/MAP kinase signalling [3].

Chemokines are divided into subfamilies by structural and functional criteria. Structurally, chemokines are classified into four groups (C, CC, CXC, and CX3C) according to the number and location of the conserved cysteine residues in the primary structure of these molecules (Figure 1). The "C" group of chemokines (containing only two cysteines) consists of two molecules (XCL), namely, XCL1/lymphotactin and XCL2/SCM-1β, both binding the receptor XCR1. Lymphotactin, coded on human chromosome 1, attracts lymphocytes but not monocytes or neutrophils.

Human "CC" chemokines (structurally characterized by four cysteines) includes 28 members, called CCL1-28 that bind at least 10 receptors (CCR1-10). CC chemokine targets include monocytes, T cells, dendritic cells, eosinophils, and basophils. Representative CC chemokines are CCL2 (also called monocyte chemotactic protein, MCP-1), CCL3 and CCL4 (macrophage inflammatory protein MIP-1α and MIP-1β), CCL5 (RANTES), and CCL11 (eotaxin).

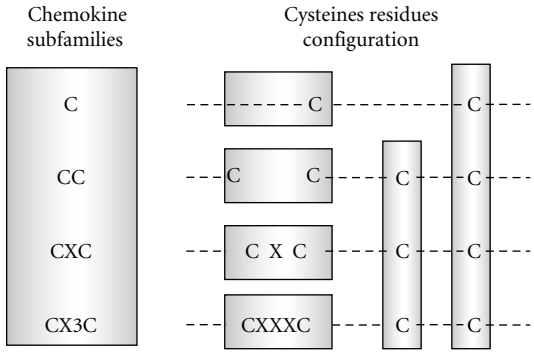


Figure 1: *Chemokine subfamilies classification.* The first cysteine (C) in the sequence forms a covalent bond with the third, the second and the fourth cysteines also form a disulfide bond to create the tertiary structure characteristic of chemokines. In the CC subfamily the first two cysteines are adjacent to each other, in the CXC group there is one amino acid between the first two cysteines, and in the CX3C group there are three amino acids between cysteines.

The "CXC" group (in which one amino acid is present between the first two cysteines) includes 21 ligands (CXCL1-21) mostly encoded on human chromosome 4. CXC chemokines bind at least 7 receptors (CXCR1-7) and mediate neutrophil chemotaxis. The CXC group can be divided into two main categories based on the presence of the tripeptide Glu-Leu-Arg (ELR) before the CXC motif (N-terminal domain). Representative CXC chemokines include CXCL8/IL-8, among the ELR-containing peptides and CXCL9/monokine-induced by IFN-γ (MIG), CXCL10/IFN-γ inducible protein-10 (IP-10), and CXCL12/stromal cell-derived factor-1 (SDF1) as ELR negative molecules.

Lastly, the "CX3C" chemokines (three amino acids between the first two cysteines) are, to date, represented by a single peptide, namely, CX3CL1/fractalkine, which is encoded on human chromosome 16, binds the CX3CR1 receptor and regulates T cell trafficking and adhesion [4].

Functionally, chemokines, released upon inflammatory stimuli that induce leukocyte recruitment to damaged/infected sites, are considered as "inflammatory" [5] while chemokines that induce migration of leukocytes to lymphoid organs are considered "homeostatic" and are usually constitutively secreted by stromal cells expressed at these sites [6]. Homeostatic chemokines, such as CXCL12, coordinate cell trafficking and homing, which is essential during development and for homeostasis and function of the immune system.

More recently, several extra-immunological functions were discovered for most of the components of the chemokine sub-families (for review see [7]). In particular, it was demonstrated that chemokines are major players during embryonic development, when their role as chemotactic mediators contribute to cell migration in the different body districts. Moreover, in the adult, chemokines play a relevant function in the central nervous system (CNS) where both ligands and receptors are expressed [8, 9]. At CNS level, chemokines control, among other functions, pain, alimentary behavior and glial responses to injuries [10–12].

2. Chemokines in Cancer

In cancer, genetic changes that accumulate in transformed cells are dependent on microenvironmental factors and control the development of the malignant process. In the past few years, a major role has been assigned to chemokines and their receptors as molecules that affect neoplastic development and progression.

Many chemokine/receptor pairs are expressed in tumors, not only by cancer cells but also by cells of the tumor microenvironment, including cells of the stroma (endothelial cells, fibroblasts) and leukocytes, thus contributing to the cross-talk between the tumor and its microenvironment to control tumor growth and progression [13].

In the malignancy context, chemokines play diverse effects, most of them deriving from their ability to induce cell migration. The ability of chemokines to enhance the motility of leukocytes, endothelial cells, and/or tumor cells is a key factor in determining the cancer establishment and progression. Depending on their specific expression pattern on target cells, on tumor type and on tumor microenvironment factors, several chemokines support malignancy, while others can at times inhibit this process [14].

The extensive research that was thus far performed on the roles of chemokines in cancer indicates that these molecules affect tumors mainly acting at four levels: (a) determine the extent and type of leukocyte infiltrates; (b) promote angiogenesis; (c) control site-specific metastasization; (d) affect tumor cells proliferation [15, 16].

The immune response induced by malignancy is clearly evident since many solid tumors are highly populated by host leukocytes that have migrated into the tumor from the systemic circulation.

In tumors, leukocyte infiltrates may have either anti-cancer or cancer-promoting effects, depending on their type, their activity, and their modes of interaction with the tumor cells. In line with their classification as leukocyte chemoattractants, chemokines are released by tumor cells or by cells of their microenvironment and are able to induce the recruitment of different hematopoietic cell subtypes to tumors (T lymphocytes, macrophages, natural killer (NK) cells, neutrophils, eosinophils, and B cells). In particular, among CXC chemokines, CXCL9, CXCL10, and CXCL11 are induced by interferon γ (IFNγ) and are typical chemoattractants of NK cells [15]. Accordingly, overexpression of these chemokines by different experimental means leads to limitations in cancer development, associated with elevation in cytotoxic responses and with the creation of long-term antitumor immunity. These chemokines have additional antitumor activities but at the same time they may exert tumorigenic functions when acting directly on the tumor cells [17].

On the other hand, some chemokines may induce proangiogenic effects, leading to highly neovascularized tumors and increased metastatic spread. A large number of studies now clearly indicate that chemokines, mainly belonging to the CXC and CC subgroups, are important regulators of tumor angiogenesis. However, also on this parameter, opposite final effects may occur; some chemokines support

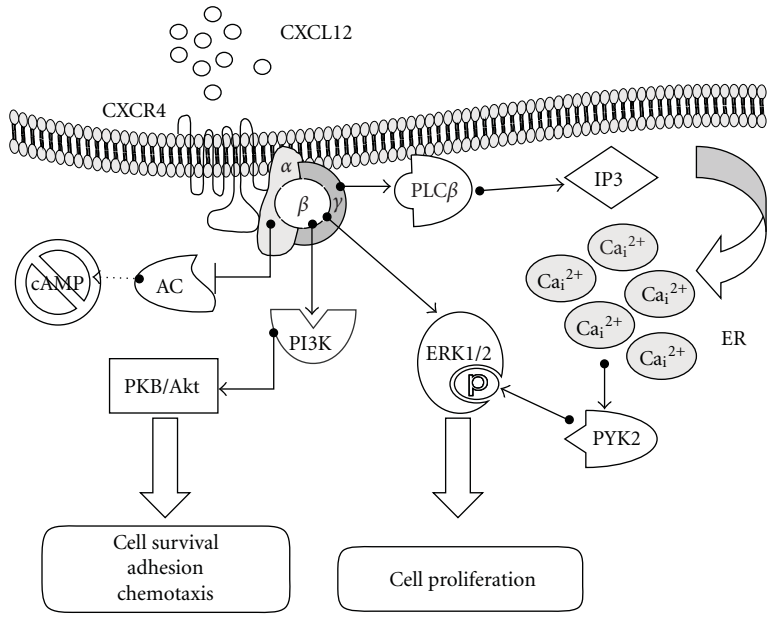


Figure 2: *Signaling pathways downstream CXCR4 receptor activation.* Upon CXCL12 binding the G protein complex dissociates into α and $\beta\gamma$ subunits that trigger parallel signal transduction cascades culminating in tumor cell proliferation, migration, or survival. AC: adenylyl cyclase; ER: endoplasmic reticulum; PLC: phospholipase C; PI3K: phosphoinositide 3-kinase; PKB/Akt: protein kinase B; PKC: protein kinase C; PYK2: proline-rich tyrosine kinase 2; ERK1/2: extracellular regulated kinase.

the formation of new blood vessels, while others are angiostatic.

Angiogenic CXC chemokines promote the migration and proliferation of endothelial cells. Accordingly, they were shown to be potent tumor-supporting factors in a large variety of tumor types. For example, CXCL8 acts on endothelial cells mainly via their high affinity CXCR2 receptor [18]. Conversely, other CXC chemokines, including CXCL4, CXCL9, CXCL10, and CXCL11 are potent angiostatic factors. Their activity, via the CXCR3, receptor inhibits the neovascularization induced by powerful angiogenic factors. This activity of CXC chemokines, altogether with their ability to recruit antitumoral immune cells, led to the hypothesis of a function for these peptides as potential antitumoral factors. In contrast, CXCL12, another relevant member of this group, promotes tumor neoangiogenesis under specific conditions, thus being regarded as one of the most powerful pro-malignancy factors [19]. Many chemokines sustain cancer cell proliferation and survival, through an interaction with receptors expressed by the tumor cells. Such an activity was reported for CXCL8 that acts as an autocrine growth factor for human ovarian cancer Hey-A8 cell line [20]. Moreover, in vivo studies showed that CXCL8-overexpressing Hey-A8 cells are able to increase tumor cell growth, microvessel density, and the tumorigenic rate [20].

The chemokine CXCL12 also exerts a direct effect on tumor cell proliferation and survival in a high variety of tumors. In fact, altogether with its receptor CXCR4, CXCL12 constitutes the chemokine/receptor axis that attracted the greatest interest in oncology. This receptor-ligand system was reported to be overexpressed in several cancer types including acute and chronic leukemias [21] and solid tumors such as breast [22], colon [23], prostate [24], and ovarian cancers [25], glioblastomas [26, 27], melanomas [28], pituitary adenomas [29], and meningiomas [30, 31].

Physiologically, the CXCR4/CXCL12 axis is involved in migration of embryonic cells participating to the development of the central nervous system, bone marrow, and heart [32, 33]. Although for many years the interaction between CXCR4 and CXCL12 was thought to be unique, more recently, it was reported that CXCL12 binds also to another receptor, named CXCR7 [34]. CXCR7 is expressed in several cell types, including endothelial cells, T and B cells, dendritic cells, chondrocytes, endometrial stromal cells. The interaction of CXCL12 with CXCR4 mainly affects chemotaxis, while the binding to CXCR7 mediates proliferation in tumor cells [35]. Thus, CXCL12 can modulate the migration capacity of tumor cells and CXCR7 can enhance tumor growth.

Chemokines interacting to specific G protein coupled receptors (GPCRs) activate several signalling pathways in both normal and cancer cells. In normal cells, upon activation, CXCR4 dimerizes and transduces several intracellular signals (Figure 2). Most of them are PTX-sensitive and therefore dependent on activation of G_i/G_o proteins, including $G\alpha$ and $G\beta\gamma$-mediated signals. $G\alpha_t$ activation implies the inhibition of adenylyl cyclase (AC) function, thereby determining a decrease in the cytosolic concentration of cyclic AMP (cAMP), and leading to the inhibition of protein kinase A (PKA). $G\alpha_q$ transduces the CXCR4 signals through the activation of phospholipase Cβ (PLC) which increases inositol triphosphate and intracellular calcium levels. Chemokine signalling activated by $G\beta\gamma$ induces a direct activation of PI3 kinase (PI3K), a survival regulator

acting on effectors of apoptosis [36]. PI3K activation is often detected in cancer as mediator of the increased survival of the tumor cells. Importantly, beside survival, chemokines in general, and CXCL12 in particular, are also powerful activators of the MAP kinase (ERK1/2) cascade, the most studied proliferative mechanism responsible of tumor growth [30, 36]. Interestingly, ERK1/2 activation may involve either the classical Ras/Raf/MEK pathway or the activation of the cytosolic, Ca^{++}-dependent tyrosine kinase Pyk2 [11]. However, in pituitary adenoma cell lines, CXCR4-induced activation of the calcium-dependent protein kinase Pyk2 was involved in a proliferative response independent by ERK1/2 and involving large conductance Ca^{2+}-dependent K^{+} channels [37, 38]. Thus, the intracellular signaling involved in CXCL12 tumor cell proliferation is extremely dependent on the cell type analyzed.

More recently, several studies proposed that CXCR4-dependent activation of ERK1/2 was mediated by transactivation of tyrosine kinase receptors. Cross-talk between growth factor and G protein-coupled receptors is now believed to play an important role in both normal and tumor responses. In particular, the transactivation of epidermal growth factor receptor (EGFR) and the activation of its downstream signaling pathways are critical for the mitogenic activity of different GPCR ligands, including chemokines. In the ovarian cancer cell line SKOV-3, CXCL8/IL-8 was shown to induce transient phosphorylation of EGFR and its association with the adaptor molecules Shc and Grb2, suggests an important cross-talk between chemokine and growth factor pathways [39]. In three other ovarian cancer cell lines (OC 314, OC 315, OC 316), it was demonstrated that CXCL12/CXCR4 interaction induces a dose-dependent cell proliferation through ERK1/2 and Akt activation that was dependent on EGFR phosphorylation caused by a mechanism involving the activity of the cytosolic tyrosine kinase c-Src [40]. Thus, a "cross-talk" between CXCL12/CXCR4 and EGFR intracellular pathways may link signals of cell migration and proliferation in ovarian cancer. A similar mechanism was also demonstrated in breast cancer cell lines, showing that estrogen-dependent proliferation involves the synthesis of CXCL12 (identified as estradiol-dependent gene), that causes an autocrine stimulation of CXCR4 and the subsequent activation of c-Src that, in turn, induces a ligand-independent activation of EGFR and ERK1/2 phosphorylation [41]. The transactivation of HER2/neu through CXCL12 stimulation of CXCR4 and Src activation cells was also demonstrated, in breast and prostate cancers [41–43].

3. Chemokines, Tumor-Specific Immune Responses, and Tumor Microenvironment

The interaction of chemokines, their receptors, growth factors, inflammatory with cancer cells forms a complex network at the tumor site, responsible for the overall progression or rejection of the tumor. In particular, chemokines play an essential role in coordinating the function of the immune system participating either in several steps of the antitumor immunity or in the regulation of the release of several mediators able to activate proangiogenic stimuli, and thus supporting tumor development.

In fact, on one hand, chronic inflammation is often associated with cancer development [44] with an inflammatory component detectable also in the microenvironment of tumors non epidemiologically related to inflammation [45]. Moreover, several chemokines (mainly of the CXC/ELR+ family) are involved in regulation and recruitment of multiple cell types within tumor microenvironment, often possessing neoangiogenetic activity (Figure 3). Thus, chemokines may exert not only a direct effect on tumor cells but may control tumor growth also through the activation of their specific receptors expressed in a large number of stromal (fibroblasts and endothelial cells) and inflammatory cells. Importantly these cells also secrete a variety of chemokines, which regulate the migration of infiltrating macrophages, lymphocytes, dendritic cells, and neutrophils in response to a chemokine gradient [13].

On the other hand, the activity of several chemokine may be detrimental for tumor growth causing the recruitment in tumor microenvironment cytotoxic T lymphocytes and NK cells responsible of the immunosurveillance against transformed cells (Figure 3).

Among CXC-chemokines, CXCL12 is known to regulate the local immune response and is a potent chemoattractant for T cells, pre-B lymphocytes, and dendritic cells and its receptor CXCR4 is expressed by T lymphocytes, monocytes, neutrophils, and endothelial cells. This chemokine produced by different cell types in the tumor microenvironment modulates the activity of immunosuppressive cells (macrophages, neutrophils, T regulatory cells) contributing to tumor progression.

The molecular pathways activated in tumor cells that control cancer-related immunity include transcription factors, such as nuclear factor-κB (NF-κB), hypoxia inducible factor α (HIF1α), and signal transducer and activator of transcription 3 (STAT3), which, in turn, control the production of other chemokines and inflammatory mediators (prostaglandins, cytokines). Altogether these factors trigger the recruitment of activated inflammatory cells generating the cancer-related inflammatory microenvironment. For example, tumor-associated leukocytes represent a source of growth factors, acting on tumor cells, and angiogenic factors.

This dual role of chemokines in tumor development, to either eliminate malignant cells or escape the host immune control, was demonstrated in several tumor types [46]. For example, in melanoma, while chemokines contribute to the recruitment of CD8+ T lymphocytes expressing CXCR3 that infiltrate the tumor leading to the improvement of patient survival [22], the lack of critical chemokines (CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10) in melanoma metastases may block the migration of activated T cells, which in turn could limit the effectiveness of antitumor immunity [47]. On the other hand, the aberrant expression of chemokines in tumors induces immunosuppression and favors tumor growth as shown in hepatocellular carcinoma, where high levels of CXCL9 and CXL10 have been associated with inhibition of CXCR3 expression by CD8+ T limphocytes,

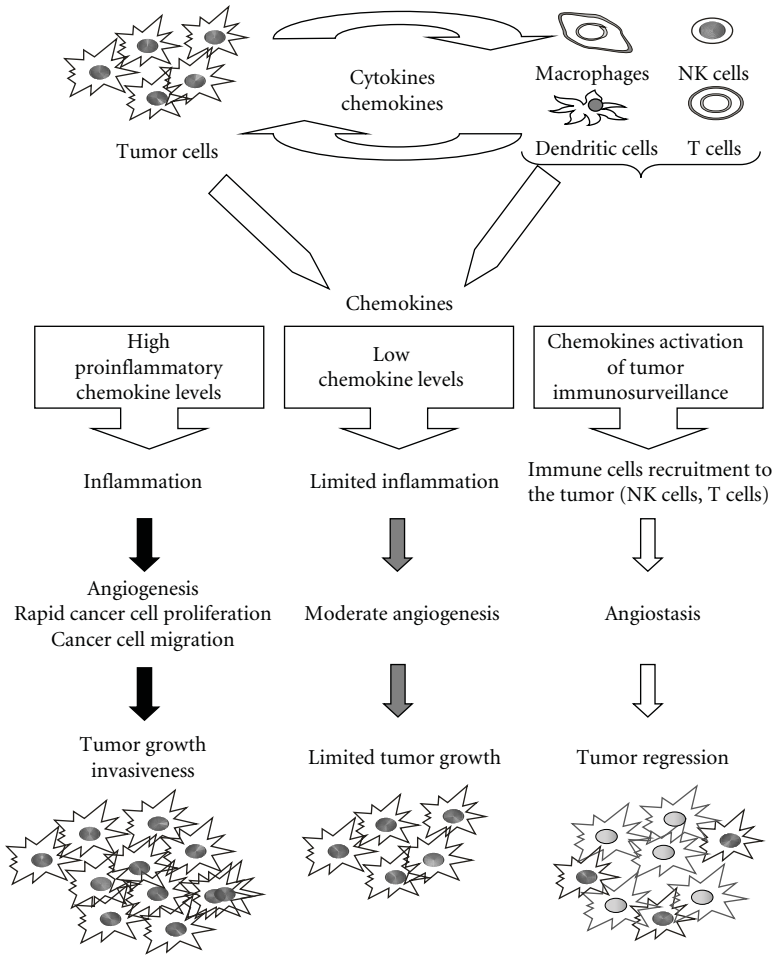


Figure 3: *Role of chemokines in the tumor-specific immune response.* The type and the amount of the chemokines secreted by tumor and inflammatory cells determine the extent and the effect of immune response leading to antitumor cytotoxic response, limited inflammatory, and vascular activation or potentiating tumor cell proliferation and neoangiogenesis.

reduction of T-cell tumor infiltration and cytotoxic functions and tumor growth [48].

Another mechanism by which certain tumors evade the immune system is through the chemorepulsive activity of high levels of CXCL12. CXCL12 at low concentration (<10 nM) acts as a T cell chemoattractant [49], while higher concentrations of the chemokine can repel T cells in vitro and in vivo via a CXCR4 receptor-mediated mechanism [50].

In conclusion, according to which chemokines are released, completely opposite events may occur; abundant production of proinflammatory chemokines (i.e., ELR+ CXC-chemokines) can lead to a strong inflammatory response that potentiates angiogenesis, thus favoring a rapid neoplastic growth. Alternatively, high levels of monocytes and/or neutrophil infiltration, for example, in response to ELR- chemokines, can be associated with angiostasis, cytotoxicity, and possible tumor regression [19] (Figure 3).

On the basis of these evidences, the characterization of the different chemokine networks in various types of cancer cells may foster better knowledge for understanding the immune-related mechanisms of cancer development and application in cancer immunotherapy.

4. Ovarian Cancer

Ovarian cancer causes more deaths than any other cancer of the female reproductive system, representing the world sixth most commonly diagnosed neoplasia among women [51]. Despite the high incidence and mortality rates, the etiology of this disease is poorly understood. Age and family history for the disease represent established risk factors for ovarian cancer; other possible risk factors include postmenopausal hormone-replacement therapy and lifestyle factors such as cigarette smoking and alcohol consumption. However, in many cases, the causes of ovarian cancer are yet to be identified.

The progression of these tumors within the peritoneal cavity results in late diagnosis and high mortality rate. Indeed, the majority of patients are diagnosed with advanced disease and treated with surgery and postoperative cis-platinum- and taxane-based chemotherapy [52]. Moreover, several patients exhibit primary resistance to chemotherapy and approximately 70% achieve remission that is generally not durable, with an overall 5-year survival rate of 20–30% [53].

Ovarian cancers are histologically diverse: about 80% originates in the epithelium (epithelial ovarian cancer, EOC); the remaining 20% arise from other cell types (germ cell, sex cord-stromal, and mixed cell tumors) or are metastases to the ovary (most commonly, from the breast or gastrointestinal tract tumors). EOC includes four histotypes (serous, mucinous, clear cell, and endometrioid) differing for epidemiologic, genetic changes, tumor markers, and response to therapy. The molecular events leading to the development of EOC are not yet clear, although if genetic and epigenetic alterations have commonly been observed. One of the most frequent genetic changes (about 70% of cases) is the mutation or loss of *TP53* function but, despite the great number of studies, its correlation with chemoresistance and prognostic impact are not yet fully proved [54]. Loss of heterozigosity and mutations *BRCA1* and *BRCA2* leading to inactivation of other genes, has been described in familial ovarian cancers [55]. Mutations, amplification, and overexpression of well-known oncogenes, such as *PI3K* subunit-α, *FGF1, MYC, EGFR, KRAS, HER2,* and *AKT2,* have been also associated with ovarian cancer [56].

The different subtypes of ovarian cancer could be further subclassified according to tumor cell type grade, taking into account the different molecular characteristics: high grade serous cancers typically contain *BRCA1* and *TP53* mutations (up to 80%); low grade serous carcinomas often have mutations in the *KRAS* and *BRAF* genes (>60%), and low-grade endometrioid cancers are associated with mutations in the beta-catenin gene, *CTNNB1, PTEN, and PI3CA* and mucinous carcinomas frequently have mutations in *KRAS* and *TP53*. This subclassification of ovarian cancers is essential because different subtypes of ovarian cancer respond differently to treatment and have different prognoses [57].

Some of the genes involved in ovarian cancer development control the activation of specific intracellular signalling pathways, particularly PI3K/Akt, EGFR, HER2/neu, PKC1, Src, and Ras, that are activated in more than half of ovarian neoplasms and thus could represent future targets for new anticancer agents.

In particular, EGFR and HER2/neu activate signaling pathways (PI3K/Akt and ERK1/2 MAP kinase), leading to different cellular processes involved in tumor development, such as cell division and migration, adhesion, differentiation, and apoptosis [58]. Aberrant EGFR and HER2 expression was reported in ovarian carcinomas [59]. This evidence prompted the development of several strategies to target EGFR and HER2: monoclonal antibodies directed against the extracellular domain of the receptors or small molecules targeting the intracellular tyrosine kinase domains (tyrosine kinase inhibitors (TKIs)) are in various stages of clinical trials for ovarian cancer [60].

However, the molecular pathways underlying ovarian cancer progression are still poorly understood and currently the signaling pathway research identified promising novel candidates for cancer treatment and thus much effort has been made to establish signal transduction as target for therapy.

One of the major challenges for ovarian cancer clinical outcome is the occurrence of metastasis. Ovarian cancer spreads by direct seeding of cells into the peritoneal cavity where they form cancer nodules, by lymphatic dissemination to the pelvis, or, less frequently, by hematological diffusion to the parenchyma of the liver or lung. Ovarian cancer metastasis in the peritoneal cavity is not limited by anatomical barriers, thus peritoneal metastatic lesions can easily implant and give rise to ascitic tumor cells growing in plasma-derived exudate. Both tumor size and accumulation of ascites are inversely associated with survival [61].

Some of the EOC clinical features (failure to early disease detection, resistance to chemotherapy, high rate of recurrence) have been recently ascribed to the presence, as in other solid human cancer types, of cancer stem cells (CSCs) a rare cell subpopulation that maintain their tumorigenic potential after cytotoxic therapy.

These tumor characteristics well fit with the known properties of CSC such as quiescence and elevated multidrug resistance activity, leading to insensitivity to cytotoxic drugs, and multipotency, resulting in diversity in histological phenotype associated with ovarian cancer. A large number of studies on tumor-initiating stem cells in hematological and solid cancers have been published (for review see [62]), although very few reports addressed the role of CSCs in EOC. Stem and progenitor-like cells able of self-renewal, pluripotency, differentiation in vitro and in vivo have been recently isolated from human epithelial ovarian cancers [63]. It was also hypothesized that putative ovarian CSC possesses an altered mitochondrial phenotype associated to its evolution towards tumorigenesis. Few studies showed the isolation of putative mouse ovarian CSC endowed with clonogenic, tumorigenic activity in vivo, and enhanced chemo-resistance in vitro [64, 65]. Another recent evidence, supporting the role of CSC in EOC, was reported by Alvero et al., describing that these cells have a distinctive genetic profile that confers them the capacity to form ascites and solid tumors, display chemoresistance, and promote tumor recurrence [66].

A further development of this research area is required to unequivocally define the contribution of CSC to human ovarian cancer development, and the signaling pathways involved. Importantly, these findings could lead to new therapeutic strategies to specifically target ovarian CSCs. Moreover, this knowledge is essential to understand the mechanisms underlying the risk factors for this important disease and is crucial for the development of effective screening protocols aimed at its early detection. On these bases, an improved understanding of the molecular biology of ovarian cancer may lead to the discovery of novel molecular targets for the treatment of ovarian cancer.

5. Chemokines in Ovarian Cancer

One novel and, possibly, extremely relevant signaling pathway in ovarian cancer development, growth, and diffusion is represented by the chemokine system. In EOC there is now evidence for a complex chemokine network regulating

autocrine/paracrine mechanisms, relevant for the biology of both normal and malignant ovarian cells [67, 68]. As outlined before, chemokines expressed in cells of tumor microenvironment can affect the type and the degree of the immune infiltrate in the tumor. Among chemokines, the CC subfamily, and particularly CCL2, is the most often expressed in ovarian cancer histotypes [69] being particularly involved in macrophage recruitment. Negus et al. [70] reported the expression of CCL2 and CCL5 in epithelial ovarian cancer cells and demonstrated the relationship between the presence of CCL2 with the extent of immune cell infiltration. More recently, the analysis of ovarian cancer ascitic fluid and ascite cells allowed the identification of the expression of CCL2,-3,-4,-5,-8, and -22, altogether with their receptors (namely, CCR1, -2a,-2b, -3,-4,-5, and-8), at mRNA and protein level [71]. However, a definite correlation between this expression pattern and the total cell counts in ascites or the stage of the disease still has not been completely reached. Indeed, tumors appear to utilize the same molecular mechanisms used by normal immune system to eliminate malignant cells. Concerning this topic, the influence of chemokines in the antitumor immune response has been described in a study that strongly supports the view that tumor-associated regulatory T cells (mediators of the immune tolerance by suppressing autoreactive T cells directed towards tumor antigens) impair the function of T effector cells in tumor-bearing patients [72]. Tumor tissue and ascites from patients with ovarian cancer contain high levels of cells with the hallmarks of regulatory T cells. These cells migrate into the tumor microenvironment in a process mediated by the chemokine CCL22 and are capable of suppressing antitumor responses. This specific recruitment of regulatory T cells represents a mechanism by which tumors may develop immune advantages and, as a consequence, the suggested inhibition of regulatory T cell migration or function using antibodies against CCL22, may represent a novel antitumor approach.

One of the main features of all solid tumors is their dependence on neovascularization. Cancer cells recruit endothelial cells through the activity of several chemokines, cytokines, and growth factors. Angiogenesis is also critical for ascites development and metastasis in ovarian cancer. The role of chemokines in tumor angiogenesis is well known, and this process is mainly controlled by chemokines of the CXC family in a negative (angiostatic chemokines, ELR-) or positive manner (angiogenic, ELR+) [73]. In particular, CXCL8/IL-8 and CXCL1-3/GROα, β and γ, and CXCL5/ENA-78 induce angiogenesis through the activation of direct mechanisms on endothelial cells [74]. In ovarian cancer, the existence of a direct relationship between the expression of angiogenic molecules and the pathological behavior of five different human ovarian cancers, xenografted in the peritoneal cavity of nude mice, was reported, demonstrating that the expression of CXCL8/IL-8 was associated with neovascularization and inversely correlated to survival [75].

Most chemokines sustain cancer cell proliferation and survival acting as autocrine factors, as reported for CXCL8 in Hey-A8 human ovarian cancer cells line blocked by specific neutralizing antibodies. Moreover, CXCL8-overexpressing cells when xenotransplanted in mice display an increased cell growth, microvessel density, and the tumorigenic rate [20]. Similarly, in vitro, IL-6 and CXCL8/IL-8 accelerate the proliferation rate of several EOC cell lines [76].

The metastatic spread of tumors is controlled by the microenvironment of the metastatic organ that supports the homing and the growth of the tumor cells. Several observations indicate that the tumor microenvironment at metastatic sites is enriched with chemokines and that tumor cells, expressing the cognate receptors, migrate and adhere in response to the chemokines promoting metastasis formation at these specific target organs.

In this respect, a very extensive research was performed on CXCL12/CXCR4 ligand/receptor pair in breast cancer, showing high expression of CXCL12 in target metastatic organs of breast tumor cells, and the expression of CXCR4 in tumor cells [77].

An important role for the CXCL12/CXCR4 axis in ovarian tumor metastasis was also identified and a correlation between the activity of this chemokine system and an enhanced intraperitoneal dissemination of EOC was described [78] (see below). However, it is clear that CXCL12 does not act by itself, and other pairs of chemokines of different families and their respective receptors are involved in metastasization. In EOC ascitic fluid and blood of patients the reduction of migration responses of monocyte/macrophages expressing several chemokine receptors (CCR1, CCR5, and CXCR4) produced in the tumor microenvironment, has been described [79]. In particular, the role of CCL11/eotaxin-1 in proliferation and invasion of ovarian cancer cells was analyzed. EOC overexpressed CCR2, 3, and 5, the cognate receptors of CCL11, with a strong positive correlation between tumor grade and the levels of each of these receptors. Interestingly, the inhibition of CXCL11 activity by neutralizing antibodies significantly increases cis-platinum response in ovarian carcinoma cells [80].

6. CXCL12/CXCR4 Expression in Ovarian Cancer and Its Role in Tumor Cell Proliferation and Metastases

In 2001 and 2002 Scotton et al. investigated, for the first time, the possible role of CXCL12 in ovarian cancer [25, 81]. In particular the expression of chemokines and their receptors was reported in ten primary ovarian tumors, six cell lines, and twenty ovarian cancer ascites. From these studies it was shown that CXCR4 was the only chemokine receptor expressed in these cells. Its expression was evidenced not only in tumor cells but also in mononuclear and endothelial cells within the tumor tissues; whereas endothelial cells in tissues adjacent to the tumors were negative. CXCR4 was also expressed in almost all the cells derived from ascites, and high concentrations of CXCL12 were detected in all ascitic fluids analyzed.

Interestingly, CXCR4 and CXCL12 are not involved in the biology of normal ovarian epithelium since their

expression was not detected in normal ovarian tissues and in healthy women with family history of ovarian cancer. In contrast, papillary serous and endometrioid ovarian tumors display a high expression of CXCL12. Low-grade ovarian cancers were reported to have a differential pattern of expression, with benign mucinous tumors negative but serous benign positive for the expression of this chemokine [25].

Subsequently, CXCR4 and CXCL12 expression in ovarian cancer have been confirmed by other studies [78, 82, 83].

More recent detailed analysis showed the expression of CXCR4 and CXCL12 also in normal ovary, but their localization was confined to the follicular cells and it was not detected in normal epithelium [82]. However, in consideration that 91% and 59% of ovarian cancers express CXCL12 and CXCR4, respectively, it was proposed that an overexpression of this chemokine system was present in ovarian malignant cells. In addition, the CXCR4/CXCL12 expression was associated with unfavorable prognosis with significantly reduced median disease progression-free survival [82]. Similarly, Kajiyama et al. [78] demonstrated that in patients whose tumors were positive for the expression of CXCR4, the overall survival was significantly worse than in patients negative for CXCR4 expression. Moreover, the level of CXCL12 in the ascites was directly related to the stages of disease.

Thus, even if the published studies individually examined a small number of ovarian cancers, all these results agree that CXCL12/CXCR4 axis may be closely associated with the development of peritoneal metastasis and the prognosis of patients with epithelial ovarian carcinoma (EOC).

In vitro studies directly demonstrated that, in the presence of CXCL12, CXCR4 controls both ovarian cancer cell proliferation and migration, through the activation of the ERK1/2 and Akt pathway [40]. In addition, it was also demonstrated that CXCL12 effects on ovarian cancer cell lines are mediated by EGFR transactivation through a mechanism involving the activity of cytosolic tyrosine kinases, belonging to the c-Src family [84].

Inhibition of CXCR4 activity reduces intraperitoneal dissemination of ovarian cancer xenografts. In vitro, CXCL12 induces cell migration and invasion of the IGROV ovarian cancer cell line. Human peritoneal mesothelial cells (HPMCs), lining the peritoneal cavity, bind to EOC in the initial step of peritoneal metastasis. CXCL12 is predominantly expressed in HPMCs rather than in EOC cells, while CXCR4 was found in both EOC and HPMCs, thus creating an extracellular chemotactic milieu for EOC migration. Moreover, coculture experiments, using HPMCs and EOC, showed a strong increase in CXCL12 release, suggesting that some tumor-derived factors upregulate CXCL12 levels in ascites. TGFβ1 may be one of these factors increasing CXCL12 production. AMD3100, a potent CXCR4 antagonist, reduced the formation of peritoneal metastasis in an in vivo experimental model, even if no significant differences in survival were observed between mice treated with AMD3100 and control mice [78]. Clinical trials should become feasible with the development of novel orally available CXCR4 inhibitors.

Ovarian cancer metastasizes preferentially to local lymph nodes and peritoneum and, in contrast with breast cancer, only rarely in other organs such as liver, lung, and bones.

Ovarian and breast cancers share the overexpression of HER2/neu, a member of the EGFR family. HER2/neu increases the metastatic potential in murine and human cell lines and induces mammary tumors and lung metastases in transgenic animal models [85]. CXCR4 plays an important role in targeting the metastasis of breast cancer. Malignant breast cancer cells express CXCR4, invade the extracellular matrix, and circulate in the blood and lymphatic vessels attracted by CXCL12 that is abundantly released by target metastatic organs. Recently, it has been demonstrated that the overexpression of HER2/neu in breast cancer enhances the metastatic potential through the upregulation of CXCR4, providing a link between CXCR4 and HER2/neu in tumor progression and metastasis [86].

A similar study was performed in a cohort of 148 ovarian tumor samples by immunohistochemistry on tissue microarrays [87]. HER2/neu overexpression was found in a quarter of malignant tumors and associated with significantly shorter overall survival, in agreement with the majority of publications. However, HER2/neu positive patients did not show a higher expression of cytoplasmic CXCR4 staining, which was positive in over half of the cases and closely correlated with CXCL12 expression [87]. The lack of influence of CXCR4 in ovarian cancer could reflect the peculiar characteristics of the metastatic process in ovarian cancer as compared to other cancers such as colon [88], non-small-cell lung cancer [89], gliomas [90], malignant melanomas [28], oral squamous carcinoma [91], and adult acute myeloid leukemia [92]. In these types of cancer, distant metastasis, favored by CXCL12 expression on target organs, significantly influences the survival, while in ovarian cancer the recurrence in the pelvis and in the peritoneum is the main cause for death. Nevertheless, it needs to take in account that, in this study, CXCR4 and HER2/neu expression was evaluated in paraffin-embedded tissues blocks and their histology and grading was related with survival of patients, without analysis of the metastatic sites.

Thus, the presence of high level of CXCL12 in the ascitic fluids as well as the inhibition of intraperitoneal dissemination of ovarian cancer xenografts by CXCR4 antagonist suggests that CXCR4/CXCL12 axis may be important in the invasion of ovarian cancer cells and further studies will be necessary to better deepen this important question.

The published studies dealing with the expression of CXCL12 and CXCR4 in human ovarian cancer cell lines, tumor biopsies and ascite cells are summarized in Tables 1 and 2, respectively.

Interestingly, also in other gynecological cancers, such as cervical and endometrial carcinomas, chemokines were reported to have a pathogenic role.

Most of the early studies on the role of CXCR4 in cervical cancer were performed using the HeLa cell line (cervical adenocarcinoma-derived cell), the first immortalized cell line developed for research purposes [95, 96]. CXCL12 stimulation of HeLa cells induces increase of intracellular calcium concentrations, activation of ERK1/2 MAP kinase,

Table 1: CXCR4 and CXCL12 expression in human ovarian cancer cell lines.

Ovarian cancer cell lines	CXCR4 expression		CXCL12 expression		References
	Protein	mRNA	Protein	mRNA	
IGROV, CAOV-3, PEO1, PEO14	X	X	X	X	[81]
OVCAR-3, SKOV-3	Not detected	Not detected	Not detected	Not detected	[81]
IGROV, CAOV-3			X	X	[25]
OC 314, OC 315, OC 316	X	X		X	[40]
SKOV-3, RMG-I, NOS-2, KOC-7C,	X		X	X	[78]
ES2, NOS-4	X				[78]
BG-1	X	X	X	X	[93, 94]

Table 2: CXCR4 and CXCL12 expression in human ovarian carcinomas and tumor ascite cells.

Ovarian carcinoma	CXCR4 expression		CXCL12 expression		References
Solid tumors (positive/total, %)	Protein	mRNA	Protein	mRNA	
8/10, 80%	X	X	X	X	[81]
19/20, 95%		X		X	[81]
16/18, 88%	X		X		[25]
26/44, 60%	X				[82]
40/44, 91%			X		[82]
27/30 (metastatic), 91%			X		[82]
23/30 (metastatic), 77%	X				[82]
12/12, 100%	X	X	X	X	[83]
119/128, 93%	X				[87]
128/128, 100%			X		[87]
16/36, 44%	X				[78]
Ascites (positive/total, %)					
63/63, 100%			X		[81]
26/26, 100%			X		[78]

PI3K-Akt and Jak-STAT pathways; all these signals cooperate in cell migration and spreading. Studies on human cervical carcinoma (HCC) demonstrated high expression of CXCR4 in HCC-derived cell lines and in tissue sections, while normal cervical epithelium was negative [97]. CXCL12 binding to CXCR4 induces cell movement, cytoskeleton reorganization, and gene activation, synergizing with hepatocyte growth factor. In fact, metastasization of cervical adenocarcinoma or squamous cell carcinomas is more frequent in tumors expressing high levels of CXCR4 than tumors that express either low levels or are negative for CXCR4 [98, 99]. Also CCR7 levels in cervical adenocarcinoma/squamous cells are associated with invasion of lymph nodes as well as tumor cell proliferation and survival. CXCR4 and CCR7 expression is significantly higher in patients with larger tumor size, deep stromal invasion, lymph-vascular space involvement, or lymph node metastasis [100]. As far as endometrial cancer, studies on CXCR4 and CXCL12 expression revealed that CXCR4 is overexpressed in endometrial cancers as compared with normal tissues, whereas CXCL12 was overexpressed in normal mucosa. In addition, in vivo cell migration may be contrasted by CXCR4 neutralizing monoclonal antibody that reduces size and number of the metastasis in all target organs (peritoneum, lung, liver) [101]. However, a different study shows that CXCR4 expression was inversely related to tumor grade and patient outcome [100]. On the contrary, no difference between cancer and normal tissues was reported as far as CXCR7 expression [101].

7. CXCR4/CXCL12 and Primordial Germ Cells Development

Ovarian germ cell tumors (OGCTs) account for about 20% of all ovarian neoplasms and constitute the second largest group of ovarian cancer mainly affecting young women (58% of all ovarian tumors in women younger than the age 20 years). They histogenetically derive from primordial germ cells and differ with regard to clinical presentation, tumor biology, and histology. OGCTs include both benign (predominantly) and malignant (MOGCT) subtypes. MOGCTs are rare but aggressive and very curable tumors, accounting for approximately 1-2% of all ovarian malignancies [102].

In most organisms primordial germ cells (PGCs) migrate through developing embryo to reach the location where the gonad develop. In this process somatic cells of the gonads support the proper development of germ cells, the lineage that gives rise to sperm and eggs.

In mice lacking CXCL12, the colonization of gonads by PGCs is impaired [103]. CXCR4 is expressed in mouse germ cells; whereas expression of its ligand is high in the genital, the target of the migrating cells. In fact, CXCL12 is essential for homing of PGCs into genital ridges but is not required for direct migration through tissues of embryos [104, 105]. Different studies demonstrated that in mouse germ cell migration and survival requires the CXCL12/CXCR4 interaction [106, 107].

Recently, it was reported that also the other CXCL12 receptor, CXCR7, takes an active part in the migration of PGCs; knockdown of CXCR7 gene results in impaired polarity and aberrant migration of PGCs [108]. Unlike CXCR4, CXCR7 function was found to be required in tissues surrounding the migrating cells (where it is found primarily in intracellular structures) rather than in PGCs. It was suggested that the key role of CXCR7 is to bind and internalize CXCL12 thereby controlling the level of the diffusible chemokine in the extracellular space [108]. Thus, CXCR7 may act as a high-affinity decoy receptor to facilitate the migration of PGCs by shaping the distribution of the chemokine in the environment [109].

CXCR4 and CXCL12 control cell migration in several normal and pathological conditions [103–105, 108, 109]. In neonatal mice CXCL12/CXCR4 signalling contributes to maintain the size and longevity of the primordial follicle pool [106]. CXCL12/SDF1α and β (but not CXCL12/SDF1γ) transcript variants were identified in cultured neonatal and adult ovary by microarray analysis and RT-PCR. In neonatal tissues both CXCL12 and CXCR4 display similar expression pattern. They are detected in primordial and primary/secondary oocytes with lower level of staining in the interstitial tissues and granulosa cells. The primordial oocytes are essentially "resting" cells with limited metabolic capability suggesting that the presence of CXCR4/CXCL12 may be with index of an essential role for this chemokin system within the follicle [106].

Recently it was proposed an innovative hypothesis concerning the cell type by which ovarian epithelial tumors may rise [110]. In fact, it was widely accepted that the origin of epithelial ovarian tumors derives from the mesothelial cell layer lining the ovary surface (ovarian coelomic epithelium). However, it was observed that ovarian epithelial neoplasms are remarkably similar to epithelial cells from extra-ovarian sites in the female reproductive tract. The three most common subtypes of these tumors, referred as serous, endometrioid, and mucinous are morphologically identical to carcinomas of the fallopian tube, endometrium, and endocervix, respectively. It has been suggested that ovarian epithelial cells could arise from tissues that are embryological derived from the Mullerian ducts.

In other types of cancer the physiological role of CXCR4/CXCL12, carried out during the embryonic development, is turned in the ability to influence cell migration and spreading in cancer. Thus, studying CXCR4/CXCL12 function in epithelial ovarian cancer, the choice of an incorrect cell controls could bring wrong conclusions. It could be possible that a deregulated CXCR4/CXCR7/CXCL12 axis could be already evident in early stage of illness.

8. CXCR4 as a Potential Therapeutic Target

All the data previously discussed provide the rationale for targeting CXCR4 in cancer. In particular, this notion is further supported by the different mechanisms resulting from CXCR4 inhibition: (a) the reversal of stromal cell interactions responsible of tumor cell survival; (b) the blockade of proangiogenic activity of CXCL12 and the reduction of the dissemination and migration ability of tumor cells; (c) the blockade of tumor growth through autocrine/paracrine signaling mediated by CXCL12/CXCR4 interaction; (d) the mobilization of tumor cells from tissues to increase their sensitivity to conventional chemoterapeutic agents.

On these premises, several molecules able to antagonize CXCR4 activity in response to CXCL12 have been identified. To date four types of CXCR4 inhibitors have been described: (1) peptide-based antagonists, (2) nonpeptidic antagonists; (3) neutralizing antibodies for CXCR4; (4) modified CXCL12 peptides, endowed of antagonistic activity.

The first small peptide antagonists of CXCR4 (named T22, T134, and T140) [111, 112] were discovered screening compounds with potential anti-HIV-1 activity (CXCR4 acts also as coreceptor in T-cell line tropic HIV infection [95, 96]). In particular T22 blocks CXCR4 activity by binding the receptor region involved in HIV-1 entry into the cell. T140, the most active antagonist [113], is a 14-residues peptide with the main limitation in the low stability in serum. Thus, this shortcome was overcome by modified structural analogs (T14003 and TC14012) [114]. Recently, T140 synthesize as cyclic peptide (FC131) [115], and a new antagonist (named POL3026) [116], with better pharmacokinetic properties and potent CXCR4 antagonist activity, have been described. In cancer, T140 efficacy to block CXCR4 has been reported in different tumor models in vivo and in vitro, including leukemia [117], breast [118] and lung cancers [119], and malignant melanoma [120]. T140 and TN14003 are currently in clinical development for B-cell homing [117].

Among nonpeptidic small molecules, cyclams and bicyclams such as AMD3100 are endowed of CXCR4 antagonistic properties [121] and weak partial agonist activity [122–124].

Antitumor efficacy of AMD3100 was demonstrated in breast cancer where it blocks CXCL12-induced HER2/neu activation in vitro [125] and inhibits tumor growth in vivo [126]. Its antineoplastic activity has been also demonstrated in pancreatic cancer cells, colorectal and glioblastoma tumor xenografts [127–129]. Moreover, playing CXCR4 a key role in cross-talk between leukemia cells and their microenvironment, the potential use of this drug in hematological cancer has been widely studied and it is currently used in clinics for the mobilization of hematopoietic stem cells [130, 131].

Indeed, in hematological malignancies, tumor cells use CXCR4 for dissemination and progression of the disease, because interactions of CXCR4 with its ligand are critical for hematopoietic cells trafficking and homing to lymphatic tissues [132]. Stromal cells within bone marrow microenvironment constitutively secrete CXCL12 and the activation of CXCR4 induces leukemia cell migration to

the marrow microenvironment, providing growth and drug resistance signals. AMD3100 is able to mobilize leukemia cells from their stromal microenvironment and inhibits adhesive tumor-stroma interactions, thus making leukemia cells accessible to conventional drugs [133].

AMD3100 has also been used as agent that disrupts interaction with the bone marrow microenvironment in multiple myeloma cells [134] and in between mantle cell lymphoma cells [135]. Therefore, targeting the CXCR4/CXCL12 axis is attractive therapeutic approach in leukemia patients. It was shown that in leukemia cells, several growth and survival factors from the tumor microenvironment, including CXCR4 activation, induce PI3K activation. Therefore, the activity of isoform-selective PI3K inhibitors was investigated to indirectly block CXCL12/CXCR4 signals in chronic cell leukemia leading migration, stromal cell interactions, and stromal cell-mediated drug resistance [136].

AMD3100 was successfully used in both in vitro and in vivo experiments carried out in ovarian cancer cells [25].

Antibodies against CXCR4 have been reported to affect HIV-1 infection and cancer cell migration [137]. The limiting point for monoclonal antibody therapeutic development is due to the high frequency of heterogeneous conformation of CXCR4 and posttranslational modification that reduce the antibody specificity and function [138].

Antichemokine activity was also identified in some natural compounds. Soybean and cruciferous vegetables have been implicated in the protection against spontaneous and carcinogen-induced cancers although the mechanisms for this anticarcinogenicity are not fully elucidated. Epidemiologic studies in Asian women indicate that consumption of a traditional diet high in soy confers significant protection against breast cancer [139].

3,3′-Diindolylmethane (DIM) and genistein, dietary phytoestrogens, belonging to isoflavone class of flavonoids, have anticarcinogenic activities [140]. Recently, Hsu et al. demonstrated that DIM inhibits the chemotactic and invasive potential of breast and ovarian cancer cells especially through an estrogen-independent mechanism, reducing the chemotaxis towards CXCL12. In addition, downregulation of CXCR4 and CXCL12 and inhibition of chemotaxis and chemoinvasion in breast and ovarian cancer cells toward CXCL12 are among of the biological effects of genistein [93] likely through the inhibition of the estrogen dependent CXCL12 mRNA synthesis.

9. Conclusions

One of the topics emerging from this review is that ovarian cancer growth and metastasis can be controlled by immunomodulatory and chemotactic chemokines. Indeed, chemokine/chemokine receptor systems attract increasing attention as anticancer strategies due to their direct involvement in almost every aspect of tumorigenesis. Thus, further biology and pharmacology studies have to be developed to fully address the chemokinergic system as an ideal target for the inhibition of tumor proliferation, angiogenesis, invasion, and metastasization.

References

[1] C. Krieg and O. Boyman, "The role of chemokines in cancer immune surveillance by the adaptive immune system," *Seminars in Cancer Biology*, vol. 19, no. 2, pp. 76–83, 2009.

[2] I. F. Charo and R. M. Ransohoff, "The many roles of chemokines and chemokine receptors in inflammation," *The New England Journal of Medicine*, vol. 354, no. 6, pp. 610–621, 2006.

[3] J. B. Rubin, "Chemokine signaling in cancer: one hump or two?" *Seminars in Cancer Biology*, vol. 19, no. 2, pp. 116–122, 2009.

[4] A. Zlotnik and O. Yoshie, "Chemokines: a new classification system and their role in immunity," *Immunity*, vol. 12, no. 2, pp. 121–127, 2000.

[5] P. Loetscher, B. Moser, and M. Baggiouni, "Chemokines and their receptors in lymphocyte traffic and HIV infection," *Advances in Immunology*, no. 74, pp. 127–180, 2000.

[6] M. Baggiolini, "Chemokines and leukocyte traffic," *Nature*, vol. 392, no. 6676, pp. 565–568, 1998.

[7] A. Bajetto, R. Bonavia, S. Barbero, T. Florio, and G. Schettini, "Chemokines and their receptors in the central nervous system," *Frontiers in Neuroendocrinology*, vol. 22, no. 3, pp. 147–184, 2001.

[8] A. Bajetto, R. Bonavia, S. Barbero, et al., "Glial and neuronal cells express functional chemokine receptor CXCR4 and its natural ligand stromal cell-derived factor 1," *Journal of Neurochemistry*, vol. 73, no. 6, pp. 2348–2357, 1999.

[9] R. J. Miller, W. Rostene, E. Apartis, et al., "Chemokine action in the nervous system," *Journal of Neuroscience*, vol. 28, no. 46, pp. 11792–11795, 2008.

[10] A. Guyon, F. Massa, C. Rovère, and J.-L. Nahon, "How cytokines can influence the brain: a role for chemokines?" *Journal of Neuroimmunology*, vol. 198, no. 1-2, pp. 46–55, 2008.

[11] A. Bajetto, S. Barbero, R. Bonavia, et al., "Stromal cell-derived factor-1α induces astrocyte proliferation through the activation of extracellular signal-regulated kinases 1/2 pathway," *Journal of Neurochemistry*, vol. 77, no. 5, pp. 1226–1236, 2001.

[12] S. Melik-Parsadaniantz and W. Rostene, "Chemokines and neuromodulation," *Journal of Neuroimmunology*, vol. 198, no. 1-2, pp. 62–68, 2008.

[13] F. Balkwill, "The significance of cancer cell expression of the chemokine receptor CXCR4," *Seminars in Cancer Biology*, vol. 14, no. 3, pp. 171–179, 2004.

[14] J. A. Burger and T. J. Kipps, "CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment," *Blood*, vol. 107, no. 5, pp. 1761–1767, 2006.

[15] J. Vandercappellen, J. Van Damme, and S. Struyf, "The role of CXC chemokines and their receptors in cancer," *Cancer Letters*, vol. 267, no. 2, pp. 226–244, 2008.

[16] A. Ben-Baruch, "Organ selectivity in metastasis: regulation by chemokines and their receptors," *Clinical and Experimental Metastasis*, vol. 25, no. 4, pp. 345–356, 2008.

[17] B. C. Sheu, W. C. Chang, C. Y. Cheng, H. H. Lin, D. Y. Chang, and S. C. Huang, "Cytokine regulation networks in the cancer microenvironment," *Frontiers in Bioscience*, vol. 13, pp. 6255–6268, 2008.

[18] R. M. Strieter, M. D. Burdick, J. Mestas, B. Gomperts, M. P. Keane, and J. A. Belperio, "Cancer CXC chemokine networks and tumour angiogenesis," *European Journal of Cancer*, vol. 42, no. 6, pp. 768–778, 2006.

[19] M. L. Balestrieri, A. Balestrieri, F. P. Mancini, and C. Napoli, "Understanding the immunoangiostatic CXC chemokine network," *Cardiovascular Research*, vol. 78, no. 2, pp. 250–256, 2008.

[20] L. Xu and I. J. Fidler, "Interleukin 8: an autocrine growth factor for human ovarian cancer," *Oncology Research*, vol. 12, no. 2, pp. 97–106, 2000.

[21] J. A. Burger and A. Burkle, "The CXCR4 chemokine receptor in acute and chronic leukaemia: a marrow homing receptor and potential therapeutic target," *British Journal of Haematology*, vol. 137, no. 4, pp. 288–296, 2007.

[22] A. Müller, B. Homey, H. Soto, et al., "Involvement of chemokine receptors in breast cancer metastasis," *Nature*, vol. 410, no. 6824, pp. 50–56, 2001.

[23] I. S. Zeelenberg, L. Ruuls-Van Stalle, and E. Roos, "The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases," *Cancer Research*, vol. 63, no. 13, pp. 3833–3839, 2003.

[24] D. J. J. Waugh, C. Wilson, A. Seaton, and P. J. Maxwell, "Multi-faceted roles for CXC-chemokines in prostate cancer progression," *Frontiers in Bioscience*, vol. 13, no. 12, pp. 4595–4604, 2008.

[25] C. J. Scotton, J. L. Wilson, K. Scott, et al., "Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer," *Cancer Research*, vol. 62, no. 20, pp. 5930–5938, 2002.

[26] J. B. Rubin, A. L. Kung, R. S. Klein, et al., "A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13513–13518, 2003.

[27] A. Bajetto, F. Barbieri, A. Dorcaratto, et al., "Expression of CXC chemokine receptors 1-5 and their ligands in human glioma tissues: role of CXCR4 and SDF1 in glioma cell proliferation and migration," *Neurochemistry International*, vol. 49, no. 5, pp. 423–432, 2006.

[28] S. Scala, A. Ottaiano, P. A. Ascierto, et al., "Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma," *Clinical Cancer Research*, vol. 11, no. 5, pp. 1835–1841, 2005.

[29] F. Barbieri, A. Bajetto, R. Stumm, et al., "Overexpression of stromal cell-derived factor 1 and its receptor CXCR4 induces autocrine/paracrine cell proliferation in human pituitary adenomas," *Clinical Cancer Research*, vol. 14, no. 16, pp. 5022–5032, 2008.

[30] A. Bajetto, F. Barbieri, A. Pattarozzi, et al., "CXCR4 and SDF1 expression in human meningiomas: a proliferative role in tumoral meningothelial cells in vitro," *Neuro-Oncology*, vol. 9, no. 1, pp. 3–11, 2007.

[31] F. Barbieri, A. Bajetto, C. Porcile, et al., "CXC receptor and chemokine expression in human meningioma: SDF1/CXCR4 signaling activates ERK1/2 and stimulates meningioma cell proliferation," *Annals of the New York Academy of Sciences*, vol. 1090, pp. 332–343, 2006.

[32] T. Nagasawa, S. Hirota, K. Tachibana, et al., "Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1," *Nature*, vol. 382, no. 6592, pp. 635–638, 1996.

[33] Q. Ma, D. Jones, P. R. Borghesani, et al., "Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 16, pp. 9448–9453, 1998.

[34] K. Balabanian, B. Lagane, S. Infantino, et al., "The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes," *The Journal of Biological Chemistry*, vol. 280, no. 42, pp. 35760–35766, 2005.

[35] J. M. Burns, B. C. Summers, Y. Wang, et al., "A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development," *Journal of Experimental Medicine*, vol. 203, no. 9, pp. 2201–2213, 2006.

[36] S. Barbero, R. Bonavia, A. Bajetto, et al., "Stromal cell-derived factor 1α stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt," *Cancer Research*, vol. 63, no. 8, pp. 1969–1974, 2003.

[37] T. Florio, S. Casagrande, F. Diana, et al., "Chemokine stromal cell-derived factor 1α induces proliferation and growth hormone release in GH4C1 rat pituitary adenoma cell line through multiple intracellular signals," *Molecular Pharmacology*, vol. 69, no. 2, pp. 539–546, 2006.

[38] A. Massa, S. Casagrande, A. Bajetto, et al., "SDF-1 controls pituitary cell proliferation through the activation of ERK1/2 and the Ca^{2+}-dependent, cytosolic tyrosine kinase Pyk2," *Annals of the New York Academy of Sciences*, vol. 1090, pp. 385–398, 2006.

[39] G. Venkatakrishnan, R. Salgia, and J. E. Groopman, "Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells," *The Journal of Biological Chemistry*, vol. 275, no. 10, pp. 6868–6875, 2000.

[40] C. Porcile, A. Bajetto, F. Barbieri, et al., "Stromal cell-derived factor-1α (SDF-1α/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation," *Experimental Cell Research*, vol. 308, no. 2, pp. 241–253, 2005.

[41] A. Pattarozzi, M. Gatti, F. Barbieri, et al., "17β-estradiol promotes breast cancer cell proliferation-inducing stromal cell-derived factor-1-mediated epidermal growth factor receptor transactivation: reversal by gefitinib pretreatment," *Molecular Pharmacology*, vol. 73, no. 1, pp. 191–202, 2008.

[42] N. Cabioglu, J. Summy, C. Miller, et al., "CXCL-12/stromal cell-derived factor-1α transactivates HER2-neu in breast cancer cells by a novel pathway involving Src kinase activation," *Cancer Research*, vol. 65, no. 15, pp. 6493–6497, 2005.

[43] S. R. Chinni, H. Yamamoto, Z. Dong, A. Sabbota, R. D. Bonfil, and M. L. Cher, "CXCL12/CXCR4 transactivates HER2 in lipid rafts of prostate cancer cells and promotes growth of metastatic deposits in bone," *Molecular Cancer Research*, vol. 6, no. 3, pp. 446–457, 2008.

[44] T. A. Gonda, S. Tu, and T. C. Wang, "Chronic inflammation, the tumor microenvironment and carcinogenesis," *Cell Cycle*, vol. 8, no. 13, pp. 2005–2013, 2009.

[45] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[46] G. P. Dunn, L. J. Old, and R. D. Schreiber, "The immunobiology of cancer immunosurveillance and immunoediting," *Immunity*, vol. 21, no. 2, pp. 137–148, 2004.

[47] H. Harlin, Y. Meng, A. C. Peterson, et al., "Chemokine expression in melanoma metastases associated with $CD8^+$ T-ceII recruitment," *Cancer Research*, vol. 69, no. 7, pp. 3077–3085, 2009.

[48] Y.-Q. Liu, R. T. Poon, J. Hughes, Q.-Y. Li, W.-C. Yu, and S.-T. Fan, "Desensitization of T lymphocyte function by CXCR3 ligands in human hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 11, no. 2, pp. 164–170, 2005.

[49] F. Vianello, N. Papeta, T. Chen, et al., "Murine B16 melanomas expressing high levels of the chemokine stromal-derived factor-1/CXCL12 induce tumor-specific T cell chemorepulsion and escape from immune control," *The Journal of Immunology*, vol. 176, no. 5, pp. 2902–2914, 2006.

[50] M. C. Poznansky, I. T. Olszak, R. Foxall, R. H. Evans, A. D. Luster, and D. T. Scadden, "Active movement of T cells away from a chemokine," *Nature Medicine*, vol. 6, no. 5, pp. 543–548, 2000.

[51] J. Permuth-Wey and T. A. Sellers, "Epidemiology of ovarian cancer," *Methods in Molecular Biology*, vol. 472, pp. 413–437, 2009.

[52] S. A. Cannistra, "Cancer of the ovary," *The New England Journal of Medicine*, vol. 351, no. 24, pp. 2519–2529, 2004.

[53] R. F. Ozols, B. N. Bundy, B. E. Greer, et al., "Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study," *Journal of Clinical Oncology*, vol. 21, no. 17, pp. 3194–3200, 2003.

[54] J. Hall, J. Paul, and R. Brown, "Critical evaluation of p53 as a prognostic marker in ovarian cancer," *Expert Reviews in Molecular Medicine*, vol. 6, no. 12, pp. 1–20, 2004.

[55] S. L. Edwards, R. Brough, C. J. Lord, et al., "Resistance to therapy caused by intragenic deletion in BRCA2," *Nature*, vol. 451, no. 7182, pp. 1111–1115, 2008.

[56] B. Aunoble, R. Sanches, E. Didier, and Y. J. Bignon, "Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer (review)," *International Journal of Oncology*, vol. 16, no. 3, pp. 567–576, 2000.

[57] R. C. Bast Jr., B. Hennessy, and G. B. Mills, "The biology of ovarian cancer: new opportunities for translation," *Nature Reviews Cancer*, vol. 9, no. 6, pp. 415–428, 2009.

[58] Y. Yarden, "The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities," *European Journal of Cancer*, vol. 37, supplement 4, pp. S3–S8, 2001.

[59] J. Reibenwein and M. Krainer, "Targeting signaling pathways in ovarian cancer," *Expert Opinion on Therapeutic Targets*, vol. 12, no. 3, pp. 353–365, 2008.

[60] A. P. G. Crijns, E. W. Duiker, S. de Jong, P. H. B. Willemse, A. G. J. van der Zee, and E. G. E. de Vries, "Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy," *International Journal of Gynecological Cancer*, vol. 16, supplement 1, pp. 152–165, 2006.

[61] M. E. van der Burg, "Advanced ovarian cancer," *Current Treatment Options in Oncology*, vol. 2, no. 2, pp. 109–118, 2001.

[62] L. L. C. Marotta and K. Polyak, "Cancer stem cells: a model in the making," *Current Opinion in Genetics and Development*, vol. 19, no. 1, pp. 44–50, 2009.

[63] S. A. Bapat, A. M. Mali, C. B. Koppikar, and N. K. Kurrey, "Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer," *Cancer Research*, vol. 65, no. 8, pp. 3025–3029, 2005.

[64] A. A. Wani, N. Sharma, Y. S. Shouche, and S. A. Bapat, "Nuclear-mitochondrial genomic profiling reveals a pattern of evolution in epithelial ovarian tumor stem cells," *Oncogene*, vol. 25, no. 47, pp. 6336–6344, 2006.

[65] P. P. Szotek, R. Pieretti-Vanmarcke, P. T. Masiakos, et al., "Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian inhibiting substance responsiveness," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 30, pp. 11154–11159, 2006.

[66] A. B. Alvero, R. Chen, H.-H. Fu, et al., "Molecular phenotyping of human ovarian cancer stem cells unravel the mechanisms for repair and chemo-resistance," *Cell Cycle*, vol. 8, no. 1, pp. 158–166, 2009.

[67] F. Burke and F. R. Balkwill, "Cytokines in animal models of cancer," *Biotherapy*, vol. 8, no. 3-4, pp. 229–241, 1996.

[68] M. A. Nash, G. Ferrandina, M. Gordinier, A. Loercher, and R. S. Freedman, "The role of cytokines in both the normal and malignant ovary," *Endocrine-Related Cancer*, vol. 6, no. 1, pp. 93–107, 1999.

[69] A. Mantovani, P. Allavena, S. Sozzani, A. Vecchi, M. Locati, and A. Sica, "Chemokines in the recruitment and shaping of the leukocyte infiltrate of tumors," *Seminars in Cancer Biology*, vol. 14, no. 3, pp. 155–160, 2004.

[70] R. P. M. Negus, G. W. H. Stamp, J. Hadley, and F. R. Balkwill, "Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines," *American Journal of Pathology*, vol. 150, no. 5, pp. 1723–1734, 1997.

[71] D. Milliken, C. Scotton, S. Raju, F. Balkwill, and J. Wilson, "Analysis of chemokines and chemokine receptor expression in ovarian cancer ascites," *Clinical Cancer Research*, vol. 8, no. 4, pp. 1108–1114, 2002.

[72] T. J. Curiel, G. Coukos, L. Zou, et al., "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival," *Nature Medicine*, vol. 10, no. 9, pp. 942–949, 2004.

[73] R. M. Strieter, J. A. Belperio, R. J. Phillips, and M. P. Keane, "CXC chemokines in angiogenesis of cancer," *Seminars in Cancer Biology*, vol. 14, no. 3, pp. 195–200, 2004.

[74] B. Homey, A. Müller, and A. Zlotnik, "Chemokines: agents for the immunotherapy of cancer?" *Nature Reviews Immunology*, vol. 2, no. 3, pp. 175–184, 2002.

[75] J. Yoneda, H. Kuniyasu, M. A. Crispens, J. E. Price, C. D. Bucana, and I. J. Fidler, "Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice," *Journal of the National Cancer Institute*, vol. 90, no. 6, pp. 447–454, 1998.

[76] Y. Wang, J. Yang, Y. Gao, et al., "Regulatory effect of e2, IL-6 and IL-8 on the growth of epithelial ovarian cancer cells," *Cellular & Molecular Immunology*, vol. 2, no. 5, pp. 365–372, 2005.

[77] M. Z. Dewan, S. Ahmed, Y. Iwasaki, K. Ohba, M. Toi, and N. Yamamoto, "Stromal cell-derived factor-1 and CXCR4 receptor interaction in tumor growth and metastasis of breast cancer," *Biomedicine and Pharmacotherapy*, vol. 60, no. 6, pp. 273–276, 2006.

[78] H. Kajiyama, K. Shibata, M. Terauchi, K. Ino, A. Nawa, and F. Kikkawa, "Involvement of SDF-1α/CXCR4 axis in the enhanced peritoneal metastasis of epithelial ovarian carcinoma," *International Journal of Cancer*, vol. 122, no. 1, pp. 91–99, 2008.

[79] R. S. Freedman, Q. Ma, E. Wang, et al., "Migration deficit in monocyte-macrophages in human ovarian cancer," *Cancer Immunology, Immunotherapy*, vol. 57, no. 5, pp. 635–645, 2008.

[80] V. Levina, B. M. Nolen, A. M. Marrangoni, et al., "Role of eotaxin-1 signaling in ovarian cancer," *Clinical Cancer Research*, vol. 15, no. 8, pp. 2647–2656, 2009.

[81] C. J. Scotton, J. L. Wilson, D. Milliken, G. Stamp, and F. R. Balkwill, "Epithelial cancer cell migration: a role for chemokine receptors?" *Cancer Research*, vol. 61, no. 13, pp. 4961–4965, 2001.

[82] Y.-P. Jiang, X.-H. Wu, B. Shi, W.-X. Wu, and G.-R. Yin, "Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression," *Gynecologic Oncology*, vol. 103, no. 1, pp. 226–233, 2006.

[83] M. Furuya, T. Suyama, H. Usui, et al., "Up-regulation of CXC chemokines and their receptors: implications for proinflammatory microenvironments of ovarian carcinomas and endometriosis," *Human Pathology*, vol. 38, no. 11, pp. 1676–1687, 2007.

[84] C. Porcile, A. Bajetto, S. Barbero, P. Pirani, and G. Schettini, "CXCR4 activation induces epidermal growth factor receptor transactivation in an ovarian cancer cell line," *Annals of the New York Academy of Sciences*, vol. 1030, pp. 162–169, 2004.

[85] Y. Yarden and M. X. Sliwkowski, "Untangling the ErbB signalling network," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 2, pp. 127–137, 2001.

[86] Y. M. Li, Y. Pan, Y. Wei, et al., "Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis," *Cancer Cell*, vol. 6, no. 5, pp. 459–469, 2004.

[87] D. Pils, A. Pinter, J. Reibenwein, et al., "In ovarian cancer the prognostic influence of HER2/neu is not dependent on the CXCR4/SDF-1 signalling pathway," *British Journal of Cancer*, vol. 96, no. 3, pp. 485–491, 2007.

[88] J. Kim, H. Takeuchi, S. T. Lam, et al., "Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival," *Journal of Clinical Oncology*, vol. 23, no. 12, pp. 2744–2753, 2005.

[89] J.-P. Spano, F. Andre, L. Morat, et al., "Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome," *Annals of Oncology*, vol. 15, no. 4, pp. 613–617, 2004.

[90] A. Salmaggi, M. Gelati, B. Pollo, et al., "CXCL12 expression is predictive of a shorter time to tumor progression in low-grade glioma: a single-institution study in 50 patients," *Journal of Neuro-Oncology*, vol. 74, no. 3, pp. 287–293, 2005.

[91] A. Almofti, D. Uchida, N. M. Begum, et al., "The clinicopathological significance of the expression of CXCR4 protein in oral squamous cell carcinoma," *International Journal of Oncology*, vol. 25, no. 1, pp. 65–71, 2004.

[92] E. J. C. Rombouts, B. Pavic, B. Lowenberg, and R. E. Ploemacher, "Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia," *Blood*, vol. 104, no. 2, pp. 550–557, 2004.

[93] E. L. Hsu, N. Chen, A. Westbrook, et al., "Modulation of CXCR4, CXCL12, and tumor cell invasion potential in vitro by phytochemicals," *Journal of Oncology*, vol. 2009, Article ID 491985, 9 pages, 2009.

[94] E. L. Hsu, N. Chen, A. Westbrook, et al., "CXCR4 and CXCL12 down-regulation: a novel mechanism for the chemoprotection of 3, 3′-diindolylmethane for breast and ovarian cancers," *Cancer Letters*, vol. 265, no. 1, pp. 113–123, 2008.

[95] Y. Feng, C. C. Broder, P. E. Kennedy, and E. A. Berger, "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor," *Science*, vol. 272, no. 5263, pp. 872–877, 1996.

[96] H. Deng, R. Liu, W. Ellmeier, et al., "Identification of a major co-receptor for primary isolates of HIV-1," *Nature*, vol. 381, no. 6584, pp. 661–666, 1996.

[97] M. Majka, J. Drukala, E. Lesko, M. Wysoczynski, A. B. Jenson, and M. Z. Ratajezak, "SDF-1 alone and in co-operation with HGF regulates biology of human cervical carcinoma cells," *Folia Histochemica et Cytobiologica*, vol. 44, no. 3, pp. 155–164, 2006.

[98] Y.-C. Yang, Z.-Y. Lee, C.-C. Wu, T.-C. Chen, C.-L. Chang, and C.-P. Chen, "CXCR4 expression is associated with pelvic lymph node metastasis in cervical adenocarcinoma," *International Journal of Gynecological Cancer*, vol. 17, no. 3, pp. 676–686, 2007.

[99] J.-P. Zhang, W.-G. Lu, F. Ye, H.-Z. Chen, C.-Y. Zhou, and X. Xie, "Study on CXCR4/SDF-1α axis in lymph node metastasis of cervical squamous cell carcinoma," *International Journal of Gynecological Cancer*, vol. 17, no. 2, pp. 478–483, 2007.

[100] J. Kodama, Hasengaowa, N. Seki, T. Kusumoto, and Y. Hiramatsu, "Expression of the CXCR4 and CCR7 chemokine receptors in human endometrial cancer," *European Journal of Gynaecological Oncology*, vol. 28, no. 5, pp. 370–375, 2007.

[101] S. Gelmini, M. Mangoni, F. Castiglione, et al., "The CXCR4/CXCL12 axis in endometrial cancer," *Clinical and Experimental Metastasis*, vol. 26, no. 3, pp. 261–268, 2009.

[102] D. Pectasides, E. Pectasides, and D. Kassanos, "Germ cell tumors of the ovary," *Cancer Treatment Reviews*, vol. 34, no. 5, pp. 427–441, 2008.

[103] E. Raz, "Guidance of primordial germ cell migration," *Current Opinion in Cell Biology*, vol. 16, no. 2, pp. 169–173, 2004.

[104] T. Ara, Y. Nakamura, T. Egawa, et al., "Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 9, pp. 5319–5323, 2003.

[105] K. A. Molyneaux, H. Zinszner, P. S. Kunwar, et al., "The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival," *Development*, vol. 130, no. 18, pp. 4279–4286, 2003.

[106] J. E. Holt, A. Jackson, S. D. Roman, R. J. Aitken, P. Koopman, and E. A. McLaughlin, "CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary," *Developmental Biology*, vol. 293, no. 2, pp. 449–460, 2006.

[107] J. Stebler, D. Spieler, K. Slanchev, et al., "Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12," *Developmental Biology*, vol. 272, no. 2, pp. 351–361, 2004.

[108] B. Boldajipour, H. Mahabaleshwar, E. Kardash, et al., "Control of chemokine-guided cell migration by ligand sequestration," *Cell*, vol. 132, no. 3, pp. 463–473, 2008.

[109] H. Mahabaleshwar, B. Boldajipour, and E. Raz, "Killing the messenger: the role of CXCR7 in regulating primordial germ cell migration," *Cell Adhesion & Migration*, vol. 2, no. 2, pp. 69–70, 2008.

[110] L. Dubeau, "The cell of origin of ovarian epithelial tumours," *The Lancet Oncology*, vol. 9, no. 12, pp. 1191–1197, 2008.

[111] M. Masuda, H. Nakashima, T. Ueda, et al., "A novel anti-HIV synthetic peptide, T-22 ([$Tyr^{5,12}$, Lys^{7}]-polyphemusin II)," *Biochemical and Biophysical Research Communications*, vol. 189, no. 2, pp. 845–850, 1992.

[112] H. Tamamura, R. Arakaki, H. Funakoshi, et al., "Effective lowly cytotoxic analogs of an HIV-cell fusion inhibitor, T22 ([$Tyr^{5,12}$, Lys^{7}]-polyphemusin II)," *Bioorganic and Medicinal Chemistry*, vol. 6, no. 2, pp. 231–238, 1998.

[113] H. Tamamura, A. Omagari, K. Hiramatsu, et al., "Certification of the critical importance of L-3-(2-naphthyl)alanine at position 3 of a specific CXCR4 inhibitor, T140, leads to an

exploratory performance of its downsizing study," *Bioorganic and Medicinal Chemistry*, vol. 10, no. 5, pp. 1417–1426, 2002.

[114] H. Tamamura, A. Omagari, K. Hiramatsu, et al., "Development of specific CXCR4 inhibitors possessing high selectivity indexes as well as complete stability in serum based on an anti-HIV peptide T140," *Bioorganic and Medicinal Chemistry Letters*, vol. 11, no. 14, pp. 1897–1902, 2001.

[115] S. Ueda, S. Oishi, Z.-X. Wang, et al., "Structure-activity relationships of cyclic peptide-based chemokine receptor CXCR4 antagonists: disclosing the importance of side-chain and backbone functionalities," *Journal of Medicinal Chemistry*, vol. 50, no. 2, pp. 192–198, 2007.

[116] S. J. DeMarco, H. Henze, A. Lederer, et al., "Discovery of novel, highly potent and selective β-hairpin mimetic CXCR4 inhibitors with excellent anti-HIV activity and pharmacokinetic profiles," *Bioorganic and Medicinal Chemistry*, vol. 14, no. 24, pp. 8396–8404, 2006.

[117] M. Burger, T. Hartmann, M. Krome, et al., "Small peptide inhibitors of the CXCR4 chemokine receptor (CD184) antagonize the activation, migration, and antiapoptotic responses of CXCL12 in chronic lymphocytic leukemia B cells," *Blood*, vol. 106, no. 5, pp. 1824–1830, 2005.

[118] H. Tamamura, M. Fujisawa, K. Hiramatsu, et al., "Identification of a CXCR4 antagonist, a T140 analog, as an anti-rheumatoid arthritis agent," *FEBS Letters*, vol. 569, no. 1–3, pp. 99–104, 2004.

[119] M. Burger, A. Glodek, T. Hartmann, et al., "Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells," *Oncogene*, vol. 22, no. 50, pp. 8093–8101, 2003.

[120] M. Takenaga, H. Tamamura, K. Hiramatsu, et al., "A single treatment with microcapsules containing a CXCR4 antagonist suppresses pulmonary metastasis of murine melanoma," *Biochemical and Biophysical Research Communications*, vol. 320, no. 1, pp. 226–232, 2004.

[121] G. A. Donzella, D. Schols, S. W. Lin, et al., "AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor," *Nature Medicine*, vol. 4, no. 1, pp. 72–77, 1998.

[122] E. De Clercq, N. Yamamoto, R. Pauwels, et al., "Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 12, pp. 5286–5290, 1992.

[123] E. De Clercq, "The bicyclam AMD3100 story," *Nature Reviews Drug Discovery*, vol. 2, no. 7, pp. 581–587, 2003.

[124] E. De Clercq, "The AMD3100 story: the path to the discovery of a stem cell mobilizer (Mozobil)," *Biochemical Pharmacology*, vol. 77, no. 11, pp. 1655–1664, 2009.

[125] H. Tamamura, A. Hori, N. Kanzaki, et al., "T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer," *FEBS Letters*, vol. 550, no. 1–3, pp. 79–83, 2003.

[126] M. C. P. Smith, K. E. Luker, J. R. Garbow, et al., "CXCR4 regulates growth of both primary and metastatic breast cancer," *Cancer Research*, vol. 64, no. 23, pp. 8604–8612, 2004.

[127] F. Marchesi, P. Monti, B. E. Leone, et al., "Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4," *Cancer Research*, vol. 64, no. 22, pp. 8420–8427, 2004.

[128] K. Yasumoto, K. Koizumi, A. Kawashima, et al., "Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer," *Cancer Research*, vol. 66, no. 4, pp. 2181–2187, 2006.

[129] A. Ottaiano, R. Franco, A. A. Talamanca, et al., "Overexpression of both CXC chemokine receptor 4 and vascular endothelial growth factor proteins predicts early distant relapse in stage II-III colorectal cancer patients," *Clinical Cancer Research*, vol. 12, no. 9, pp. 2795–2803, 2006.

[130] H. E. Broxmeyer, S. Cooper, G. Hangoc, and C. H. Kim, "Stromal cell-derived factor-1/CXCL12 selectively counteracts inhibitory effects of myelosuppressive chemokines on hematopoietic progenitor cell proliferation in vitro," *Stem Cells and Development*, vol. 14, no. 2, pp. 199–203, 2005.

[131] A. F. Cashen, B. Nervi, and J. DiPersio, "AMD3100: CXCR4 antagonist and rapid stem cell-mobilizing agent," *Future Oncology*, vol. 3, no. 1, pp. 19–27, 2007.

[132] D. A. Sipkins, X. Wei, J. W. Wu, et al., "In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment," *Nature*, vol. 435, no. 7044, pp. 969–973, 2005.

[133] J. A. Burger and A. Peled, "CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers," *Leukemia*, vol. 23, no. 1, pp. 43–52, 2009.

[134] A. K. Azab, J. M. Runnels, C. Pitsillides, et al., "CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy," *Blood*, vol. 113, no. 18, pp. 4341–4351, 2009.

[135] A. V. Kurtova, A. T. Tamayo, R. J. Ford, and J. A. Burger, "Mantle cell lymphoma cells express high levels of CXCR4, CXCR5, and VLA-4 (CD49d): importance for interactions with the stromal microenvironment and specific targeting," *Blood*, vol. 113, no. 19, pp. 4604–4613, 2009.

[136] M. Niedermeier, B. T. Hennessy, Z. A. Knight, et al., "Isoform-selective phosphoinositide 3′-kinase inhibitors inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukemia: a novel therapeutic approach," *Blood*, vol. 113, no. 22, pp. 5549–5557, 2009.

[137] D. R. Kuritzkes, "HIV-1 entry inhibitors: an overview," *Current Opinion in HIV and AIDS*, vol. 4, no. 2, pp. 82–87, 2009.

[138] M. Farzan, G. J. Babcock, N. Vasilieva, et al., "The role of post-translational modifications of the CXCR4 amino terminus in stromal-derived factor 1α association and HIV-1 entry," *The Journal of Biological Chemistry*, vol. 277, no. 33, pp. 29484–29489, 2002.

[139] C. A. Lamartiniere, "Protection against breast cancer with genistein: a component of soy," *American Journal of Clinical Nutrition*, vol. 71, supplement 6, pp. 1705S–1709S, 2000.

[140] Y. Guo, S. Wang, D. R. Hoot, and S. K. Clinton, "Suppression of VEGF-mediated autocrine and paracrine interactions between prostate cancer cells and vascular endothelial cells by soy isoflavones," *Journal of Nutritional Biochemistry*, vol. 18, no. 6, pp. 408–417, 2007.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 285191, 12 pages
doi:10.1155/2010/285191

Research Article

Expression of Fatty Acid Synthase Depends on NAC1 and Is Associated with Recurrent Ovarian Serous Carcinomas

Stefanie M. Ueda,[1,2] Kai Lee Yap,[3] Ben Davidson,[4,5] Yuan Tian,[3] Vivek Murthy,[3] Tian-Li Wang,[1,2] Kala Visvanathan,[2] Francis P. Kuhajda,[3] Robert E. Bristow,[1,2] Hui Zhang,[3] and Ie-Ming Shih[1,2,3]

[1] *Department of Gynecology and Obstetrics, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA*
[2] *Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA*
[3] *Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA*
[4] *Division of Pathology, Norwegian Radium Hospital, Rikshospitalet University Hospital, N-0310 Oslo, Norway*
[5] *Faculty Division Radiumhospitalet, The Medical Faculty, University of Oslo, N-0310 Oslo, Norway*

Correspondence should be addressed to Ie-Ming Shih, shihie@yahoo.com

Received 2 January 2010; Accepted 21 February 2010

Academic Editor: Ben Davidson

Our previous reports demonstrated that NAC1, a BTB/POZ domain-containing nuclear protein, upregulates in recurrent ovarian serous carcinoma and participates in developing drug resistance in cancer cells. The current study applies quantitative proteomics to identify the proteins controlled by NAC1 by comparing the proteomes of SKOV3 cells with and without expression of a dominant negative NAC1 construct, N130. From the proteins that are downregulated by N130 (upregulated by NAC1), we chose to further characterize fatty acid synthase (FASN). Similar to change in protein level, the FASN transcript level in SKOV3 cells was significantly reduced by N130 induction or by NAC1 knockdown. Immunohistochemistry showed that NAC1 and FASN immunointensities in ovarian serous carcinoma tissues had a highly significant correlation ($P < .0001$). Moreover, we found that recurrent serous carcinomas exhibited higher FASN immunointensities than their matched primary tumors ($P < .001$). Multivariate analysis showed that an FASN staining score of >1 in serous carcinomas was associated with a worse overall survival time ($P < .01$). Finally, C93, a new FASN inhibitor, induced massive apoptosis in carboplatin/paclitaxel resistant ovarian cancer cells. In conclusion, we show that NAC1 is essential for FASN expression in ovarian serous carcinomas and the expression of FASN significantly correlates with tumor recurrence and disease aggressiveness. The dependence of drug resistant tumor cells on FASN suggests a potential application of FASN-based therapeutics for recurrent ovarian cancer patients.

1. Introduction

Ovarian cancer is a neoplastic disease that exemplifies many of the major issues underlying current chemotherapy regimens in clinical oncology [1, 2]. Although most ovarian carcinomas at advanced stages are responsive to initial carboplatin and paclitaxel treatment, tumor clones resistant to these drugs eventually evolve as recurrent diseases. As a consequence, the main contributors to the mortality and morbidity of advanced stage ovarian cancer patients are chemoresistant tumors. In an effort to elucidate the molecular mechanisms underlying chemoresistance, we have studied ovarian cancer genome and transcriptome and have identified several genes and pathways that are potentially involved in this phenotype. One of these genes, *NACC1* encoding NAC1 (or NAC-1) protein, shows significantly higher expression in recurrent chemoresistant ovarian serous carcinomas than in primary untreated tumors [3]. *NACC1* belongs to the BTB/POZ domain gene family and contains the BEN domain that potentially mediates protein-DNA and protein-protein interactions during chromatin organization and transcription [4]. Biologically, NAC1 has been demonstrated to be an embryonic stem cell marker that controls proliferation and pluriopotency in embryonic stem cells

[5–7]. NAC1 homodimerizes through the highly conserved BTB/POZ domain (a.a. from 1–129) [8] and its complex formation is essential for a variety of its biological functions.

In our previous study, we found that recurrent ovarian serous carcinomas showed a higher NAC1 expression level than their matched primary untreated tumors [3, 9]. Ectopic expression of NAC1 increased paclitaxel resistance while knockdown of NAC1 or disruption of NAC1 homodimerization sensitized cancer cells to chemotherapeutic drugs [3, 9]. In order to understand how NAC1 contributes to drug resistance, we previously compared the gene expression profiles of SKOV3 ovarian cancer cells to those of NAC1 inactivated SKOV3 cells where the inactivation was induced by expression of N130, a mutant protein containing only the BTB/POZ domain of NAC1 that competitively inhibits NAC1 homodimerization. We found that NAC1 negatively regulated the components of the Gadd45 tumor suppressor pathway including Gadd45α and its binding protein, Gadd45gip1 [9, 10]. However, suppressing the Gadd45 tumor suppressor pathway did not completely rescue ovarian cancer cells after NAC1 inactivation, thus suggesting that other mechanisms also play a role.

In this study, we identified proteins that are potentially regulated by NAC1 by applying a quantitative proteomic method using tandem mass spectrometry (MS/MS) and spectral count [11]. A total of 2914 proteins were identified. To reduce the sampling error and increase the quantification accuracy, 208 proteins identified by at least 20 spectra and two unique peptides were quantified to identify candidate proteins controlled by N130 expression. By comparing the protein quantity in the proteomes between N130-induced and noninduced SKOV3 cells, we identified new NAC1 regulated proteins that could be responsible for the NAC1-mediated drug resistance and for other biological functions. Among the proteins found to be downregulated in N130-induced cells, we selected fatty acid synthase (FASN) for further characterization because it has been shown to be associated with tumor progression in a variety of human cancers and its inhibitors are available for potential translation studies.

2. Methods

2.1. Cell Lines and Clinical Tissue Samples. High-grade ovarian carcinoma cell lines, SKOV3, A2780, and OVCAR3, were obtained from the American Type Culture Collection (Rockville, MD), and a low-grade serous cell line, MPSC1, was previously established by us [12]. OSE10 was as SV-40-immortalized ovarian surface epithelial cell line. All cell lines were maintained in RPMI media with 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 2% penicillin/streptomycin (Gibco, Rockville, MD).

A total of 427 ovarian carcinoma tumors collected between January 1990 and December 2006 were arranged in tissue microarrays. These included 269 high-grade serous, 45 low-grade serous, 45 endometrioid, and 68 clear cell carcinomas. Fifteen histologically normal ovarian tissues and 20 benign ovarian serous cystadenomas collected over the same period were also analyzed. For Kaplan-Meier survival analysis, we included 184 primary high-grade serous carcinoma patients who underwent optimal primary cytoreductive surgery followed by platinum-based chemotherapy at the Johns Hopkins Hospital. The paraffin tissues were obtained from the surgical pathology repository at the Johns Hopkins Hospital and clinical data was obtained from medical records, including age, race, stage, histologic subtype, and date of patient's current status (alive or deceased). Median follow-up in those patients was 44.7 months. A subset of 56 high-grade serous carcinomas including 28 pairs of matched primary and recurrent tumors was analyzed to correlate FASN expression levels and to evaluate primary/recurrent tumor status. Another subset of 162 high-grade serous carcinomas was analyzed for correlation of NAC1 and FASN expression levels because the NAC1 immunostaining slides from these cases were available from our previous study [3]. Collection of tissue samples was in accordance with the guidelines of the institutional review board.

2.2. Quantitative Proteomics. Cell lysates were collected 48 hours after induction (removal of doxycyclin) or mock induction [3]. Protein concentration was measured by BCA assay. The same amounts of proteins (1 mg) from each condition were reduced, alkylated, and digested with 1 to 50 Trypsin/protein ratio at 37°C overnight. The peptides were purified with C18 columns and resuspended in water with a final concentration of 10 μg/μL.

For protein identification and quantification, each peptide mixture was analyzed twice by the LTQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). For each analysis, 2 μg of peptides were injected into a peptide cartridge packed with C18 resin, and then passed through a 10 cm $\times$ 75 μm i.d. microcapillary HPLC (μLC) column packed with C18 resin. A linear gradient of acetonitrile from 5%–32% over 100 minutes at flow rate of ~300 nL/min was applied. During the LC-MS mode, data was acquired in the m/z range of 400 and 2000. The MS/MS was also turned on to collect CID using data dependent mode.

MS/MS spectra were searched with SEQUEST against the human IPI protein database (version 2.28). The peptide mass tolerance is 3.0 Da. Other parameters of database searching are as follows: cysteine modification (add cysteine 57) and oxidized methionine (add methionine with 16 Da). The output files were evaluated by INTERACT [13] and Peptide Prophet [14]. The identified peptides with a probability score $\geq$ 0.9 were used for the spectral count. To determine the number of MS/MS spectra used for identification of each protein in different conditions using our in-house developed software tool and to reduce the error for protein identification and quantification using MS/MS spectra, we only quantified proteins identified by at least 20 spectra and 2 independent peptides from the N130-induced and-noninduced cells.

2.3. Western Blot and Real Time PCR. Western blot analysis was performed on the protein lysates prepared from ovarian cancer cell lines and OSE10. Similar amounts of total

protein from each lysate were separated on 10% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and then electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes. The membranes were probed with an anti-FASN mouse monoclonal antibody (1 : 100) (FASgen, Baltimore, MD) followed by a peroxidase-conjugated goat antimouse immunoglobulin (1 : 6,000). Western blots were developed by chemiluminescence (Pierce, Rockford, IL) utilizing glyceraldehyde-3-phosphate dehydrogenase as the loading control. To determine the mRNA levels of FASN, we performed quantitative real-time PCR using a BioRad iCycler. Total RNA was isolated with the TRIzol method (Invitrogen) and cDNA was synthesized from 2 to 5 μg total RNA. FASN primer sequences were 5′-CATCCAGATAGG-CCTCATAGAC-3′ (forward) and 5′-CTCCATGAAGTA-GGAGTGGAAG-3′ (reverse). The expression of FASN was normalized to that of human amyloid beta precursor protein based with threshold cycle numbers calculated from duplicate measurements. Mean fold expression differences were further normalized to those of ovarian surface epithelium, OSE10.

2.4. Immunohistochemistry. For immunohistochemistry, paraffin sections after deparaffinization were incubated with a primary antimouse FASN antibody at a dilution of 1 : 50 in a 4°C moist chamber overnight. Negative controls included benign serous cystadenomas and normal ovaries. Two independent observers scored the FASN immunoreactivity using a categorical scoring system from 0 (not detectable) to 3 (intense) with the mean score recorded from triplicates. Among the FASN stained cases, there were 162 that had been previously stained with an anti-NAC1 antibody [3, 15].

2.5. Cell Number and Apoptosis Detection. Cancer cells were seeded in 96-well plates at 2.5×10^3 cells/well for 24 hours and then were incubated with 100 μL of the FASN inhibitor, C93, at concentrations ranging from 2.5 μg/mL to 50 μg/mL for 48 hours. After incubation, the number of viable cells was measured by the CellTiter-Blue assay (Promega, Madison, WI). These numbers were plotted against FASN inhibitor concentrations, and the value of IC_{50} (i.e., the C93 concentration at which cell growth dropped to 50% of the control level) was estimated. To detect early apoptotic cells, we grew ovarian cancer cell lines in 6 well plates (5×10^5 cells/well) and treated them with C93 at their IC_{50} and with DMSO of equal concentration. The early apoptotic cells were quantified utilizing the Annexin V-FITC detection kit (Biovision, Mountain View, CA) and annexin V-stained cells were determined by flow cytometry.

After treating cells with C93 or DMSO, cells were trypsinized, washed, and resuspended in a solution containing 0.6% NP-40, 3.7% formaldehyde, and 11 mg/mL Hoechst 33258 in a phosphate buffered solution. The stained cells were then quantified by the BD LSR cytometer (Becton Dickinson, Franklin Lakes, NJ). Cell cycle analysis was performed using the CellQuest software (Becton Dickinson) and cells in the $subG_1$ phase that represent the late phase of apoptosis were also measured.

2.6. Lentivirus Production and Transduction. Two NAC1 targeting shRNA plasmids previously cloned into the pLKO.1 (Sigma) and the packaging lentivirus plasmids were cotransfected into 293FT cells using Lipofectamine 2000 (Invitrogen. The sequences of NAC1 targeting shRNAs were 5′-CCGGCCCAAGTGAGATTG CACATTTCTCGAGAAA-TGTG CAATCTCACTTGGGTTTTTTG-3′ (shRNA-A) and 5′-CCGG CAAGTACTACTGCC AGAACTTCTCGAG AA-GTTCTGGCAGTAGTACTTGTTTTTTG-3′ (shRNA-C). Five hours after incubation, the transfection reagents were replaced with 10% FBS RPMI culture media supplemented with bovine serum albumin. The virus-containing supernatant was collected, the mixture was centrifuged, and polybrene was added to a final concentration of 8 μg/mL. Transduction was carried out by adding 0.25 mL of virus supernatant to the SKOV3 cells. A second transduction was performed 24 hours later using the same protocol. shRNA or control (vector only) virus transduced cells were enriched by adding 2 μg/mL of puromycin. Following this second round of transduction, cells were collected at 48 and 72 hours for quantitative real-time PCR analysis of NAC1 and FASN mRNA expression levels.

3. Results

3.1. High-Throughput Quantitative Proteomics Identifies FASN as an NAC1-Regulated Protein. To determine the possible mechanism by which NAC1 promotes drug resistance, we used the quantitative proteomics to compare the proteomes of N130-induced and-noninduced SKOV3 ovarian cancer cells using tandem mass spectrometry (MS/MS) and spectral count [11]. We were able to identify proteins that corresponded to a total of 2914 proteins. To reduce the sampling error and increase the quantification accuracy, 208 proteins identified by at least 20 spectra and two unique peptides were quantified to identify candidate proteins controlled by N130 expression. Based on the protein expression ratio of N130 noninduced to N130 induced cells (N130 OFF versus N130 ON), we listed the 19 proteins whose abundances were at least increased 50% (OFF/ON $\geq$ 1.5, Table 1) and identified 18 proteins whose levels were at least increased 50% (ON/OFF $\geq$ 1.5) by N130 induction (Table 1). Cells with induced expression of N130 showed significantly higher levels NAC1 expression than N130-noninduced cells (ON/OFF = 76.5), and all the identified peptides from NAC1 matched the N130 (BTB/POZ) domain, indicating the robustness of N130 induction. Since our primary interest was to identify the proteins that are upregulated by NAC1 (i.e., downregulated by N130), we selected fatty acid synthase (FASN) for validation and characterization since its level in noninduced cells is 1.57 times that in induced cells. We made this choice because, more than other proteins in the list, FASN has been associated with tumor progression and because specific inhibitors of FASN are available for further studies. The levels of FASN are less abundant in N130 induced cells than in N130-noninduced cells, suggesting that NAC1 inactivation by N130 suppresses FASN protein expression in SKOV3 cells.

Table 1: Proteins that are differentially expressed in N130-induced and-noninduced SKOV3 cells.

(a) Proteins downregulated by N130 induction

IPI	Protein name	ON-spectra	ON-peptides	OFF-spectra	OFF-peptides	Total spectra	Off/on
IPI00013881	Heterogeneous nuclear ribonucleoprotein H	4	5	20	7	24	4.62
IPI00012388	Transcription intermediary factor 1-beta	8	7	23	9	31	3.07
IPI00220985	Keratin, type I cytoskeletal 18	8	4	18	5	26	2.35
IPI00298547	RNA-binding protein regulatory subunit	10	7	23	7	33	2.30
IPI00299571	Protein disulfide isomerase A6	11	3	21	8	32	1.91
IPI00216976	aldolase C, fructose-bisphosphate	10	3	19	4	29	1.90
IPI00012074	Heterogeneous nuclear ribonucleoprotein R	9	5	17	7	26	1.89
IPI00013917	40S ribosomal protein S12	10	3	19	3	29	1.85
IPI00297779	T-complex protein 1, beta subunit	10	5	18	8	28	1.80
IPI00027434	Transforming protein RhoC	8	5	14	5	22	1.75
IPI00141318	P63 protein	8	6	13	5	21	1.73
IPI00219217	lactate dehydrogenase B	11	6	18	8	28	1.67
IPI00219096	high-mobility group box 1	15	4	25	5	40	1.67
IPI00009236	Caveolin-1	9	2	15	2	24	1.67
IPI00328343	Probable ATP-dependent RNA helicase p47	11	6	18	6	29	1.64
IPI00302927	T-complex protein 1, delta subunit	8	3	13	6	21	1.63
IPI00328188	fatty acid synthase	21	7	33	11	54	1.57
IPI00015027	AHNAK-related protein	11	7	17	11	27	1.55
IPI00017617	Probable RNA-dependent helicase p68	11	8	17	7	28	1.50

(b) Proteins upregulated by N130 induction

IPI	Protein name	ON-spectra	ON-peptides	OFF-spectra	OFF-peptides	Total spectra	On/off
IPI00017454	Hypothetical protein FLJ13940	23	4	0	0	23	?
IPI00045207	NAC1 protein	153	9	2	1	155	76.50
IPI00395440	Unknown	26	5	5	4	31	5.13
IPI00107117	Peptidylprolyl isomerase B	14	5	6	3	20	2.33
IPI00290566	T-complex protein 1, alpha subunit	14	6	6	3	20	2.33
IPI00176692	similar to Heterogeneous nuclear ribonucleoprotein A1	27	5	12	6	39	2.25
IPI00221088	ribosomal protein S9	17	6	8	6	25	2.13
IPI00215918	ADP-ribosylation factor 4	17	7	9	7	26	1.89
IPI00015786	Spectrin alpha chain, brain	53	27	30	18	83	1.77
IPI00152412	Hypothetical protein	14	4	8	5	22	1.75
IPI00020984	Calnexin	29	10	17	7	46	1.71
IPI00010896	Chloride intracellular channel protein 1	17	5	10	4	27	1.70
IPI00027626	T-complex protein 1, zeta subunit	18	9	11	9	29	1.64
IPI00216587	40S ribosomal protein S8	13	6	8	6	21	1.63

(b) Continued.

IPI	Protein name	ON-spectra	ON-peptides	OFF-spectra	OFF-peptides	Total spectra	On/off
IPI00216318	tyrosine 3-monooxygenase	40	10	25	9	65	1.58
IPI00329351	60 kDa heat shock protein, mitochondrial	66	21	44	17	110	1.52
IPI00217468	H1 histone family, member 5	21	5	14	4	35	1.50
IPI00291006	Malate dehydrogenase, mitochondrial	15	6	10	4	25	1.50

Table 2: Immunointensities of NAC1 and FASN in high-grade serous carcinoma.

	FASN 2+/3+	FASN 0/1+
NAC1 intense	51	34
NAC1 weak	19	58
Total (n)	70	92

Table 3: FASN immunointensity in primary and recurrent ovarian serous carcinomas.

	Score	
Tumor	0/1+	2+/3+
Primary	18	10
Recurrent	4	24
Total (n)	22	34

$\chi^2 P < .0001$.

3.2. Validation of NAC1-Dependent FASN Expression. To validate the proteomic result above, we used quantitative real-time PCR to determine FASN transcript levels in two independent experimental systems. In our first system, we used the same NAC1 dominant negative (N130) cell model that was used in the proteomic analysis to assess whether disruption of NAC1 homodimerization by N130 leads to a decrease in FASN mRNA level. We observed that N130 mRNA, as detected by a PCR primer pair that amplifies the N130 region, increases 48 and 72 hours after induction (Figure 1(a)). At these same time points, SKOV3 cells with N130 induction exhibit a downregulation of FASN mRNA. Of note, at 48 hours after N130 induction, the decrease in FASN expression is similar between mRNA (46%) and protein (36%) levels. In our second system, we knocked down NAC1 in SKOV3 cells to determine the FASN levels. Two NAC1 shRNAs (shRNA-A and -C) that target different coding regions of *NACC1* were designed and packaged into lentivirus. We found that both shRNAs effectively reduce NAC1 transcript levels (to less than 10% of control) after transduction with shRNA lentivirus (Figure 1(b)). Correspondingly, FASN expression levels also decrease as compared to control. The above findings from cell culture systems suggest that NAC1 expression and its dimerization domain are essential for maintaining FASN expression in tumor cells.

In order to assess the biological significance of NAC1-dependent FASN expression, we stained tumor samples obtained from ovarian cancer ascites and tissues for NAC1 and FASN and correlated their immunointensities. The specificity of the anti-NAC1 has been demonstrated previously [3] and the specificity of the anti-FASN monoclonal antibody is shown in Figure 2(a). Western blot analysis shows a single protein band at the molecular mass of FASN protein in all three ovarian cancer cell lines (OVCAR3, A2780, and SKOV3) but not in a low-grade serous carcinoma cell line, MPSC1, nor in ovarian surface epithelial cells (OSE10). Analyzing 162 high-grade serous carcinomas showed a significant positive correlation in NAC1 and FASN immunointensities ($P < .0001$, Fisher Exact test) (Table 2). These immunointensities for four representative high-grade serous carcinomas are shown in Figure 2(b). Our observations support the view that FASN expression is at least in part regulated by NAC1.

3.3. FASN Expression in Ovarian Serous Tumors. To extend the above immunostaining findings, we investigated FASN immunoreactivity in various types of ovarian tumors. All benign cystadenomas and normal ovarian surface epithelium ($n = 35$) display undetectable to very low FASN staining (mean score $= 0$) whereas ovarian carcinomas of various histologic subtypes show FASN immunoreactivity (mean score ≥ 1) in most cases (Figures 2(c) and 2(d)). The FASN staining score of high-grade serous carcinomas is significantly higher than that of low-grade serous carcinomas ($P < .0001$, t-test) and normal ovaries and benign cystadenomas ($P < .0001$). The FASN score in high-grade serous carcinomas is marginally higher than that in clear cell carcinoma ($P = .024$) and there is no statistical significance of FASN score between high-grade serous and endometrioid carcinoma ($P = .099$). Since NAC1 has been shown to be highly expressed in recurrent rather than primary high-grade ovarian serous carcinomas, we expect that FASN expression levels will follow the same pattern. To test this possibility, we assessed the FASN immunoreactivity of matched primary and first recurrent tumors from the same individuals. As expected, the FASN staining score is significantly higher in recurrent than in primary tumors ($P < .0001$, paired t-test) (Figure 3(a)). Based on a 2×2 contingency table and chi-square analysis, we also found that recurrent tumors exhibit a higher percentage of cases with intense FASN immunoreactivity than primary tumors do (Table 3). Figure 3(b) illustrates representative pairs of primary and recurrent tumors with FASN stain.

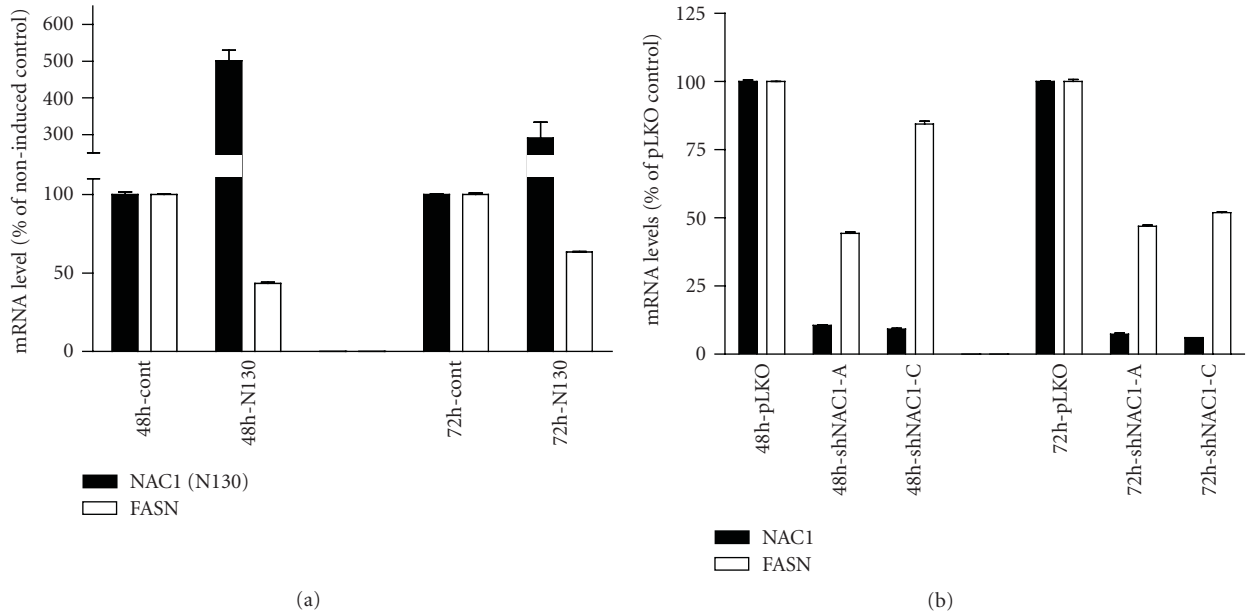


Figure 1: NAC1-dependent FASN expression in ovarian cancer cells. (a) N130 mRNA, as detected by a PCR primer pair that amplifies the N130 region, increases after N130 induction. In contrast, FASN mRNA decreases 48 and 72 hours after induction. (b) After shRNA lentivirus transduction, both shRNAs (-A and -C) effectively reduce NAC1 transcript levels to less than 10% of control and reduce FASN expression.

3.4. Clinical Significance of FASN Expression in High-Grade Ovarian Serous Carcinomas. To evaluate the clinical significance of FASN expression, we correlated the expression levels in primary high-grade serous carcinoma tumors with overall survival of the patient. We found that higher FASN staining scores (scores > 1) correlated with worse overall survival ($P < .002$) (Figure 3(c)). Patients with primary tumors that showed minimal or undetectable FASN immunoreactivity (score ≤ 1) had a median survival time of 60.4 months (range 1–193 months), whereas those with tumors that showed positive immunoreactivity (score > 1) had a median survival time of 36.9 months (range 1–140 months, $P < .01$). After adjusting for age, stage, and race in a multivariate analysis, we found that, in patients with primary serous carcinomas, FASN expression remains an independent marker for prognosis with a hazard ratio of 1.87 (95% CI: 1.12–3.11, $P = .02$).

3.5. C93 Suppresses Growth of Paclitaxel-Resistant and Carboplatin-Resistant Ovarian Cancer Cells. The above findings suggest that FASN preferentially expresses in recurrent and in most aggressive types of ovarian serous carcinomas, raising the possibility that FASN expression contributes to this phenotype. Thus, to determine if FASN expression is essential for cell growth and survival of high-grade serous carcinoma cells (including those that are resistant to paclitaxel or carboplatin), we applied C93, a second generation FASN inhibitor, to ovarian cancer cell lines (including SKOV3, A2780, and OVCAR3). We first used the cell number counted by CellTiter Blue assay to determine the IC_{50} of C93 for each ovarian cancer cell line. The IC_{50} of C93 was 7.4 μg/mL, 7.4 μg/mL, and 8.7 μg/mL for parental, paclitaxel, and carboplatin resistant SKOV3 cells, respectively; 7.4 μg/mL, 7.5 μg/mL, and 8.4 μg/mL for parental, paclitaxel, and carboplatin resistant A2780 cells, respectively; and 8.6 μg/mL, 6.5 μg/mL, and 8.8 μg/mL for parental, paclitaxel, and carboplatin resistant OVCAR3 cells, respectively. When applied to each cell line at its IC_{50} concentration, C93 significantly increases the percentage of annexin V stained cells (representing the early phase of apoptosis) and the sub-G_1 fraction in cell cycle analyses (representing the late phase) (Figure 4). Thus, C93 induces apoptosis in all three ovarian cancer cell lines. The number of annexin V-stained cells increases in a time dependent fashion in all cancer cell lines including those resistant to carboplatin and paclitaxel (Figure 4(b)). Moreover, paclitaxel resistant cells are more sensitive to C93 than carboplatin resistant cells, especially in the A2780 and SKOV3 cell lines. In all three cell lines, the C93-treated group shows a significantly higher percentage of sub-G_1 cells than the DMSO-treated group ($P < .01$). These sub-G_1 cells can be detected as early as 12 hours after C93 treatment and become more pronounced by 48 hours (Figure 4(c)). C93 fails to affect cell cycle progression in SKOV3 and OVCAR3 cells by 96 hours after treatment but it arrests A2780 cells in the G_1 phase as early as 24 hours after treatment (data not shown).

4. Discussion

One of the major challenges facing ovarian cancer patients is the development of chemoresistant tumors after cytoreduction surgery and chemotherapy. In this study, we provide new evidence that homodimerization of NAC1, a drug resistance-associated nuclear protein, is essential for maintaining FASN

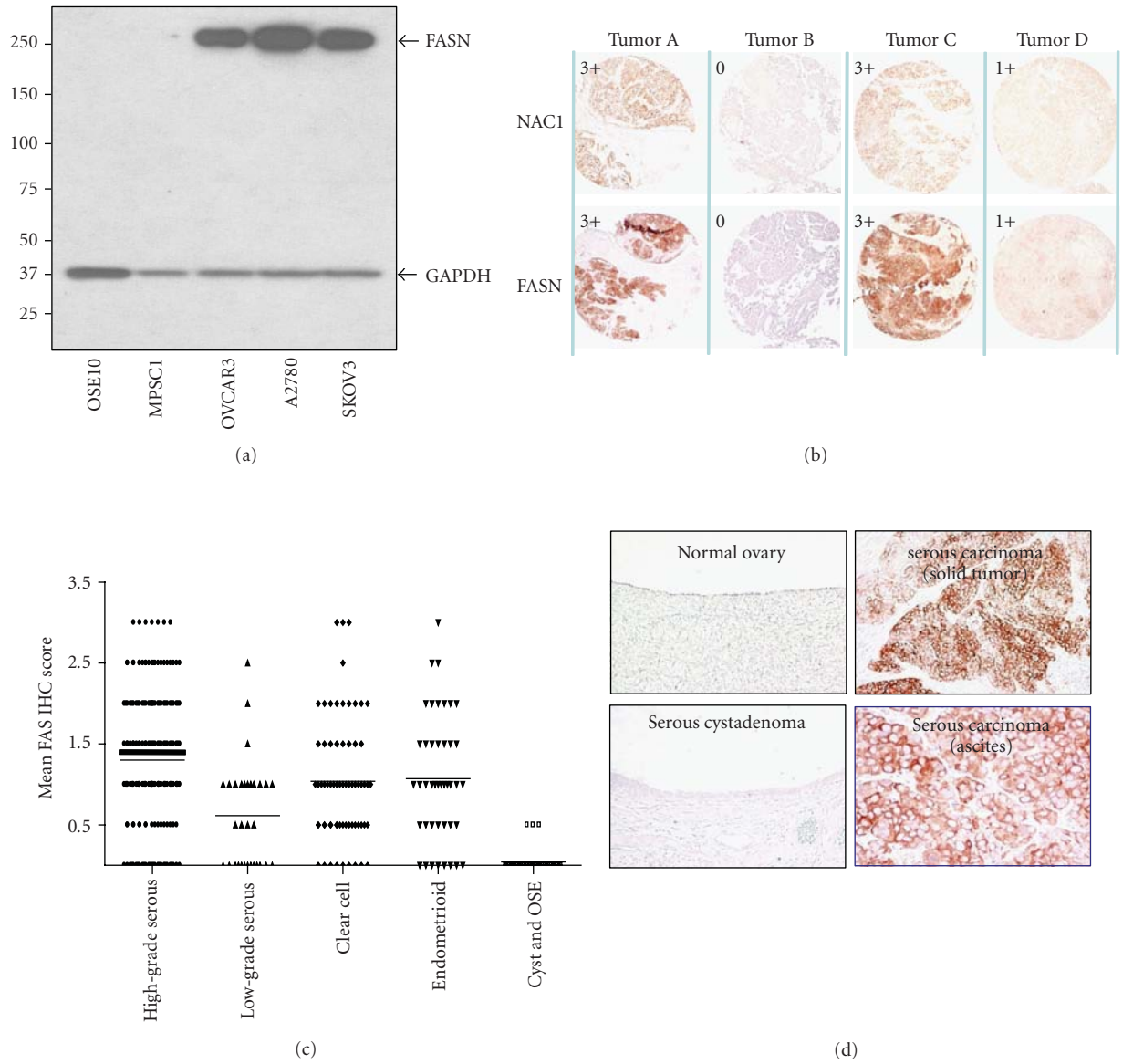


Figure 2: Expression of NAC1 and FASN in ovarian tumor tissues. (a) Western blot analysis shows a single protein band that corresponds to FASN protein in all three ovarian cancer cell lines (OVCAR3, A2780, and SKOV3) but not in a low-grade serous carcinoma cell line (MPSC1) nor in ovarian surface epithelial cells (OSE10). (b) Immunoreactivity of NAC1 (upper panels) and FASN (bottom panels) in four representative high-grade serous carcinomas. For each staining, the immunostaining scores are shown in the upper left corner (c)-(d). FASN expression in different types of ovarian carcinomas. (c) FASN immunointensity scores for different types of ovarian carcinoma including high-grade serous, low-grade serous, clear cell, and endometrioid carcinomas. The mean and standard deviation of the immunostaining score are shown. (d) Representative tumor sections of FASN immunostaining are illustrated. FASN immunoreactivity is detectable in high-grade serous carcinoma cells but not in normal ovarian surface epithelium nor in cystadenomas.

expression at both protein and mRNA levels in ovarian cancer cells. We also demonstrate that FASN expression is highly correlated with the status of recurrent chemoresistant ovarian serous carcinomas and is independently correlated with poor overall survival. Suppressing FASN enzyme activity with its inhibitor induces apoptosis in cancer cells that are resistant to paclitaxel and carboplatin. Thus, molecular studies that illuminate the fundamental properties of chemoresistance should provide new therapeutic targets for treating recurrent ovarian cancers.

Mammalian FASN is a ~260 kD cytoplasmic enzyme that is responsible for all the steps of *de novo* fatty acid synthesis. It catalyzes the NADPH-dependent condensation of malonyl-CoA and acetyl-CoA to palmitate [16]. Importantly, normal adult tissues express minimal amounts of FASN due to the presence of abundant dietary lipids. In contrast, a variety of tumors including breast, colon, ovary, and prostate cancer express elevated FASN levels [17–23], since tumor cells become less sensitive to regulatory nutritional signals and prefer the *de novo* lipogenesis pathway. Furthermore,

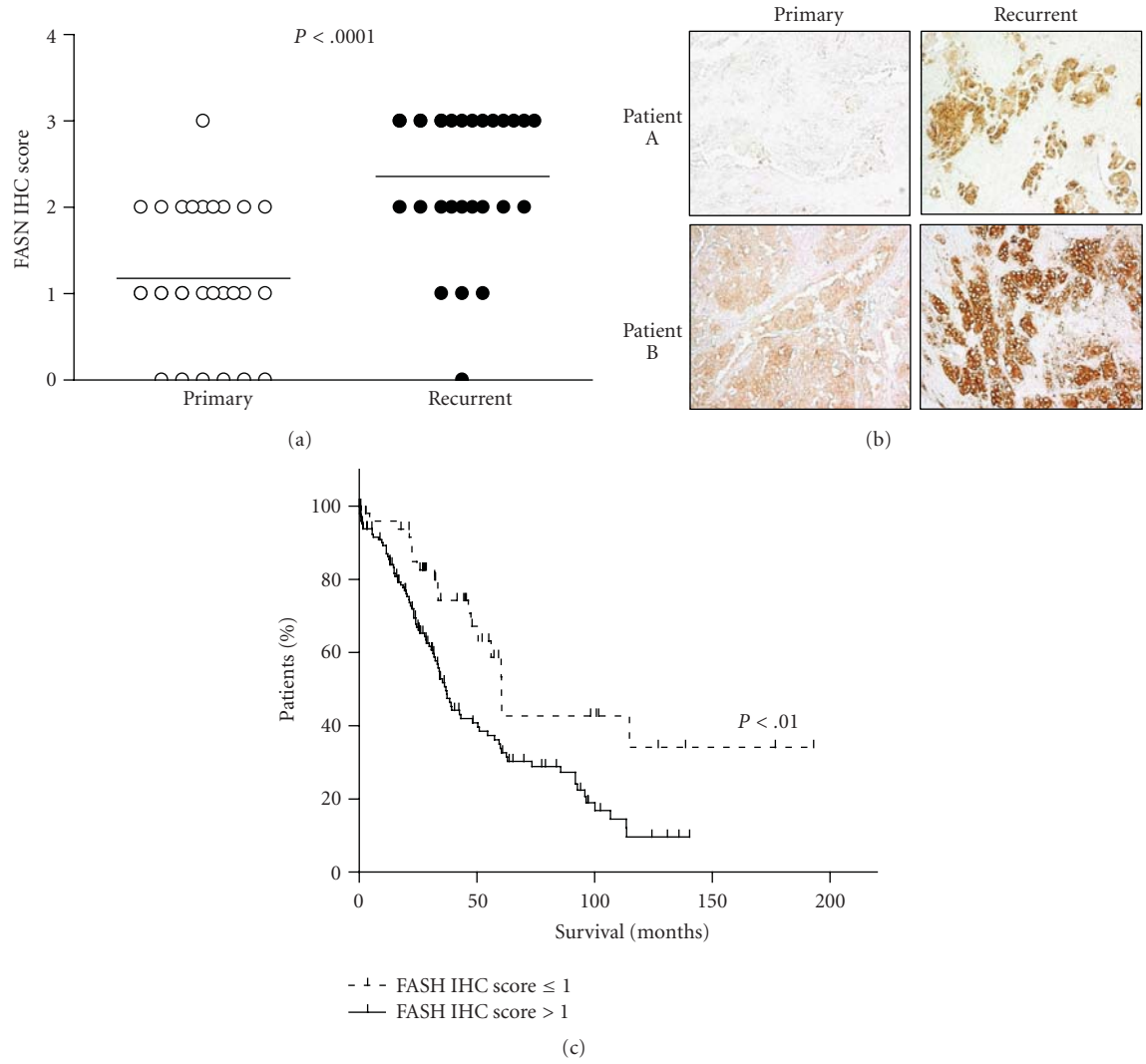


Figure 3: Clinical significance of FASN expression in high-grade ovarian serous carcinoma. (a) Comparison of FASN immunointensity scores for 28 pairs of matched primary and recurrent tumors from the same patients. Recurrent tumors exhibit elevated expression when compared to primary specimens from the same patient ($P < .0001$). (b) FASN staining of the primary and recurrent tumors from two representative patients. (c) Kaplan-Meier curve analysis shows that patients whose tumors exhibit higher FASN immunostaining scores have significantly shorter survival times than those whose tumors show undetectable or very low FASN immunoreactivity (60.4 versus 36.9 months, $P < .01$).

FASN expression level highly correlates with the clinical aggressiveness of tumors [17, 18, 24–27].

Although previous reports have shown the role of FASN expression in ovarian cancer [18, 28], the current study provides new findings that should have several biological and clinical implications. First, we use in vitro cell culture studies using NAC1 targeting shRNAs and the dominant negative NAC1 (N130) approach to demonstrate that FASN is one of the proteins regulated at least in part by NAC1. This NAC1-dependent FASN expression in protein level is further supported by its mRNA level and the positive correlation between NAC1 and FASN immunointensities in ovarian serous carcinoma samples. The mechanism underlying upregulation of FASN in human cancer is not clear and it likely involves multiple pathways. Previous studies have shown that, in prostate cancer, caveolin-1 and KLF5/SREBP-1 function upstream of FASN [29, 30]. Thus, at least in ovarian cancer cells, the NAC1 pathway represents another mechanism for controlling FASN expression. Unlike other members of the BTB/POZ family, NAC1 lacks the zinc finger DNA-binding domain. Rather, it has been reported to act as a transcription corepressor with other BTB/POZ proteins [31]. Moreover, it has also been shown to interact with nuclear proteins potentially involved in tumorigenesis, including Nanog [7], CoREST [32], and HDAC3 and HDAC4 [33]. Thus, it is possible that FASN expression is indirectly controlled by NAC1 through binding with its specific partner(s). Identification of the NAC1-FASN pathway sheds new light on the molecular mechanism by which NAC1 promotes tumor progression. Further studies are required to elucidate the transcriptional regulation of FASN by NAC1.

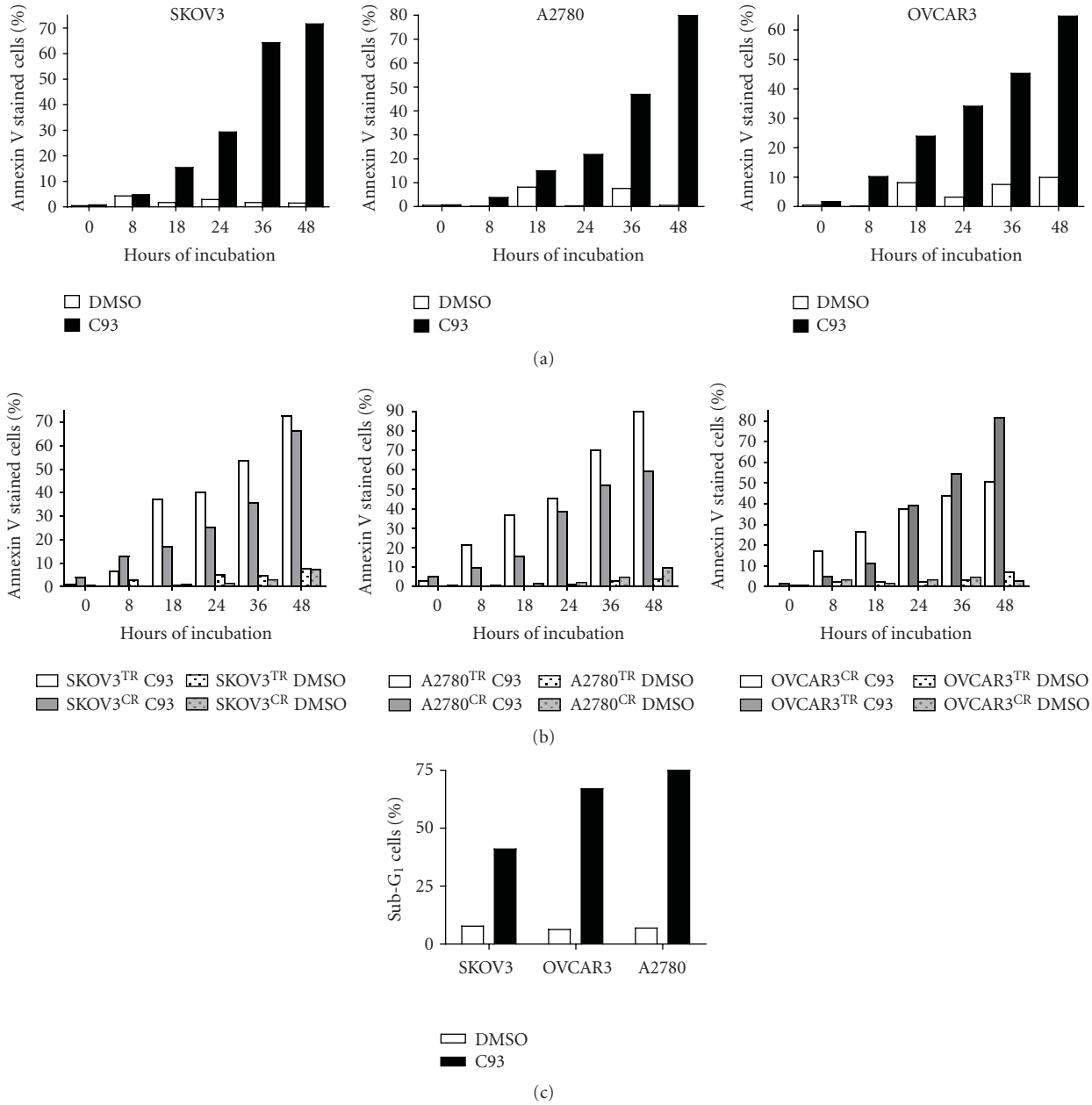


Figure 4: C93 treatment promotes apoptosis in ovarian cancer cells including cells that are resistant to carboplatin and paclitaxel. (a) In three ovarian cancer cell lines, C93 at its IC_{50} concentration increases the percentage of annexin V stained cells in a time dependent fashion. DMSO was used as the vehicle control. (b) C93 also increases annexin V stained cells in carboplatin resistant (CR) and paclitaxel resistant (TR) cell lines. (c) In all three cell lines, C93 treatment leads to an increase in sub-G_1 cells when compared to the DMSO-treated group ($P < .001$). The percentage of sub-G_1 cells is measured 48 hours after C93 treatment.

The second finding in the current study involves the positive correlation between FASN expression and recurrence status in ovarian serous carcinoma tissue. It has been shown that ectopic overexpression of FASN results in drug resistance and that reducing the FASN expression increased the drug sensitivity in breast cancer cell lines [34]. At low concentrations, FASN inhibitor also sensitized tumor cells with FASN overexpression to chemotherapeutic agents [34, 35]. The FASN-mediated drug resistance appears to be due to a decrease in drug-induced apoptosis due to abundant palmitic acid as a result of FASN overexpression [34]. Despite recent advances in discovering drug-resistance biomarkers that are associated with recurrent and chemoresistant ovarian cancer [1, 3, 15, 36–41], reversing drug resistance by targeting these markers remains a tantalizing objective. The problem lies in the lack of reagents that can be used to inhibit these genes in clinical studies. Thus, therapeutic targeting of FASN provides an attractive option since selective small compound inhibitors for this protein are available. The first generation of FASN inhibitors, including C75 and Orlistat,

potently inhibited tumor growth in a mouse xenograft model but the adverse effects associated with these drugs prevented their further consideration for clinical applications [23]. On the other hand, C93, the second generation FASN inhibitor used in this study, pharmacologically eliminates concomitant CPT-1 stimulation and does not induce the anorexia and feeding behavior changes in mice that were caused by earlier generation FASN inhibitors [28, 42]. These features are important for any FASN inhibitor before it can be considered for clinical testing. Our in vitro studies demonstrate that C93 affects both carboplatin and paclitaxel resistant ovarian cancer cells. Thus, ovarian cancer cells that overexpress FASN are molecularly dependent on it for cell survival. This observation is significant because FASN inhibitors provide an alternative treatment for ovarian cancer patients who have developed recurrent tumors after initial paclitaxel and carboplatin treatment.

The antitumor effects of FASN inhibitors, like C93, are thought to result from depletion of end product fatty acids with accumulation of toxic intracellular malonyl-CoA and altered production of phospholipids with diminished membrane synthesis [23, 43]. Alternatively, FASN inhibitors may suppress tumors through metabolism-independent mechanisms. For example, FASN inhibition has been shown to selectively activate AMP-activated protein kinase (AMPK) in ovarian cancer cells causing cytotoxicity while sparing most normal human tissues from these pleiotropic effects of AMPK activation [28]. Moreover, a positive feedback regulation has been reported in ovarian carcinoma cells between AKT activation and FASN expression [44]. Phosphorylated AKT significantly correlates with FASN expression and FASN inhibition by either C75 or cerulenin downregulates phosphorylated AKT [44–46]. Therefore, FASN inhibitors may contribute to antioncogenesis by suppressing tumor-promoting signaling pathways such as AKT, a pathway that is frequently activated in ovarian serous carcinoma [47]. Thus, the C93-induced apoptosis in ovarian cancer cells may be related to FASN inactivation and/or suppression of AKT activity. It has been demonstrated that FASN inhibition initiates apoptosis more effectively in neoplastic cells with mutant *TP53* than in those with wild-type *TP53* [27, 48]. Our current finding supports this view as the A2780 cell line that harbors wild-type *TP53* [49] responds to C93 with cell cycle arrest in the G_1 phase in addition to apoptosis, while both the SKOV3 and OVCAR3 cell lines with their deleted and mutated *TP53*, respectively, respond to C93 with massive apoptosis [50]. Since mutated *TP53* appears in the majority of ovarian high-grade serous carcinomas [1, 51], it is likely that apoptosis is the predominant antitumor mechanism of FASN inhibition.

As with other epithelial cancers of the breast, colon, and prostate [20, 21, 52], FASN overexpression in ovarian cancer, as this study shows, appears to be associated with the most malignant type, that is, high-grade serous carcinoma. More importantly, in patients with this cancer, the level of FASN expression significantly correlates with worse clinical outcome, supporting the view that FASN expression contributes to disease aggressiveness in cancer cells. A similar observation has been reported for NAC1 [3, 15], hence suggesting that the NAC1-FASN pathway constitutes one of the mechanisms that propels ovarian cancer progression. How FASN contributes to disease aggressiveness in ovarian cancer remains speculative. Besides endowing drug resistance, FASN may enhance oncogenesis via cellular mechanisms such as enhancing the Wnt [53], c-Met [54], and proteosome pathways [55]. Moreover, upregulation of FASN gives cancer cells a growth and survival advantage by blocking apoptosis under hypoxia, a common condition in solid tumors and tumor effusions [56, 57].

In summary, this study identified candidate proteins controlled by NAC1 and provides new evidence that FASN expression is at least in part regulated by NAC1. Thus the NAC1-FASN pathway may represent a new mechanism for tumor progression that creates ovarian tumor cells that are resistant to chemotherapy. Our findings also indicate that FASN is a novel biomarker for recurrent ovarian serous carcinoma and its enzyme activity is essential for the survival of chemoresistant tumor cells. New generation FASN inhibitors, like C93, deserve consideration in future clinical trials involving advanced ovarian serous carcinomas, particularly those that are refractory to paclitaxel and platinum drugs. Further studies will be required to delineate the biological and translational roles of FASN in drug resistance in ovarian and perhaps other types of cancers.

Abbreviations

FASN: Fatty acid synthase.
NAC1: Nucleus accumbens associated 1.

Acknowledgment

This study is in memory of Ms. Sean Patrick, founder of the HERA Women's Cancer Foundation, who courageously fought against recurrent ovarian cancer. The work is supported by NIH/NCI RO1CA103937 (IMS), the HERA Women's Cancer Foundation, and the Entertainment Industry Foundation (SMU).

References

[1] K. R. Cho and I. M. Shih, "Ovarian cancer," *Annual Review of Pathology: Mechanisms of Disease*, vol. 4, pp. 287–313, 2009.

[2] S. B. Kaye, "Reversal of drug resistance in ovarian cancer: where do we go from here?" *Journal of Clinical Oncology*, vol. 26, no. 16, pp. 2616–2618, 2008.

[3] K. Nakayama, N. Nakayama, B. Davidson, et al., "A BTB/POZ protein, NAC-1, is related to tumor recurrence and is essential for tumor growth and survival," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 49, pp. 18739–18744, 2006.

[4] S. Abhiman, L. M. Iyer, and L. Aravind, "BEN: a novel domain in chromatin factors and DNA viral proteins," *Bioinformatics*, vol. 24, no. 4, pp. 458–461, 2008.

[5] J. Kim, J. Chu, X. Shen, J. Wang, and S. H. Orkin, "An extended transcriptional network for pluripotency of embryonic stem cells," *Cell*, vol. 132, no. 6, pp. 1049–1061, 2008.

[6] J. Wang, S. Rao, J. Chu, et al., "A protein interaction network for pluripotency of embryonic stem cells," *Nature*, vol. 444, no. 7117, pp. 364–368, 2006.

[7] T. Ma, Z. Wang, Y. Guo, and D. Pei, "The C-terminal pentapeptide of Nanog tryptophan repeat domain interacts with Nac1 and regulates stem cell proliferation but not pluripotency," *Journal of Biological Chemistry*, vol. 284, no. 24, pp. 16071–16081, 2009.

[8] M. A. Stead, S. B. Carr, and S. C. Wright, "Structure of the human Nac1 POZ domain," *Acta Crystallographica Section F*, vol. 65, no. 5, pp. 445–449, 2009.

[9] N. Jinawath, C. Vasoontara, K.-L. Yap, et al., "NAC-1, a potential stem cell pluripotency factor, contributes to paclitaxel resistance in ovarian cancer through inactivating Gadd45 pathway," *Oncogene*, vol. 28, no. 18, pp. 1941–1948, 2009.

[10] K. Nakayama, N. Nakayama, T.-L. Wang, and I.-M. Shih, "NAC-1 controls cell growth and survival by repressing transcription of Gadd45GIP1, a candidate tumor suppressor," *Cancer Research*, vol. 67, no. 17, pp. 8058–8064, 2007.

[11] H. Liu, R. G. Sadygov, and J. R. Yates III, "A model for random sampling and estimation of relative protein abundance in shotgun proteomics," *Analytical Chemistry*, vol. 76, no. 14, pp. 4193–4201, 2004.

[12] G. Pohl, C.-L. Ho, R. J. Kurman, R. Bristow, T.-L. Wang, and I.-M. Shih, "Inactivation of the mitogen-activated protein kinase pathway as a potential target-based therapy in ovarian serous tumors with KRAS or BRAF mutations," *Cancer Research*, vol. 65, no. 5, pp. 1994–2000, 2005.

[13] D. K. Han, J. Eng, H. Zhou, and R. Aebersold, "Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry," *Nature Biotechnology*, vol. 19, no. 10, pp. 946–951, 2001.

[14] A. Keller, A. I. Nesvizhskii, E. Kolker, and R. Aebersold, "Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search," *Analytical Chemistry*, vol. 74, no. 20, pp. 5383–5392, 2002.

[15] B. Davidson, A. Berner, C. G. Trope', T.-L. Wang, and I.-M. Shih, "Expression and clinical role of the bric-a-brac tramtrack broad complex/poxvirus and zinc protein NAC-1 in ovarian carcinoma effusions," *Human Pathology*, vol. 38, no. 7, pp. 1030–1036, 2007.

[16] F. P. Kuhajda, "Fatty acid synthase and cancer: new application of an old pathway," *Cancer Research*, vol. 66, no. 12, pp. 5977–5980, 2006.

[17] F. P. Kuhajda, K. Jenner, F. D. Wood, et al., "Fatty acid synthesis: a potential selective target for antineoplastic therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 14, pp. 6379–6383, 1994.

[18] T. S. Gansler, W. Hardman III, D. A. Hunt, S. Schaffel, and R. A. Hennigar, "Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival," *Human Pathology*, vol. 28, no. 6, pp. 686–692, 1997.

[19] J. I. Epstein, M. Carmichael, and A. W. Partin, "OA-519 (fatty acid synthase) as an independent predictor of pathologic stage in adenocarcinoma of the prostate," *Urology*, vol. 45, no. 1, pp. 81–86, 1995.

[20] P. L. Alo', P. Visca, A. Marci, A. Mangoni, C. Botti, and U. Di Tondo, "Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients," *Cancer*, vol. 77, no. 3, pp. 474–482, 1996.

[21] A. Rashid, E. S. Pizer, M. Moga, et al., "Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia," *American Journal of Pathology*, vol. 150, no. 1, pp. 201–208, 1997.

[22] L. Z. Milgraum, L. A. Witters, G. R. Pasternack, and F. P. Kuhajda, "Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma," *Clinical Cancer Research*, vol. 3, no. 11, pp. 2115–2120, 1997.

[23] E. S. Pizer, F. D. Wood, H. S. Heine, F. E. Romantsev, G. R. Pasternack, and F. P. Kuhajda, "Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer," *Cancer Research*, vol. 56, no. 6, pp. 1189–1193, 1996.

[24] H. Orita, J. Coulter, E. Tully, F. P. Kuhajda, and E. Gabrielson, "Inhibiting fatty acid synthase for chemoprevention of chemically induced lung tumors," *Clinical Cancer Research*, vol. 14, no. 8, pp. 2458–2464, 2008.

[25] V. Jensen, M. Ladekarl, P. Holm-Nielsen, F. Melsen, and F. B. Soerensen, "The prognostic value of oncogenic antigen 519 (OA-519) expression and proliferative activity detected by antibody MIB-1 in node-negative breast cancer," *Journal of Pathology*, vol. 176, no. 4, pp. 343–352, 1995.

[26] M. S. Shurbaji, J. H. Kalbfleisch, and T. S. Thurmond, "Immunohistochemical detection of a fatty acid synthase (OA-519) as a predictor of progression of prostate cancer," *Human Pathology*, vol. 27, no. 9, pp. 917–921, 1996.

[27] J. A. Menendez and R. Lupu, "Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis," *Nature Reviews Cancer*, vol. 7, no. 10, pp. 763–777, 2007.

[28] W. Zhou, F. H. Wan, L. E. Landree, et al., "Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells," *Cancer Research*, vol. 67, no. 7, pp. 2964–2971, 2007.

[29] D. Di Vizio, F. Sotgia, T. M. Williams, et al., "Caveolin-1 is required for the upregulation of fatty acid synthase (FASN), a tumor promoter, during prostate cancer progression," *Cancer Biology and Therapy*, vol. 6, no. 8, pp. 1263–1268, 2007.

[30] M.-Y. Lee, J.-S. Moon, S. W. Park, Y.-K. Koh, Y.-H. Ahn, and K.-S. Kim, "KLF5 enhances SREBP-1 action in androgen-dependent induction of fatty acid synthase in prostate cancer cells," *Biochemical Journal*, vol. 417, no. 1, pp. 313–322, 2009.

[31] L. Korutla, P. Wang, T. G. Jackson, and S. A. Mackler, "NAC1, a POZ/BTB protein that functions as a corepressor," *Neurochemistry International*, vol. 54, no. 3-4, pp. 245–252, 2009.

[32] L. Korutla, R. Degnan, P. Wang, and S. A. Mackler, "NAC1, a cocaine-regulated POZ/BTB protein interacts with CoREST," *Journal of Neurochemistry*, vol. 101, no. 3, pp. 611–618, 2007.

[33] L. Korutla, P. J. Wang, and S. A. Mackler, "The POZ/BTB protein NAC1 interacts with two different histone deacetylases in neuronal-like cultures," *Journal of Neurochemistry*, vol. 94, no. 3, pp. 786–793, 2005.

[34] H. Liu, Y. Liu, and J.-T. Zhang, "A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction," *Molecular Cancer Therapeutics*, vol. 7, no. 2, pp. 263–270, 2008.

[35] A. Vazquez-Martin, S. Ropero, J. Brunet, R. Colomer, and J. A. Menendez, "Inhibition of fatty acid synthase (FASN) synergistically enhances the efficacy of 5-fluorouracil in breast carcinoma cells," *Oncology Reports*, vol. 18, no. 4, pp. 973–980, 2007.

[36] J. Song, I.-M. Shih, R. Salani, D. W. Chan, and Z. Zhang, "Annexin XI is associated with cisplatin resistance and related to tumor recurrence in ovarian cancer patients," *Clinical Cancer Research*, vol. 13, no. 22, pp. 6842–6849, 2007.

[37] I.-M. Shih, J. J.-C. Sheu, A. Santillan, et al., "Amplification of a chromatin remodeling gene, Rsf-1/HBXAP, in ovarian carcinoma," *Proceedings of the National Academy of Sciences of*

the United States of America, vol. 102, no. 39, pp. 14004–14009, 2005.

[38] A. Sorrentino, C.-G. Liu, A. Addario, C. Peschle, G. Scambia, and C. Ferlini, "Role of microRNAs in drug-resistant ovarian cancer cells," *Gynecologic Oncology*, vol. 111, no. 3, pp. 478–486, 2008.

[39] D. Gupta, M. Venkatesh, H. Wang, et al., "Expanding the roles for pregnane X receptor in cancer: proliferation and drug resistance in ovarian cancer," *Clinical Cancer Research*, vol. 14, no. 17, pp. 5332–5340, 2008.

[40] M. Ishibashi, K. Nakayama, S. Yeasmin, et al., "A BTB/POZ gene, NAC-1, a tumor recurrence-associated gene, as a potential target fortaxol resistance in ovarian cancer," *Clinical Cancer Research*, vol. 14, no. 10, pp. 3149–3155, 2008.

[41] J. Wang, J.-Y. Zhou, and G. S. Wu, "ERK-dependent MKP-1-mediated cisplatin resistance in human ovarian cancer cells," *Cancer Research*, vol. 67, no. 24, pp. 11933–11941, 2007.

[42] F. P. Kuhajda, L. E. Landree, and G. V. Ronnett, "The connections between C75 and obesity drug-target pathways," *Trends in Pharmacological Sciences*, vol. 26, no. 11, pp. 541–544, 2005.

[43] E. S. Pizer, F. J. Chrest, J. A. DiGiuseppe, and W. F. Han, "Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines," *Cancer Research*, vol. 58, no. 20, pp. 4611–4615, 1998.

[44] H. Q. Wang, D. A. Altomare, K. L. Skele, et al., "Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells," *Oncogene*, vol. 24, no. 22, pp. 3574–3582, 2005.

[45] T. W. Grunt, R. Wagner, M. Grusch, et al., "Interaction between fatty acid synthase- and ErbB-systems in ovarian cancer cells," *Biochemical and Biophysical Research Communications*, vol. 385, no. 3, pp. 454–459, 2009.

[46] S. Uddin, A. K. Siraj, M. Al-Rasheed, et al., "Fatty acid synthase and AKT pathway signaling in a subset of papillary thyroid cancers," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 10, pp. 4088–4097, 2008.

[47] J. Q. Cheng, A. K. Godwin, A. Bellacosa, et al., "AKT2, a putative oncogene encoding a member of a subfamily of protein- serine/threonine kinases, is amplified in human ovarian carcinomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 19, pp. 9267–9271, 1992.

[48] J.-N. Li, M. Gorospe, F. J. Chrest, et al., "Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53," *Cancer Research*, vol. 61, no. 4, pp. 1493–1499, 2001.

[49] X. Lu, J. Errington, N. J. Curtin, J. Lunec, and D. R. Newell, "The impact of p53 status on cellular sensitivity to antifolate drugs," *Clinical Cancer Research*, vol. 7, no. 7, pp. 2114–2123, 2001.

[50] P. M. O'Connor, J. Jackman, I. Bae, et al., "Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents," *Cancer Research*, vol. 57, no. 19, pp. 4285–4300, 1997.

[51] R. Salani, R. J. Kurman, R. Giuntoli II, et al., "Assessment of TP53 mutation using purified tissue samples of ovarian serous carcinomas reveals a higher mutation rate than previously reported and does not correlate with drug resistance," *International Journal of Gynecological Cancer*, vol. 18, no. 3, pp. 487–491, 2008.

[52] S. Rossi, E. Graner, P. Febbo, et al., "Fatty acid synthase expression defines distinct molecular signatures in prostate cancer," *Molecular Cancer Research*, vol. 1, no. 10, pp. 707–715, 2003.

[53] M. Fiorentino, G. Zadra, E. Palescandolo, et al., "Overexpression of fatty acid synthase is associated with palmitoylation of Wnt1 and cytoplasmic stabilization of β-catenin in prostate cancer," *Laboratory Investigation*, vol. 88, no. 12, pp. 1340–1348, 2008.

[54] D. T. Coleman, R. Bigelow, and J. A. Cardelli, "Inhibition of fatty acid synthase by luteolin post-transcriptionally down-regulates c-Met expression independent of proteosomal/lysosomal degradation," *Molecular Cancer Therapeutics*, vol. 8, no. 1, pp. 214–224, 2009.

[55] J. L. Little, F. B. Wheeler, C. Koumenis, and S. J. Kridel, "Disruption of crosstalk between the fatty acid synthesis and proteasome pathways enhances unfolded protein response signaling and cell death," *Molecular Cancer Therapeutics*, vol. 7, no. 12, pp. 3816–3824, 2008.

[56] E. Furuta, S. K. Pai, R. Zhan, et al., "Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1," *Cancer Research*, vol. 68, no. 4, pp. 1003–1011, 2008.

[57] A. L. Harris, "Hypoxia—a key regulatory factor in tumour growth," *Nature Reviews Cancer*, vol. 2, no. 1, pp. 38–47, 2002.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 821717, 12 pages
doi:10.1155/2010/821717

Research Article

Loss of miR-200c: A Marker of Aggressiveness and Chemoresistance in Female Reproductive Cancers

Dawn R. Cochrane, Erin N. Howe, Nicole S. Spoelstra, and Jennifer K. Richer

Department of Pathology, Denver School of Medicine, University of Colorado, Aurora CO, 80045, USA

Correspondence should be addressed to Jennifer K. Richer, jennifer.richer@ucdenver.edu

Received 1 June 2009; Accepted 26 September 2009

Academic Editor: Phillip J. Buckhaults

We focus on unique roles of miR-200c in breast, ovarian, and endometrial cancers. Members of the miR-200 family target ZEB1, a transcription factor which represses E-cadherin and other genes involved in polarity. We demonstrate that the double negative feedback loop between miR-200c and ZEB1 is functional in some, but not all cell lines. Restoration of miR-200c to aggressive cancer cells causes a decrease in migration and invasion. These effects are independent of E-cadherin status. Additionally, we observe that restoration of miR-200c to ovarian cancer cells causes a decrease in adhesion to laminin. We have previously reported that reintroduction of miR-200c to aggressive cells that lack miR-200c expression restores sensitivity to paclitaxel. We now prove that this ability is a result of direct targeting of class III beta-tubulin (TUBB3). Introduction of a TUBB3 expression construct lacking the miR-200c target site into cells transfected with miR-200c mimic results in no change in sensitivity to paclitaxel. Lastly, we observe a decrease in proliferation in cells transfected with miR-200c mimic, and cells where ZEB1 is knocked down stably, demonstrating that the ability of miR-200c to enhance sensitivity to paclitaxel is not due to an increased proliferation rate.

1. Introduction

Specific miRNAs have been found to be expressed in cell type-specific manner, at specific developmental stages, and in disease states including cancer [1, 2]. During the initiation and progression of cancer, miRNAs have been observed to act as oncogenes or tumor suppressors [3, 4]. While some miRNAs are overexpressed in cancers, the majority appear to be lost and often localize to fragile sites [5]. Differences in the miRNA expression profiles of normal compared to cancerous tissue of the endometrium, breast and ovary have been documented [6–11]. MiRNAs can affect the expression of a large number of proteins, including those involved in pathways relevant to cancer, such as apoptosis, migration and metastatis. Thus, miRNAs hold promise as biomarkers for several types of cancer [12, 13].

Epithelial to mesenchymal transition (EMT) is a normal process that occurs during development in which individual cells or groups of cells become motile. The same process is thought to be used by cancer cells during tumor progression to enable them to become more motile and thus more metastatic [14]. EMT involves reprogramming of the cells by transcription factors such as ZEB1, SIP1 (ZEB2), Twist, Snail, and Slug [15]. A hallmark of EMT is loss of E-cadherin expression, loss of polarity, acquisition of mesenchymal markers, and increased motility [16, 17]. Both ZEB1 and the closely related ZEB2 bind E-box like sequences in the E-cadherin promoter, recruit the corepressor CtBP and thereby repress E-cadherin [18]. ZEB1 also represses additional genes involved in polarity [16]. ZEB1 expression is confined to cells of mesenchymal origin, while normal epithelial cells and low grade carcinomas do not express ZEB1. However, we and others have shown that in high grade, aggressive carcinomas that have undergone EMT, ZEB1 can be expressed, leading to loss of E-cadherin [19–22].

Several miRNAs have been implicated in the process of EMT, among them are the members miR-200 family [23–25]. This family contains five members (miR-200a, -200b, -200c, -141 and, -429) which are highly homologous. Originally, miR-200c was reported to directly bind ZEB1 and cause degradation of the mRNA, resulting in an upregulation of E-cadherin [26]. Subsequently, other reports have shown

that all members of the miR-200 family, since they share a high degree of homology especially in their seed sequence, are capable of repressing both ZEB1 and ZEB2 [27–29]. We have demonstrated that miR-200c represses not only ZEB1/2, but a program of transcripts normally expressed only in cells of mesenchymal origin [30]. Since members of the miR-200 family are responsible for repressing ZEB1 and ZEB2 as well as other mesenchymal genes, these miRNAs are considered "guardians of the epithelial phenotype." MiR-200 family members are therefore thought to be expressed in an epithelial cell-specific manner in normal tissues. Recently, the ability of ZEB1 to transcriptionally repress expression of miR-200 family members has been documented [31, 32]. This double negative feedback loop between miR-200 family members and ZEB1 allows for plasticity between the epithelial and mesenchymal states [33].

In this paper, we focus on the role of miR-200c in breast, ovarian, and endometrial cancers. The mutual repression between ZEB1 and miR-200c is functional in some, but not all cells that we have tested. Increasing miR-200c levels causes a decrease in adhesion to laminin. We demonstrate that the decrease in migration and invasion observed when miR-200c is reintroduced to cancer cells that lack it is independent of restoration of E-cadherin. Lastly, we have previously demonstrated that class III beta tubulin (TUBB3) is directly controlled by miR-200c. Expression of TUBB3 is known to be a common mechanism of resistance to microtubule-targeting agents in many types of cancer. Here, we present conclusive data that repression of TUBB3 is the mechanism whereby miR-200c restores sensitivity to paclitaxel. Taken together, these data demonstrate that loss of miR-200c is a marker for chemoresistance and aggressiveness in breast, ovarian, and endometrial cancers.

2. Materials and Methods

2.1. Cell Culture. Hec50 cells, representing the more aggressive Type II endometrial cancers [19], were cultured in DMEM with 10% FBS, and 2 mM L-glutamine. MDA-MB-231 are a triple negative breast cancer cell line and were grown in media containing 5% FBS, HEPES, nonessential amino acids, L-glutamine, penicillin, streptomycin, and insulin. Hey cells were grown in RPMI with 5% FBS. All cells were grown in a 37°C incubator with 5% CO_2. The identity of all the cell lines was confirmed by DNA profiling using the Identifiler Kit (Applied Biosystems).

2.2. Transfections. Lipofectamine 2000 (Invitrogen) was combined with pre-200c (miRNA mimic) or scrambled negative control (Ambion) at a concentration of 60 nM and incubated in serum free RPMI for 20 minutes prior to addition to Hey cells. Cells were incubated at 37°C for 4 hours before replacement of FBS to 10%. Protein and RNA were harvested 48 hours posttransfection.

TUBB3 (from Fernando Cabral, University of Texas - Houston Medical School) was cloned into pCI-neo. Transient transfection of 3.3 μg of TUBB3 plasmid or empty vector (pCI-neo, Promega) per well in a 6-well plate was performed using lipofectamine 2000.

2.3. Generation of Stable Cell Lines. Transduction of cells was performed using SMARTvectorTM shRNA Lentivral Particles (Thermo Scientific Dharmacon). Each cell line was transduced with 3 separate lentiviral constructs targeting ZEB1 as well as two controls: SMARTvector Empty Vector particles and SMARTvector Firefly Luciferase Control particles. The former is a negative control and does not correlate with gene silencing and the latter is a positive control targeting firefly luciferase plasmids PGL2 and PGL3. All vectors are packaged and contain a TurboGFP and an SCMV promoter, as well as a puromycin-resistance selectable marker.

MDA231 and Hec50 cells were plated at 3000 cells/well and 1500 cells/well, respectively, in triplicate using 96 well plates. The following day, media was replaced with 80 μl of fresh media containing 10 μg/mL polybrene (Sigma). The amount of viral particles/well was determined using the following calculation: (MOI × CN)/VT, where MOI (multiplicity of infection) = 10 TU/cell, CN = number of cells/well, and VT = stock viral titer of 10^4 TU/μL. Viral particles were added in a total volume of 20 μL to each well. The following day, transduction media was removed and wells were rinsed with PBS and replaced with regular media. Once confluent, cells were trypsinized and replated in 48 well plates. At this point, antibiotic selection was initiated and cells were ultimately expanded and maintained using 1 μg/mL of puromycin (Sigma).

2.4. Immunoblotting. Whole cell protein extracts were denatured and 50 μg separated on 8% SDS PAGE gels and transferred to PVDF membranes. The membranes were blocked in 5% milk in TBS-T, and then probed overnight at 4°C. Primary antibodies were diluted in 5% milk in TBS-T. The primary antibodies used were ZEB1 (rabbit polyclonal from Dr. Doug Darling, University of Kentucky, 1 : 1500 dilution), E-cadherin (clone NCH-38 from DAKO, 1 μg/mL), TUBB3 (rabbit polyclonal PRB-435P from Covance, 1 : 5000 dilution), and α-tubulin (clone B-5-1-2 from Sigma, 1 : 20000 dilution). After incubation with appropriate HRP-conjugated secondary antibody, bands were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

2.5. Real Time RT-PCR. RNA was harvested from cells using Trizol (Invitrogen) as per the manufacturer's instructions. Prior to generating cDNA, mRNA was treated with DNase1 (Invitrogen) for 15 minutes at room temperature. RNA was reverse transcribed into cDNA in a reaction containing reaction buffer, dNTPs, RNAse inhibitor (Applied Biosystems), random hexamers, and 200 U of MuLV-RT (Applied Biosystems). The reaction proceeded at 22°C for 10 minutes, then at 37°C for one hour. For normalization, real time RT-PCR was performed on the cDNA using eukaryotic 18S rRNA endogenous control primers and FAM-MGB probe (Applied Biosystems). TaqMan MicroRNA Reverse Transcription kit was used to generate cDNA for real time RT-PCR reaction in conjunction with a miR-200c specific primer and probe (ABI, assay ID 002300). The reverse transcription primer for miR-200c is a hairpin primer which is specific for the mature miRNA and will not bind to the

precursor molecules. Reported values are the means and standard errors of 3 biological replicates.

The relative mRNA or miRNA levels were calculated using the comparative Ct method (ΔΔCt). Briefly, the cycle threshold (Ct) values for the rRNA were subtracted from Ct values of the target gene to achieve the ΔCt value. The $2^{-\Delta Ct}$ was calculated for each sample and then each of the values was then divided by a control sample to achieve the relative miRNA levels (ΔΔCt).

2.6. Migration and Invasion Assays. The assays were performed on MDA-MB-231 stable empty vector or shZEB1 #2, or Hey cells transiently transfected with the miR-200c mimic for 48 hours. Cells were serum starved for 12 hours prior to performing the assay. BD BioCoat Control Insert Chambers 24-well plate with 8 micron pore size and BD BioCoat Matrigel Invasion Chambers were used for migration and invasion assays, respectively. After starvation, cells were removed from the plate and 50000 Hey cells or 250000 MDA-MB-231 cells were plated in 0.5 mL media with 0.5% FBS in the upper chamber. In the lower chamber 0.8 mL of 50% conditioned media plus 50% complete media containing an additional 10% FBS was used as an attractant. Hey cells were incubated for 24 hours and MDA-MB-231 cells for 48 hours at 37°C. Migrating or invading cells on the lower surface of the membranes were stained with Diff-Quik stain (Fisher) and counted manually using ImagePro Plus software (Mediacybernetics Inc.).

2.7. Adhesion Assays. Adhesion assays were performed using InnoCyte ECM Cell Adhesion Assays (Calbiochem) for Collagen IV, Fibronectin, Basement Membrane Complex and Laminin. To each well 50000 cells were added and the plates were incubated at 37°C for 1.5 hour. The wells were gently washed with PBS before adding the Calcein-AM solution. The plates were incubated for 1 hour at 37°C, and fluorescence was read with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The relative fluorescent units were plotted, and the error bars represent standard error of the mean over four replicates.

2.8. Clonogenic Assay. Hey cells were plated into 6-well plates at a density of 2000 cells per well. Twenty-four hours after plating, the cells were treated with 0, 1, 2, 3, 4, or 5 nM paclitaxel (Sigma) in triplicate. The cells were incubated at 37°C for 8 days before fixing and staining with crystal violet. Photos were taken of the plates and the images analyzed using ImageJ software (NIH). The average number of colonies and the average total area was plotted, with error bars representing the standard error of the mean over the three replicates.

2.9. Cell Death ELISAs. Hec50 cells were transfected with the miR-200c mimic as described previously for the Hey cells. Twenty-four hours after transfection, cells were treated with 0, 15, 20 or 25 nM paclitaxel (Sigma). Twenty-four hours after treatment, the Cell Death ELISA (Roche) which recognizes mono- and oligonucleosomes in the cytoplasm of dying cells was performed as per manufacturer's instructions.

2.10. Proliferation Assays. Cells were plated into 6-well plates (4000 Hey cells/well and 6000 MDA-MB-231 or Hec50 cells/well). At time points indicated, cells were trypsinized and counted using the Vi-cell Cell Viability Counter (Beckman Coulter).

3. Results

3.1. Reciprocal Repression of miR-200c and ZEB1 Occurs in Some but Not All Cell Types. Just as members of the miR-200 family can repress ZEB1 by degradation of its transcript, ZEB1 can repress expression of the miR-200 family members by binding to E-boxes within their promoter regions [31, 32]; see Figure 1(a). Directly increasing miR-200c levels in Hey cells (aggressive serous ovarian cell line) by transfection of a miR-200c mimic (pre-200c) results in repression of ZEB1 expression (Figure 1(b)). Although ZEB1 is a repressor of E-cadherin, we did not observe E-cadherin expression induced by the repression of ZEB1 in these cells (data not shown). However there are several other mechanisms through which E-cadherin can be lost including methylation of the promoter [34, 35] and chromosomal deletion [36, 37]. In contrast, we have previously shown that transient transfection of the miR-200c mimic into MDA-MB-231 (an aggressive triple negative breast cancer cell line) and Hec50 (an aggressive Type 1 endometrial cancer cell line) causes a marked repression of ZEB1 and a restoration of E-cadherin expression [30]. Presently, we stably transduced lentiviral shRNAs targeting ZEB1 into these two cell lines (Hec50 and MDA-MB-231). While two of the shRNAs did not decrease ZEB1 protein, shRNA #2 caused an almost complete repression of ZEB1 expression resulting in re-expression of E-cadherin in both cell lines (Figures 2(c) and 2(d)). Intriguingly, while knock down of ZEB1 in MDA-MB-231 cells causes the expected increase in miR-200c levels (indicative of the reciprocal regulation), no such increase is observed in Hec50 cells. This suggests that while reciprocal repression of miR-200c and ZEB1 occurs in some cell lines, it does not occur in all.

3.2. Restoration of miR-200c to Aggressive Cancer Cells Results in Decreased Migration, Invasion, and Cell Adhesion. It has been previously shown that the miR-200 family members cause a decrease in cell migration and invasion [27, 29, 30]. We observe a decrease in migration and invasion in the MDA-MB-231 cells in which ZEB1 has been knocked down, resulting in an increase in miR-200c levels. In the MDA-MB-231 cells there is 52% decrease in migration and a 50% decrease in invasion in the shZEB1 #2 containing cells in which ZEB1 is completely knocked down versus luciferase control (Figures 2(a) and 2(b)). We show here that the same holds true in the aggressive ovarian cancer Hey cell line. This cell line is highly migratory and invasive, and reintroduction of miR-200c to these cells results in an 83% decrease in migration and a 79–86% decrease in invasion compared to negative controls (Figures 2(c) and 2(d)). However, it is interesting to note that the effect on migration and invasion caused by miR-200c is independent of the E-cadherin status of the cells, since unlike the MDA-MB-231 cells, Hey cells

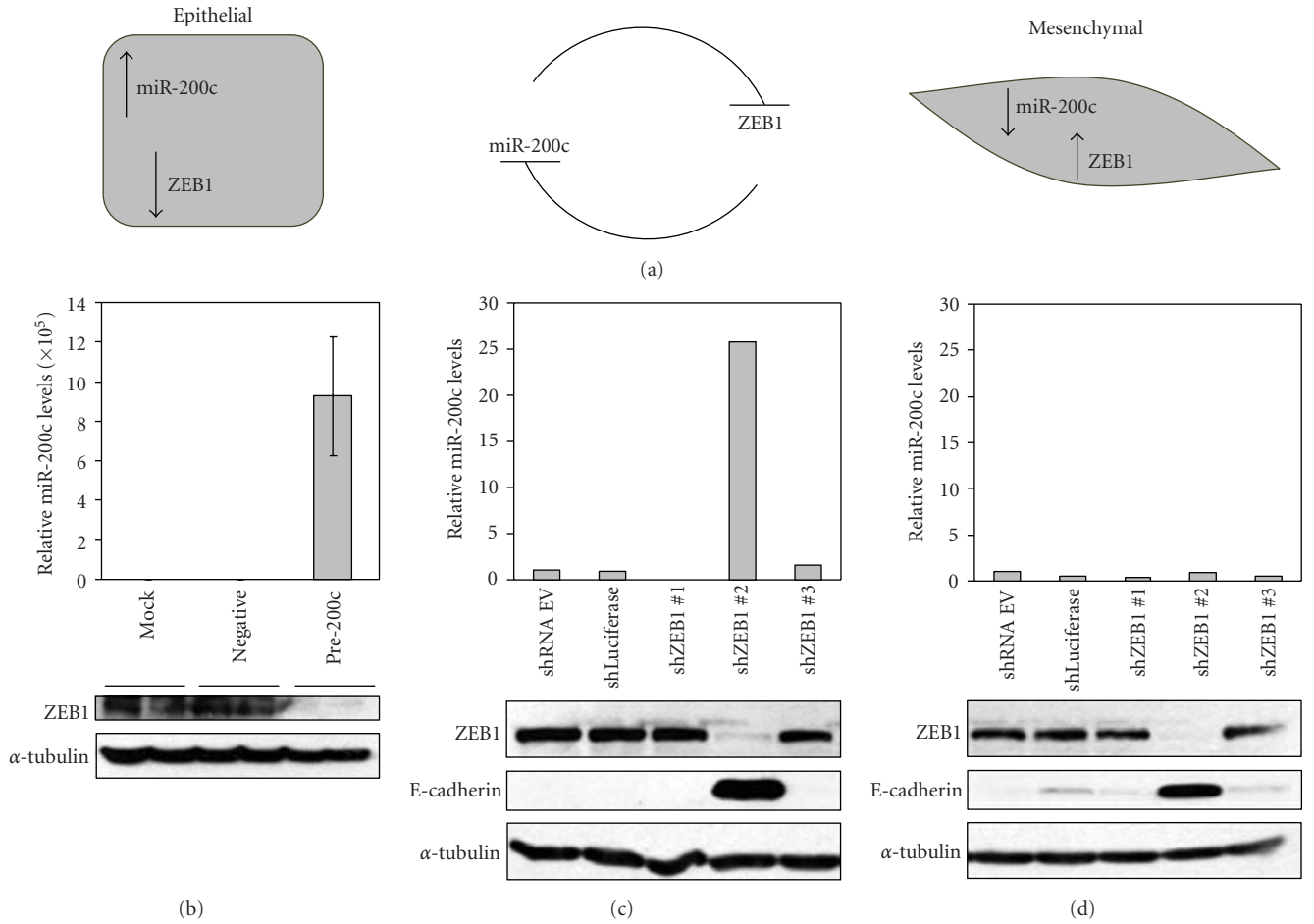


Figure 1: Reciprocal repression between ZEB1 and miR-200c occurs in some, but not all cell types. (a) Schematic of the mutual repression of ZEB1 and miR-200c. (b) Western blotting for ZEB1 and α-tubulin loading control, and real time RT-PCR for miR-200c in Hey ovarian cells transiently transfected with a miR-200c mimic. Real time RT-PCR for miR-200c and western blotting for ZEB1, E-cadherin, and α-tubulin in MDA-MB-231 breast cancer cells (c), and Hec50 endometrial cancer cells (d) stably transfected with shRNA lentiviral vector targeting ZEB1 (shZEB), luciferase (shLuciferase), or the empty vector (shRNA EV).

do not regain E-cadherin expression in response to decreased ZEB1 levels.

While E-cadherin protein affects epithelial cell-cell contact, we also wished to determine if miR-200c affects adhesion to substrates as measured by fluorescent adhesion assays. Hey cells transiently transfected with the miR-200c mimic showed a small but statistically significant decrease in adhesion to basement membrane complex (BMC) and laminin (Figures 3(a) and 3(b)). There is also a trend towards decreased adhesion to collagen IV (Figure 3(c)); however, this did not reach statistical significance. No difference in adhesion to fibronectin was observed (Figure 3(d)). Since there was an affect on adhesion to BMC and laminin in ovarian cancer cells with high miR-200c levels, we performed the adhesion assay with the Hec50 and MDA-MB-231 cells in which ZEB1 had been stably knocked down. We again see a decrease in adhesion to BMC and laminin in the MDA-MB-231 cells; however, only the decrease in BMC binding is statistically significant (Figures 3(e) and 3(f)). In contrast to the Hey and MDA-MB-231 cells, there was no decrease in adhesion to either substrate in the Hec50 cells in which ZEB1 is knocked down (data not shown), but there is not a concomitant increase in miR-200c, as shown in Figure 1(d). This result suggests that the effects on adhesion may be mediated through miR-200c.

3.3. Increased Chemosensitivity to Paclitaxel with miR-200c Expression. We have previously demonstrated that miR-200c expression causes increased chemosensitivity to microtubule targeting agents such as paclitaxel. While the ELISA cell death assay that we have used previously to demonstrate this property of miR-200c is a short-term assay, we confirm here, in a relatively long-term clonogenic assay, that there is increased sensitivity of Hey cells to paclitaxel when transfected with pre-200c (Figure 4(a)). We observe a 49–55% decrease in total area and a 67–70% decrease in the number of colonies in the pre-200c treated cells versus the negative control with 5 nM paclitaxel treatment (Figures 4(b)

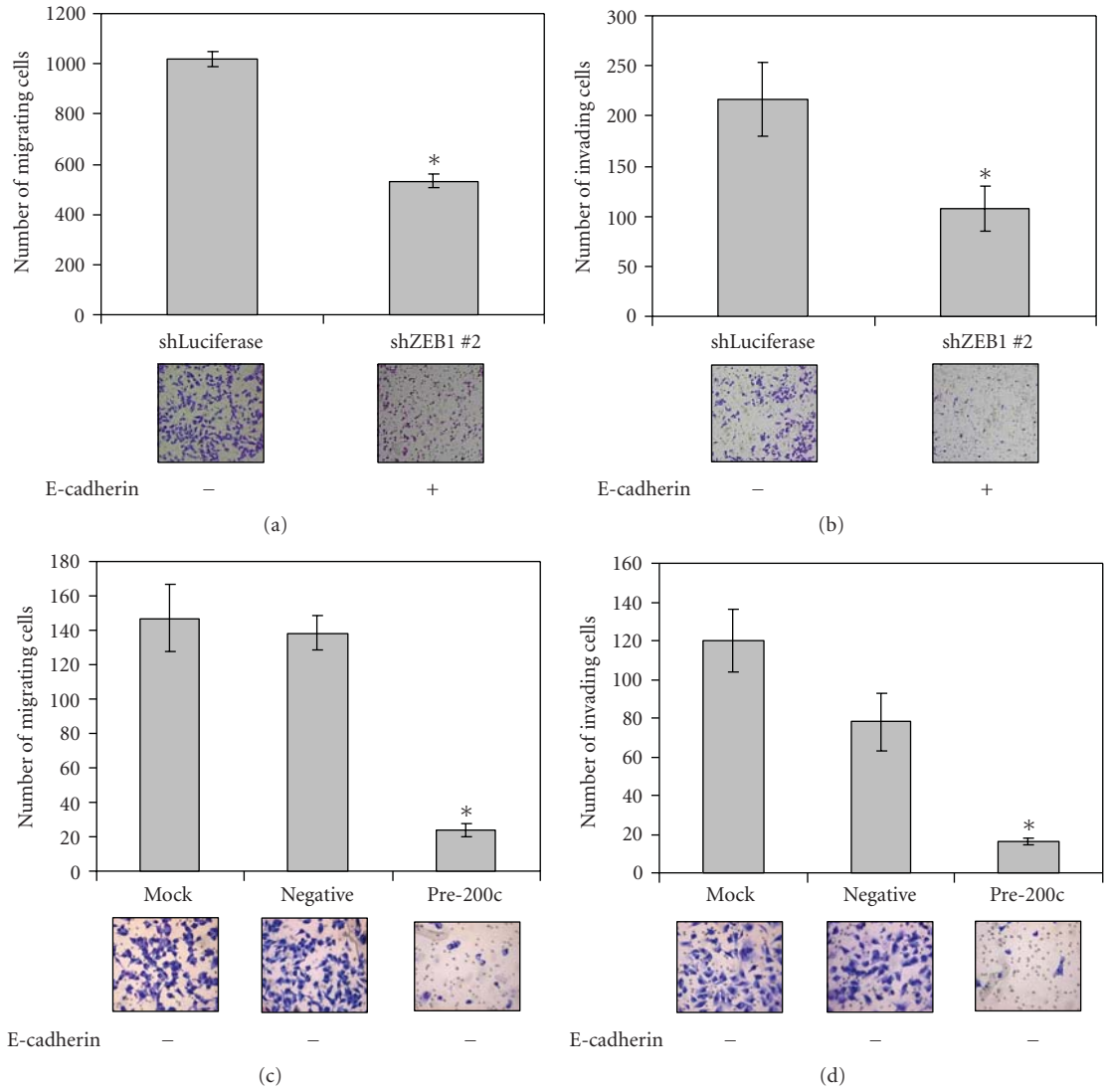


Figure 2: Increased miR-200c decreases migration and invasion, not necessarily dependent on restoration of E-cadherin. Migration (a) and invasion (b) assays for MDA-MB-231 cells stably expressing an shRNA targeting ZEB1 with representative images below. Migration (c) and invasion (d) assays in Hey cells transiently transfected with a miR-200c mimic. Asterisks indicate a statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

and 4(c)). As the assay is conducted over a relatively long period of time, the maximum dose of paclitaxel used is relatively small compared to what is used in the assays that look at acute toxicity (i.e., 24 hours). At doses of paclitaxel of 10 nM and higher, no colonies are formed in the assay (data not shown).

We have previously implicated the ability of miR-200c to directly target *TUBB3* (class III beta tubulin) as being the mechanism responsible for the increased chemosensitivity to microtubule targeting agents. TUBB3 is normally only expressed in neuronal cells; however aberrant expression of TUBB3 in several different types of cancers has been shown to cause resistance to paclitaxel [38–43]. We demonstrated that miR-200c directly targets *TUBB3* for degradation. To definitively test whether TUBB3 is responsible for the miR-200c-mediated increase in chemosensitivity to paclitaxel, we transfected cells with a TUBB3 construct lacking its 3' UTR (containing the miR-200c binding site) which is therefore not able to be targeted by miR-200c. Transfection of this exogenous TUBB3 construct does not affect the transfection of the miR-200c mimic, nor its ability to downregulate ZEB1 and upregulate E-cadherin (Figures 5(a) and 5(b)) in Hec50 cells. When the Hec50 cells are transfected with an empty vector (no exogenous TUBB3) in addition to miR-200c mimic, there is a statistically significant increase in sensitivity to paclitaxel as measured in a cell death ELISA; see Figure 5(c). However, when the cells are transfected with the TUBB3 expression vector lacking its 3' UTR, the enhanced sensitivity to paclitaxel is lost; see Figure 5(d). Therefore, expression of exogenous TUBB3 lacking the miR-200c target site reverses the chemosensitivity to paclitaxel caused by increased miR-200c expression.

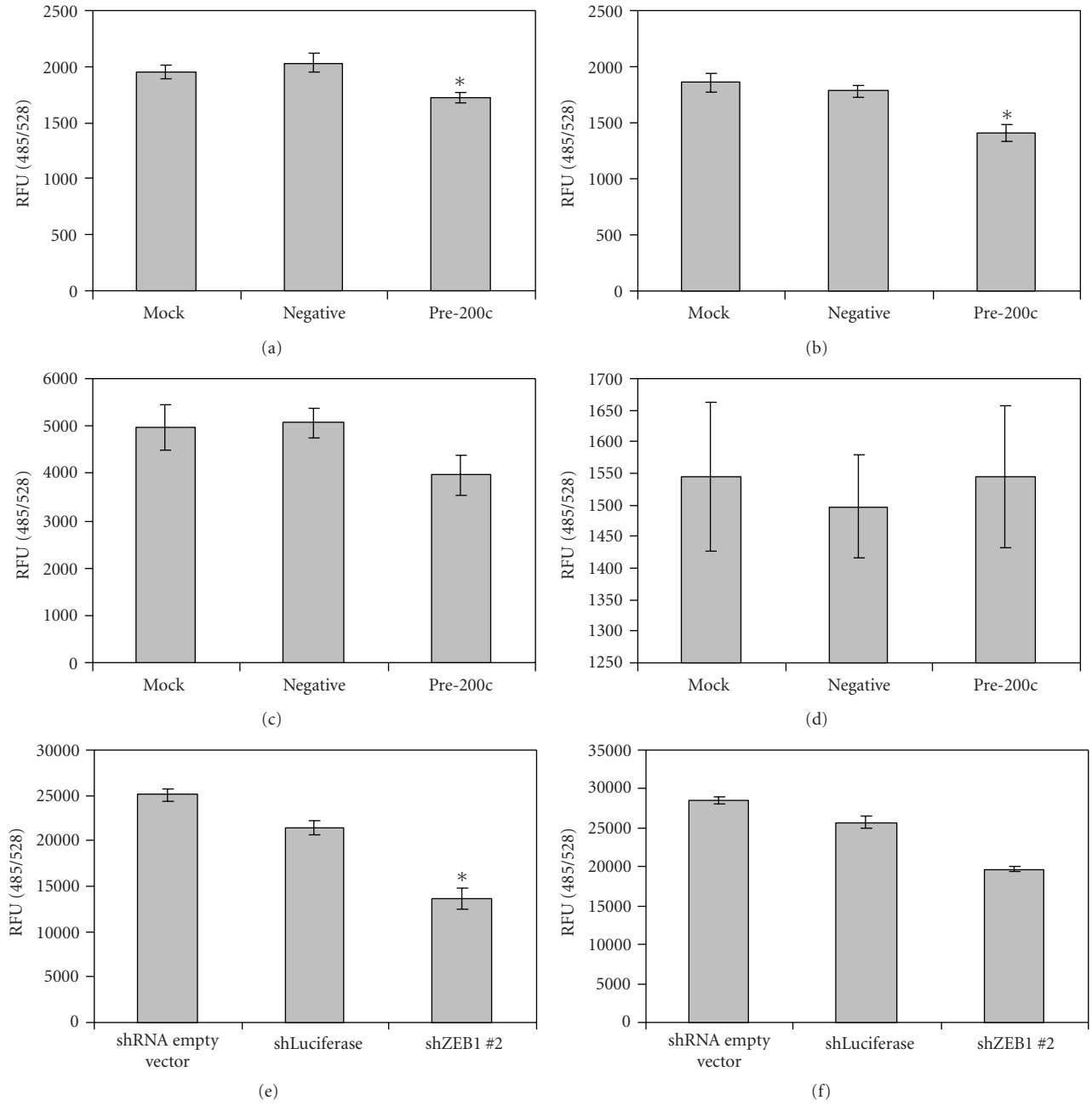


Figure 3: Increased miR-200c levels decrease adhesion to substrates. Fluorescent adhesion assays of Hey cells to (a) basement membrane complex, (b) laminin, (c) collagen type IV, and (d) fibronectin. Adhesion of MDA-MB-231 cells to (e) basement membrane complex and (f) laminin. Asterisks indicate a statistically significant difference ($P < .05$, Student's t-test) versus the negative controls.

It can be argued that cells with increased proliferation would be more sensitive to microtubule poisons and that could be an alternative explanation for the observed chemosensitivity upon restoration of miR-200c. We therefore performed proliferation assays in the three cell types and found decreased proliferation in all three (Figures 6(a), 6(b), and 6(c)). Since the decrease in proliferation is observed in all three cell types, including the Hec50s where there was no increase in miR-200c levels, it is likely that the effects on proliferation occur via ZEB1 and not by miR-200c. The fact that the increase in chemosensitivity is found in cells that are proliferating more slowly than the negative controls demonstrates that increased proliferation is not the mechanism behind the increase in chemosensitivity.

4. Discussion

In this paper we build on our previous work to further characterize the role that loss of miR-200c plays in generating an aggressive cancer phenotype. We focus on ovarian,

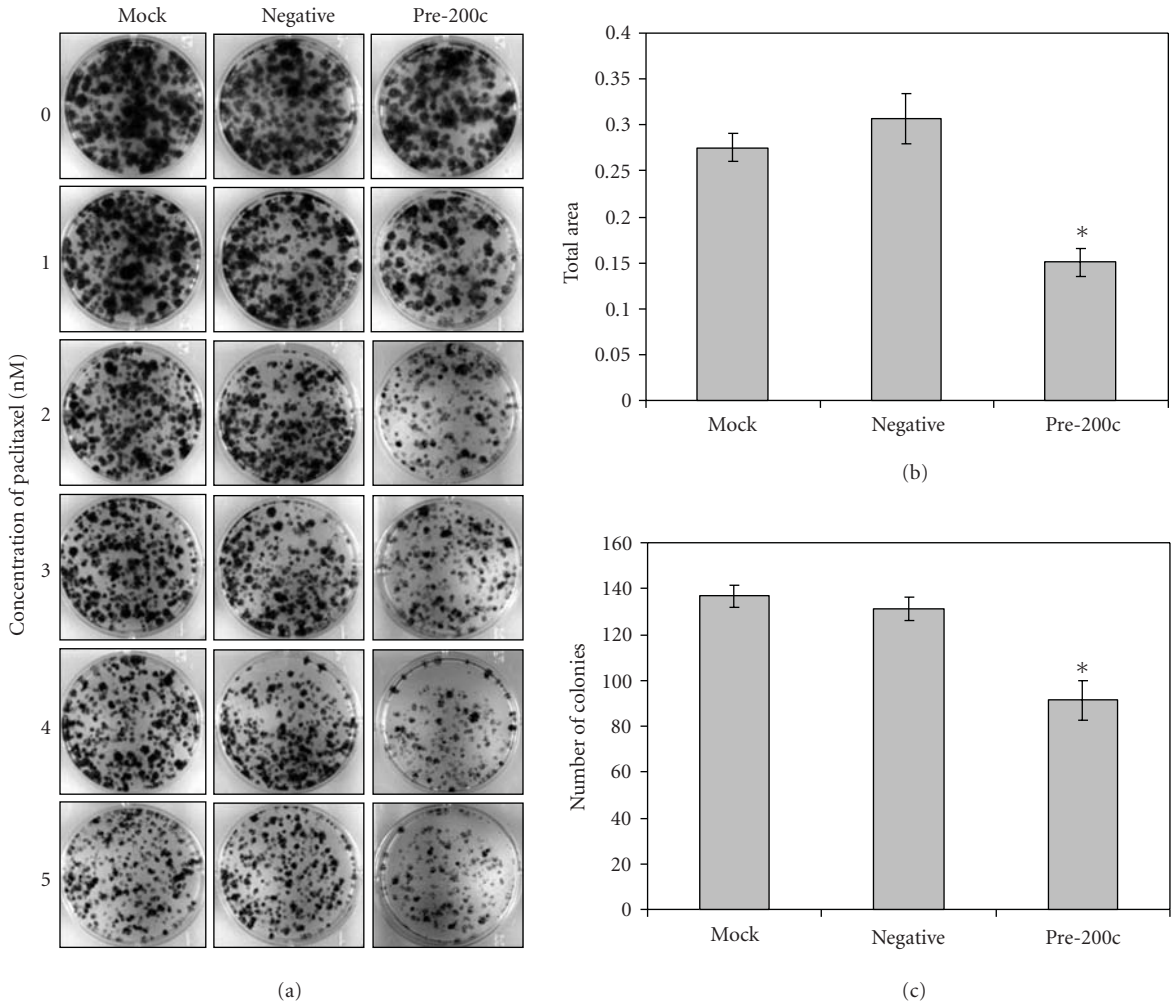


Figure 4: Chemosensitivity to paclitaxel induced by miR-200c expression. (a) Clonogenic assay in Hey cells either mock transfected, transiently transfected with a negative control, or miR-200c mimic and treated with 0–5 nM paclitaxel. The total area (b) and number of colonies (c) at 5 nM paclitaxel are quantified. Asterisks indicate statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

endometrial, and breast cancer. The miR-200 family is crucial for the maintenance of the epithelial phenotype. ZEB1 is normally only expressed in cells of mesenchymal origin; however, its aberrant expression is observed in cancers that have undergone EMT. ZEB1 (and the closely related ZEB2) transcripts are targeted by miR-200c and the other miR-200 family members. Interaction of any of the miR-200 family members with the ZEB transcripts results in degradation and inhibition of translation. Therefore the maintenance of miR-200c expression in normal epithelial cells serves to prevent ZEB1 and ZEB2 from being expressed. Since both ZEB1 and ZEB2 repress genes involved in polarity, repression of these proteins serves to maintain polarity, an important epithelial cell characteristic. We have recently shown that in addition to repressing ZEB1 and 2, miR-200c represses a program of transcripts normally only expressed in cells of mesenchymal and neuronal origin, such as fibronectin (FN1), neurotrophic tyrosine kinase (NTRK2), quaking 1 (QKI), and TUBB3 [30]. Thus, miR-200c maintains epithelial cell characteristics not only by maintaining polarity via repression of ZEB1 and ZEB2, but also by repressing additional non-epithelial genes.

It has been recently demonstrated that miR-200c and ZEB1 regulate each other in a double-negative feedback loop [31, 32]. The miR-200 family of miRNAs is expressed in two clusters, one on choromosome 1p36.33 and the other on chromosome 12p12.31. E-boxes are located in the promoter region of each of these clusters. ZEB1 can bind these E-boxes and directly repress all miR-200 family members [31]. Therefore, in cells that have undergone EMT, ZEB1 and 2 not only serve to repress genes involved in polarity, but also repress the miR-200 family and thereby release the repression of many genes characteristic of the mesenchymal phenotype. Central to the double feedback loop between miR-200c and ZEB1 is TGF-β. During TGF-β-induced EMT, there is an increase in Ets1 which binds to and activates the promoter of ZEB1 [44]. Therefore, in a tumor microenvironment, increased TGF-β levels are thought to result in an increase of ZEB1 transcription to a point where it can overcome the

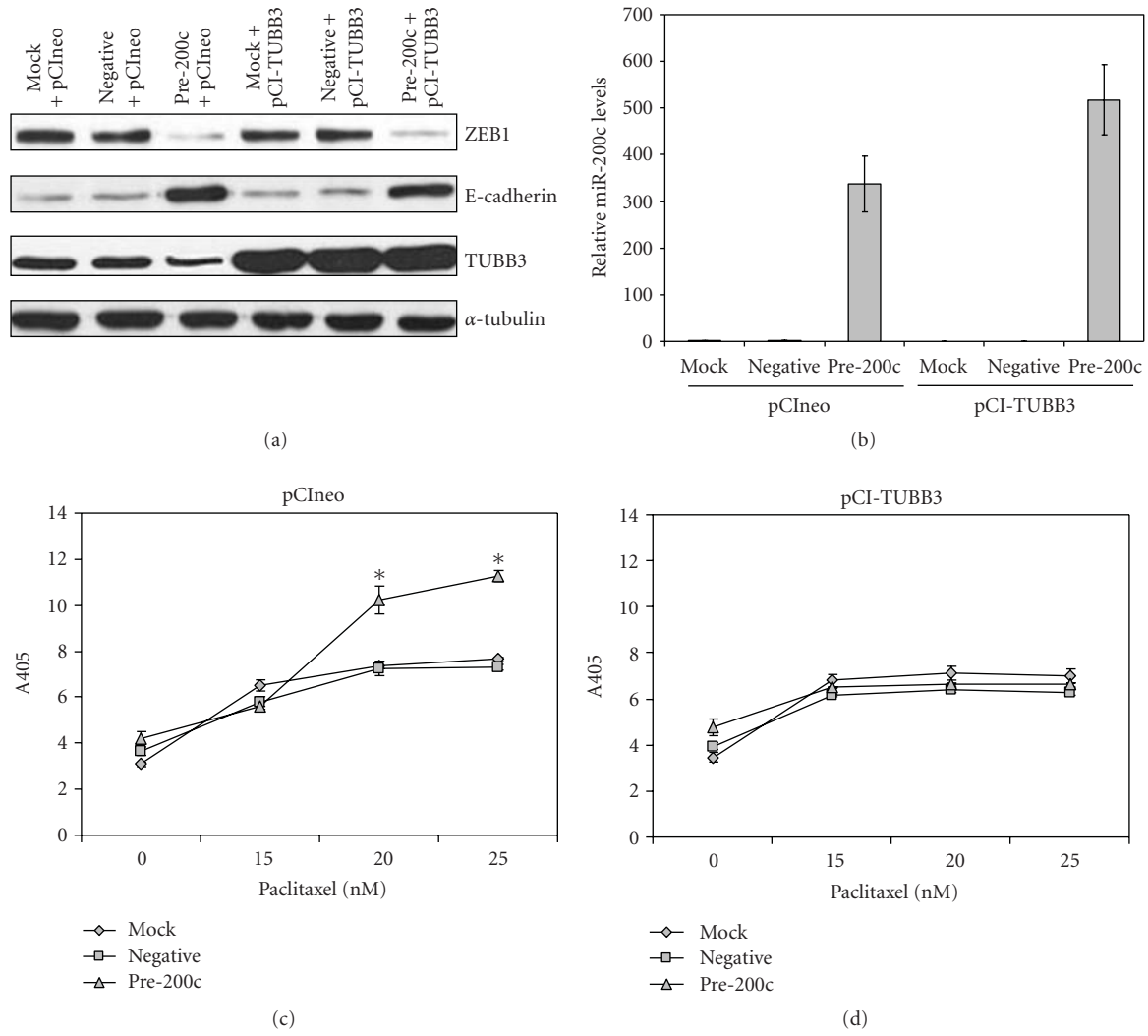


Figure 5: Restoration of TUBB3 reverses miR-200c-mediated enhanced chemosensitivity to paclitaxel. (a) Western blots for ZEB1, E-cadherin, TUBB3, and α-tubulin in Hec50 cells transiently transfected with a miR-200c mimic or negative controls and an expression vector for TUBB3 or empty vector. (b) Real time RT-PCR for miR-200c. Cell death ELISA for cells transfected with a miR-200c mimic and an empty vector (c) or TUBB3 expression vector (d) treated with various concentrations of paclitaxel. Asterisks indicate statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

repression caused by miR-200c. As ZEB1 protein begins to be made, it can then repress the miR-200 family members, resulting in progression through EMT [32].

We have previously shown that restoration of miR-200c in Hec50 endometrial cells and MDA-MB-231 breast cancer cells causes repression of ZEB1 and re-expression of E-cadherin protein. Here we show that transfection of miR-200c mimic into Hey cells, an aggressive serous ovarian cell line, also causes a dramatic repression of ZEB1; however no expression of E-cadherin was observed. To test whether the double-negative feedback loop is intact in the Hec50 and MDA-MB-231 cells, these cells were infected with lentivirus expressing an shRNA against ZEB1. In both cell lines, efficient knock down of ZEB1 was achieved, as was re-expression of E-cadherin. In MDA-MB-231 cells, ZEB1 knock down resulted in an increase in miR-200c levels, as would be expected from the negative feedback loop. However, this was not the case in Hec50 cells, where there was no increase in miR-200c. Whether the break in the negative feedback loop is an anomaly of this particular cell line remains to be tested. The mechanism behind the phenomenon is also unknown; however, it does offer an opportunity to dissect the contribution of ZEB1 versus that of miR-200c to the phenotype of the cells. For example, significantly decreased proliferation was observed in the Hey cells transiently transfected with the miR-200c mimic as well as in the MDA-MB-231 and Hec50 cells that have ZEB1 stably knocked down, although miR-200c levels did not rise in the Hec50s. Therefore it is likely that the decrease in proliferation is due to the lack of ZEB1, not an increase in miR-200c. Conversely, the decrease in adhesion to the basement membrane complex and laminin was only

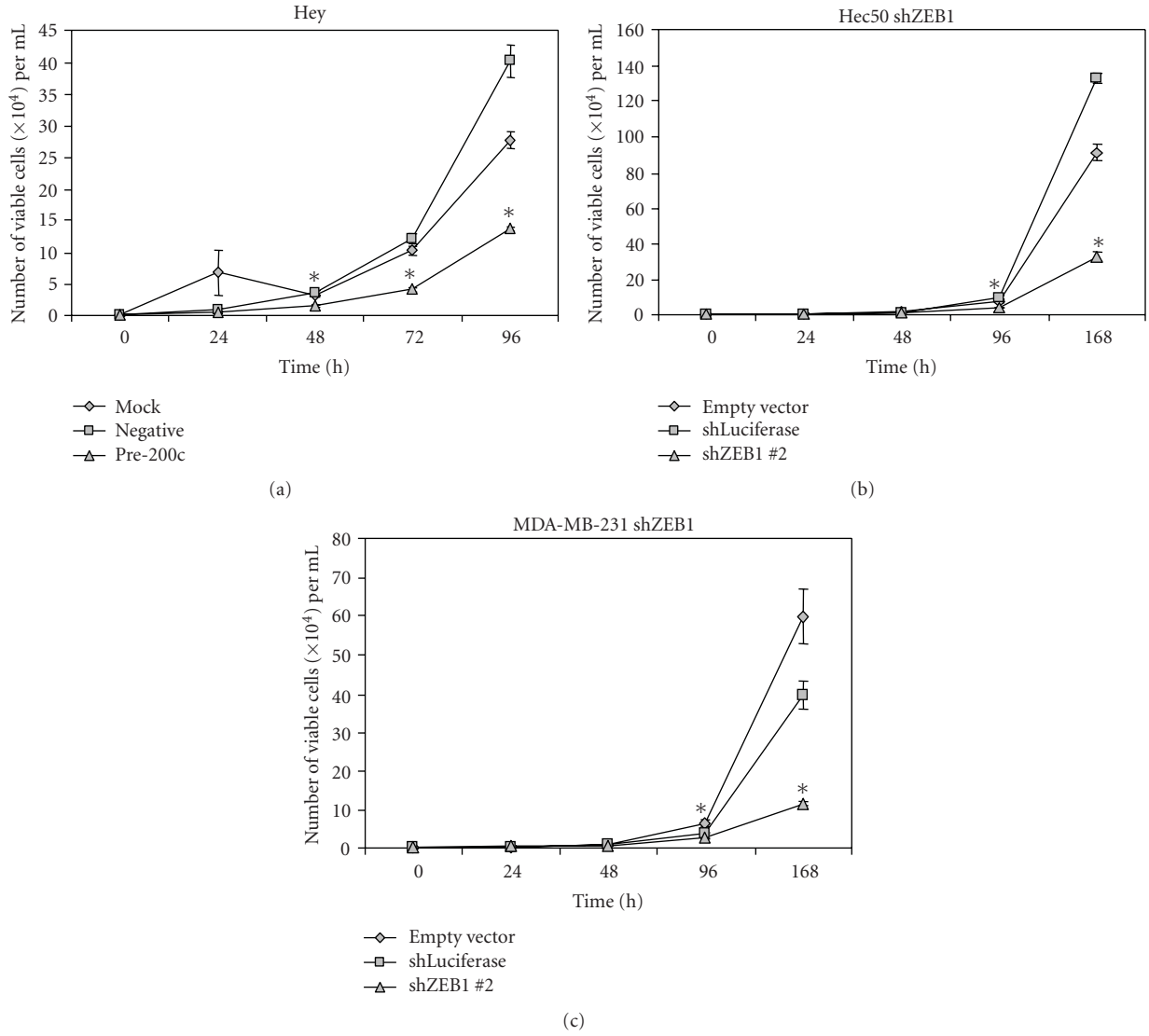


Figure 6: Proliferation assay in Hey cells transiently transfected with a miR-200c mimic (a), or Hec50 cells (b), and MDA-MB-231 cells (c) stably expressing an shRNA against ZEB1. Asterisks indicate statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

observed in the Hey and MDA-MB-231 cells, and not the Hec50s, suggesting that this phenotype is a function of miR-200c expression rather than ZEB1.

We and others have previously shown that restoration of miR-200c to cancer cells that do not express it causes a decrease in invasion and migration [27, 30]. Here we show that knock down of ZEB1 in MDA-MB-231 cells, which causes an increase in miR-200c, negatively affects migration and invasion. Furthermore, we show that restoration of miR-200c in Hey ovarian cancer cells results in a dramatic decrease in migration and invasion even though E-cadherin is not restored in these cells, despite complete repression of ZEB1. Loss of E-cadherin expression can result from mechanisms other than ZEB1 transcription repression, including chromosomal deletion and promoter hypermethylation [34–37]. Possibilities for its continued absence in these cells include promoter methylation such that even when repression by ZEB1 is relieved, E-cadherin will not be expressed, or perhaps levels of another transcriptional repressor such as Snail or Twist remain high and repress E-cadherin. Regardless of the mechanism, the effects of miR-200c on invasion and migration appear to be independent of E-cadherin status. While E-cadherin is involved in epithelial cell-cell adhesion and its expression has been shown to negatively affect migration and invasion [45, 46], increased miR-200c is able to decrease migration and invasion on its own. We have previously observed that restoration of miR-200c affects genes involved in cell motility and invasion such as ARHGDIB, NTRK2, EPHB1, and FN1 [30].

We demonstrate that miR-200c causes a decrease in adhesion to basement membrane complex, laminin, and perhaps collagen type IV. This observation is particularly

relevant to ovarian cancer because the cancerous cells adhere to sites within the peritoneal cavity. During the progression of cancer there is switching of the expression patterns of the cell surface adhesion molecules, such as the cadherins and integrins [47–49]. Although the change in the number of adherent cells appears modest, this might play a significant role in developing a potential treatment for ovarian cancer. The ability of ovarian cancer cells to spread and adhere to the peritoneal cavity is one of the major phenotypes of this disease. A small change in ability of the cells to adhere might reflect a great decrease in the tumor burden and/or increase the ability to debulk the tumor. These results are independent of E-cadherin expression since the decrease in adhesion is observed in both the MDA-MB-231 cells (where E-cadherin expression is regained with increased miR-200c) and in Hey cells, where it is not.

Clonogenic assays reveal that there is an increase in chemosensitivity to paclitaxel with increased miR-200c levels. Indeed, acquired resistance to paclitaxel in ovarian cancer cells has been shown to be associated with EMT, resulting in an aggressive phenotype [50]. Clinically, aberrant expression of TUBB3 (not normally expressed in epithelial cells) has been found to be associated with resistance to taxanes [38–41]. We have previously shown that TUBB3 is a direct target of miR-200c and suggested that its repression by miR-200c is the mechanism behind the ability of miR-200c to increase chemosensitivity to microtubule targeting agents [30]. Here, we perform the definitive experiment to prove that miR-200c-mediated TUBB3 downregulation is indeed the cause of the enhanced chemosensitivity. We utilized exogenous TUBB3 lacking its 3'UTR such that it cannot be targeted by miR-200c and show that resistance to paclitaxel is maintained even in the presence of miR-200c. In contrast, endogenous TUBB3 is reduced when miR-200c is added, resulting in enhanced chemosensitivity to paclitaxel.

Microtubule targeting agents such as paclitaxel work more efficiently in cells that are rapidly dividing. Consequently, it could be argued that the increase in chemosensitivity caused by miR-200c is due to increased proliferation. However, we show that increase of miR-200c or direct knockdown of ZEB1 results in decreased proliferation in three different types of cancer cells. It is therefore the downregulation of TUBB3, not an increase in proliferation that is responsible for the enhanced chemosensitivity to taxanes observed with restoration of miR-200c to resistant cancer cells.

5. Conclusions

MiR-200c expression serves to maintain the epithelial phenotype in well-differentiated, low-grade, breast, ovarian, and endometrial cancer cells. This phenotype includes decreased adhesion to laminin and decreased migration and invasion. Furthermore we find that not all of miR-200c's actions can be attributed to the restoration of E-cadherin via targeting of ZEB1. We further prove that miR-200c-mediated repression of TUBB3 is the cause of enhanced chemosensitivity to microtubule targeting agents. Lastly we demonstrate that not all cells exhibit the double negative feedback loop between miR-200c and ZEB1 and that this can be exploited to identify the distinct roles of miR-200c as compared to ZEB1.

Acknowledgments

The support of this paper was granted from Department of Defense Breast Cancer Research Program Idea Award BC084162 and Career Development Award (National Cancer Institute Cancer Center Support Grant P30-CA046934) to the fourth author from M. D. Anderson Uterine Specialized Program of Research Excellent Grant CA098258. D. R. Cochrane was supported by a Thorkildsen Research Fund Endowment and the DOD Breast Cancer Idea Award BC084162.

References

[1] R. Yi, M. N. Poy, M. Stoffel, and E. Fuchs, "A skin microRNA promotes differentiation by repressing 'stemness'," *Nature*, vol. 452, no. 7184, pp. 225–229, 2008.

[2] L. F. Sempere, M. Christensen, A. Silahtaroglu, et al., "Altered microRNA expression confined to specific epithelial cell subpopulations in breast cancer," *Cancer Research*, vol. 67, no. 24, pp. 11612–11620, 2007.

[3] W. C. S. Cho, "OncomiRs: the discovery and progress of microRNAs in cancers," *Molecular Cancer*, vol. 6, article 60, 2007.

[4] J. B. Cowland, C. Hother, and K. Gronbaek, "MicroRNAs and cancer," *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, vol. 115, no. 10, pp. 1090–1106, 2007.

[5] G. A. Calin, C.-G. Liu, C. Sevignani, et al., "MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 32, pp. 11755–11760, 2004.

[6] M. V. Iorio, R. Visone, G. Di Leva, et al., "MicroRNA signatures in human ovarian cancer," *Cancer Research*, vol. 67, no. 18, pp. 8699–8707, 2007.

[7] L. Zhang, S. Volinia, T. Bonome, et al., "Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 19, pp. 7004–7009, 2008.

[8] D. C. Corney and A. Y. Nikitin, "MicroRNA and ovarian cancer," *Histology and Histopathology*, vol. 23, no. 7–9, pp. 1161–1169, 2008.

[9] T. Daikoku, Y. Hirota, S. Tranguch, et al., "Conditional loss of uterine Pten unfailingly and rapidly induces endometrial cancer in mice," *Cancer Research*, vol. 68, no. 14, pp. 5619–5627, 2008.

[10] M. V. Iorio, M. Ferracin, C.-G. Liu, et al., "MicroRNA gene expression deregulation in human breast cancer," *Cancer Research*, vol. 65, no. 16, pp. 7065–7070, 2005.

[11] C. Blenkiron, L. D. Goldstein, N. P. Thorne, et al., "MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype," *Genome Biology*, vol. 8, no. 10, article R214, 2007.

[12] C. L. Bartels and G. J. Tsongalis, "Mini-reviews micrornas:novel biomarkers for human cancer," *Clinical Chemistry*, vol. 55, no. 4, pp. 623–631, 2009.

[13] M. Osaki, F. Takeshita, and T. Ochiya, "MicroRNAs as biomarkers and therapeutic drugs in human cancer," *Biomarkers*, vol. 13, no. 7-8, pp. 658–670, 2008.

[14] J. P. Thiery, "Epithelial-mesenchymal transitions in development and pathologies," *Current Opinion in Cell Biology*, vol. 15, no. 6, pp. 740–746, 2003.

[15] H. Peinado, D. Olmeda, and A. Cano, "Snail, ZEB and bHLH factors in tumour progression: an alliance against the epithelial phenotype?" *Nature Reviews Cancer*, vol. 7, no. 6, pp. 415–428, 2007.

[16] K. Aigner, B. Dampier, L. Descovich, et al., "The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity," *Oncogene*, vol. 26, no. 49, pp. 6979–6988, 2007.

[17] K. Aigner, L. Descovich, M. Mikula, et al., "The transcription factor ZEB1 (deltaEF1) represses Plakophilin 3 during human cancer progression," *FEBS Letters*, vol. 581, no. 8, pp. 1617–1624, 2007.

[18] A. Eger, K. Aigner, S. Sonderegger, et al., "DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells," *Oncogene*, vol. 24, no. 14, pp. 2375–2385, 2005.

[19] M. Singh, N. S. Spoelstra, A. Jean, et al., "ZEB1 expression in type I vs type II endometrial cancers: a marker of aggressive disease," *Modern Pathology*, vol. 21, pp. 912–923, 2008.

[20] N. S. Spoelstra, N. G. Manning, Y. Higashi, et al., "The transcription factor ZEB1 is aberrantly expressed in aggressive uterine cancers," *Cancer Research*, vol. 66, no. 7, pp. 3893–3902, 2006.

[21] T. R. Graham, H. E. Zhau, V. A. Odero-Marah, et al., "Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells," *Cancer Research*, vol. 68, no. 7, pp. 2479–2488, 2008.

[22] C. Pena, J. M. Garcia, J. Silva, et al., "E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations," *Human Molecular Genetics*, vol. 14, no. 22, pp. 3361–3370, 2005.

[23] M. Korpal and Y. Kang, "The emerging role of miR-200 family of microRNAs in epithelial-mesenchymal transition and cancer metastasis," *RNA Biology*, vol. 5, no. 3, pp. 115–119, 2008.

[24] M. E. Peter, "Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression," *Cell Cycle*, vol. 8, no. 6, pp. 843–852, 2009.

[25] A. Cano and M. A. Nieto, "Non-coding RNAs take centre stage in epithelial-to-mesenchymal transition," *Trends in Cell Biology*, vol. 18, no. 8, pp. 357–359, 2008.

[26] G. J. Hurteau, J. A. Carlson, S. D. Spivack, and G. J. Brock, "Overexpression of the MicroRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin," *Cancer Research*, vol. 67, no. 17, pp. 7972–7976, 2007.

[27] S.-M. Park, A. B. Gaur, E. Lengyel, and M. E. Peter, "The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2," *Genes and Development*, vol. 22, no. 7, pp. 894–907, 2008.

[28] P. A. Gregory, A. G. Bert, E. L. Paterson, et al., "The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1," *Nature Cell Biology*, vol. 10, no. 5, pp. 593–601, 2008.

[29] M. Korpal, E. S. Lee, G. Hu, and Y. Kang, "The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2," *Journal of Biological Chemistry*, vol. 283, no. 22, pp. 14910–14914, 2008.

[30] D. R. Cochrane, N. S. Spoelstra, E. N. Howe, S. K. Nordeen, and J. K. Richer, "MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents," *Molecular Cancer Therapeutics*, vol. 8, no. 5, pp. 1055–1066, 2009.

[31] C. P. Bracken, P. A. Gregory, N. Kolesnikoff, et al., "A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition," *Cancer Research*, vol. 68, no. 19, pp. 7846–7854, 2008.

[32] U. Burk, J. Schubert, U. Wellner, et al., "A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells," *EMBO Reports*, vol. 9, no. 6, pp. 582–589, 2008.

[33] P. A. Gregory, C. P. Bracken, A. G. Bert, and G. J. Goodall, "MicroRNAs as regulators of epithelial-mesenchymal transition," *Cell Cycle*, vol. 7, no. 20, pp. 3112–3118, 2008.

[34] J. R. F. Caldeira, E. C. Prando, F. C. Quevedo, F. A. Moraes Neto, C. A. Rainho, and S. R. Rogatto, "CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer," *BMC Cancer*, vol. 6, article 48, 2006.

[35] M. Shinozaki, D. S. B. Hoon, A. E. Giuliano, et al., "Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis," *Clinical Cancer Research*, vol. 11, no. 6, pp. 2156–2162, 2005.

[36] J. E. Etzell, S. Devries, K. Chew, et al., "Loss of chromosome 16q in lobular carcinoma in situ," *Human Pathology*, vol. 32, no. 3, pp. 292–296, 2001.

[37] C. B. J. Vos, A. M. Cleton-Jansen, G. Berx, et al., "E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumerigenesis," *British Journal of Cancer*, vol. 76, no. 9, pp. 1131–1133, 1997.

[38] C. Ferlini, G. Raspaglio, L. Cicchillitti, et al., "Looking at drug resistance mechanisms for microtubule interacting drugs: does TUBB3 work?" *Current Cancer Drug Targets*, vol. 7, no. 8, pp. 704–712, 2007.

[39] N. Izutsu, C. Maesawa, M. Shibazaki, et al., "Epigenetic modification is involved in aberrant expression of class III beta-tubulin, TUBB3, in ovarian cancer cells," *International Journal of Oncology*, vol. 32, no. 6, pp. 1227–1235, 2008.

[40] K. M. Lee, D. Cao, A. Itami, et al., "Class III beta-tubulin, a marker of resistance to paclitaxel, is overexpressed in pancreatic ductal adenocarcinoma and intraepithelial neoplasia," *Histopathology*, vol. 51, no. 4, pp. 539–546, 2007.

[41] L. Pusztai, "Markers predicting clinical benefit in breast cancer from microtubule-targeting agents," *Annals of Oncology*, vol. 18, supplement 12, pp. xii15–xii20, 2007.

[42] P. Sève and C. Dumontet, "Is class III beta-tubulin a predictive factor in patients receiving tubulin-binding agents?" *The Lancet Oncology*, vol. 9, no. 2, pp. 168–175, 2008.

[43] A. Paradiso, A. Mangia, A. Chiriatti, et al., "Biomarkers predictive for clinical efficacy of taxol-based chemotherapy in advanced breast cancer," *Annals of Oncology*, vol. 16, supplement 4, pp. iv14–iv19, 2005.

[44] T. Shirakihara, M. Saitoh, and K. Miyazono, "Differential regulation of epithelial and mesenchymal markers by deltaEF1 proteins in epithelial mesenchymal transition induced by TGF-beta," *Molecular Biology of the Cell*, vol. 18, no. 9, pp. 3533–3544, 2007.

[45] I. Molina-Ortiz, R. A. Bartolome, P. Hernandez-Varas, G. P. Colo, and J. Teixido, "Overexpression of E-cadherin on melanoma cells inhibits chemokine-promoted invasion involving p190RhoGAP/p120ctn-dependent inactivation of RhoA," *Journal of Biological Chemistry*, vol. 284, no. 22, pp. 15147–15157, 2009.

[46] Y. Imamichi and A. Menke, "Signaling pathways involved in collagen-induced disruption of the E-cadherin complex during epithelial-mesenchymal transition," *Cells Tissues Organs*, vol. 185, no. 1–3, pp. 180–190, 2007.

[47] D. Monniaux, C. Huet-Calderwood, F. Le Bellego, S. Fabre, P. Monget, and D. A. Calderwood, "Integrins in the ovary," *Seminars in Reproductive Medicine*, vol. 24, no. 4, pp. 251–261, 2006.

[48] A. P. Skubitz, "Adhesion molecules," *Cancer Treatment and Research*, vol. 107, pp. 305–329, 2002.

[49] I. S. Patel, P. Madan, S. Getsios, M. A. Bertrand, and C. D. MacCalman, "Cadherin switching in ovarian cancer progression," *International Journal of Cancer*, vol. 106, no. 2, pp. 172–177, 2003.

[50] H. Kajiyama, K. Shibata, M. Terauchi, et al., "Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells," *International Journal of Oncology*, vol. 31, no. 2, pp. 277–283, 2007.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 969084, 14 pages
doi:10.1155/2010/969084

Research Article

Breast Carcinoma Cells in Primary Tumors and Effusions Have Different Gene Array Profiles

Sophya Konstantinovsky,[1] Yoav Smith,[2] Sofia Zilber,[3] Helene Tuft Stavnes,[4] Anne-Marie Becker,[4] Jahn M. Nesland,[4,5] Reuven Reich,[1,6] and Ben Davidson[4,5]

[1] *Department of Pharmacology and Experimental Therapeutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel*
[2] *Genomic Data Analysis Unit, The Hebrew University of Jerusalem, Jerusalem 91120, Israel*
[3] *Department of Pathology, Rabin Medical Center, Petach Tikva 49100, Israel*
[4] *Division of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, 0310 Oslo, Norway*
[5] *Faculty Division Norwegian Radium Hospital, The Medical Faculty, University of Oslo, 0316 Oslo, Norway*
[6] *David R. Bloom Center for Pharmacy, The Hebrew University of Jerusalem, Israel*

Correspondence should be addressed to Reuven Reich, reich@cc.huji.ac.il

Received 22 April 2009; Accepted 2 June 2009

Academic Editor: Tian-Li Wang

The detection of breast carcinoma cells in effusions is associated with rapidly fatal outcome, but these cells are poorly characterized at the molecular level. This study compared the gene array signatures of breast carcinoma cells in primary carcinomas and effusions. The genetic signature of 10 primary tumors and 10 effusions was analyzed using the Array-Ready Oligo set for the Human Genome platform. Results for selected genes were validated using PCR, Western blotting, and immunohistochemistry. Array analysis identified 255 significantly downregulated and 96 upregulated genes in the effusion samples. The majority of differentially expressed genes were part of pathways involved in focal adhesion, extracellular matrix-cell interaction, and the regulation of the actin cytoskeleton. Genes that were upregulated in effusions included *KRT8, BCAR1, CLDN4, VIL2*, while *DCN, CLDN19, ITGA7*, and *ITGA5* were downregulated at this anatomic site. PCR, Western blotting, and immunohistochemistry confirmed the array findings for *BCAR1, CLDN4, VIL2*, and *DCN*. Our data show that breast carcinoma cells in primary carcinomas and effusions have different gene expression signatures, and differentially express a large number of molecules related to adhesion, motility, and metastasis. These differences may have a critical role in designing therapy and in prognostication for patients with metastatic disease localized to the serosal cavities.

1. Introduction

Breast cancer is the most common malignancy in women, constituting more than 25% of cancers in this group. The incidence of breast cancer around the world greatly varies, with highest rates in the North America, Western, and Northern Europe and Australia/New Zealand (82.5–99.4 per 100,000 women). Mortality in these areas of high incidence is 19.2–22.6 per 100,000 women [1]. Breast cancer metastasizes most often to axillary lymph nodes, but may involve any organ. Metastasis to serosal surfaces involves primarily the pleural cavity [2, 3], and infrequently, the pericardial and peritoneal cavities [4]. Pleural effusions may occur at any point of time in the clinical course and may be the sole manifestation of metastatic disease [5]. This condition is associated with a poor median survival of less than 1 year [5, 6].

In recent years, we have reported on the differential expression of metastasis-related molecules, including proteases, angiogenic molecules, signaling molecules, inhibitors of apoptosis, and transcription factors, in breast carcinoma effusions compared to patient-matched primary carcinomas [7–10]. However, these studies focused on a limited number of genes. The global view of cellular transcriptional activity

using gene array technology is required to identify clusters of genetic markers that explain the complex biological processes involved in carcinoma progression to effusions. At present, only one study in which breast carcinoma effusions were compared with primary carcinomas is available. Dupont et al. analyzed the gene expression signature of 19 effusions and compared them to 4 primary carcinomas, 8 cells lines and 4 specimens consisting of benign breast tissue. Effusions could be differentiated into two categories, one resembling cells lines and expressing CD24, CD44 and cytokeratins 8, 18 and 19, the other expressing metastasis-associated genes, such as S100A4, uPA receptor, vimentin and CXCR4 [11].

The present study compared the gene expression signatures of 10 effusions and 10 primary breast carcinomas. Selected differentially expressed genes of pathways related to adhesion, interaction with the extracellular matrix (ECM) and regulation of the actin cytoskeleton were validated on mRNA and protein level, and their clinical relevance was analyzed in a larger series of breast carcinoma effusions. Our data demonstrate that in agreement with our previous observations, breast carcinoma cells in effusions are markedly different from their counterparts in primary carcinomas. This bears relevance to validation of novel therapeutic targets and stratification of patients with respect to treatment response and survival.

2. Materials and Methods

2.1. Patients and Material

2.1.1. Effusions. Ten effusions (7 pleural, 2 peritoneal, 1 pericardial) from patients with primary breast carcinoma (9 of infiltrating ductal type, 1 lobular) were submitted for routine diagnostic purposes to the Department of Pathology at the Norwegian Radium Hospital during the period 1999–2005. Submitted specimens were processed immediately upon arrival, and pellets were used for preparation of paraffin-embedded cell blocks and for freezing in equal volumes of RPMI supplemented with 20% fetal calf serum and 20% DMSO. All specimens underwent morphological evaluation and were further characterized using immunohistochemistry, as previously detailed [12]. All effusions had >50% carcinoma cells of the total cellular content, ranging between 80–100% of cells in 8/10 specimens. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

2.1.2. Primary Tumors. Ten primary breast carcinomas of infiltrating duct type were retrieved from snap-frozen archival material stored at −70°C at the Department of Pathology, Norwegian Radium Hospital. Morphological evaluation of frozen sections from the studied specimens was performed in all cases in order to ensure the presence of a predominant carcinoma cell population and absence of necrosis.

2.2. Gene Array. Eleven samples were prepared from each group (one effusion and one primary carcinoma with two samples each). Analysis of differentially expressed genes was performed by two experimental designs. (1) Eleven pair-wise competitive hybridizations of cDNA target sample from randomly chosen patients from the effusion group and sample from randomly chosen patients from primary solid tumor group. (2) Six pair-wise hybridizations of cDNA samples from randomly chosen patients from the effusion group and pooled reference sample from four primary solid tumors.

2.2.1. RNA Extraction. Biopsies from primary tumors and effusions were lysed using SV- total isolation kit (Promega, Madison, Wis, USA) and total RNA was extracted according to the manufacturer's guidelines. The quantity and quality of the total RNA preparations were assessed using a NanoDrop ND-1000 (NanoDrop, Wilmington, De, USA) combined with agarose gel electrophoresis and only samples with ribosomal 28S/18S ratio near 2 were selected for array analysis.

2.2.2. Reverse-Transcription Reaction. Twenty micrograms total RNA was mixed with 2 μg oligo dT (Amersham Biosciences, Piscataway, NJ, USA) and RNAse-free water to a total volume of 16.9 μL, incubated 10 minutes at 75°C and cooled on ice. Six microliters of first strand buffer, 3 μL of 0.1 M DTT, 2 μL of Superscript II RT (Invitrogen, Carlsbad, Calif, USA), 1.2 μL 25X Aminoallyl (aa-aUTP)/d-NTP mix (Sigma, Haverhill, UK) and 1 μL of RNAse inhibitor (Amersham Biosciences) were added to each reaction tube. The mixture was centrifuged briefly and incubated 60 minutes at 42°C. Additional 1 μL of Superscript II RT was added following incubation for 60 minutes at 42°C.

2.2.3. RNA Hydrolysis. Free RNA was disassembled with mixture of 10 μL 1 M NaOH and 10 μL 0.5M EDTA and underwent neutralization with 25 μL 1 M Hepes.

2.2.4. cDNA Purification. Unincorporated aa-aUTP and free amines were removed using Microcon YM-30 spin column (Millipore, Bedford, Mass, USA) and cDNA yield were measured by Nanodrop spectrophotometer. The sample was dried at RT using speed-vac.

2.2.5. Coupling Cyanine Dye Ester to aa-cDNA. The cDNA target samples were labeled by Cy3 or Cy5 fluorescent dyes (Amersham Biosciences) according to the sample group and resuspended in 0.1M Carbonate buffer (pH 8.6). The samples were incubated in the dark for 1 hour in RT and diluted in 35 μL NaOAc 100 mM (pH 5.2). The free dyes were removed using QIAquik PCR clean up kit (Qiagen, Valencia, CA, USA), washed with 5 mM phosphate buffer (pH 8.0, 80% ethanol) and eluted with 4 mM Phosphate elution buffer (pH 8.5). The hybridization efficiency and the yield of the dye incorporation were calculated using Labeled cDNA Calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html). Equal amount of Cy3/Cy5 labeled cDNA were mixed and dried in the speed-vac.

2.2.6. Hybridization. The slides, with printed Array-Ready Oligo set for the Human Genome Version 3.0 (Qiagen) containing 34,580 longmer probes, representing 24,650 genes and 37,123 gene transcripts, were blocked with 1%BSA in 0.1% SDS 5XSSC buffer, washed in distilled water and dried at 1000 rpm for 2 minutes. The mixed pair of samples was resuspended in hybridization buffer (25% Formamide, 0.1% SDS in 5XSSC) supplied with 0.02 μg t-RNA and denatured at 95°C. The arrays were hybridized in a water bath, in sealed, watertight hybridization chambers (DieTech, Ford City, Pa, USA) for 16–18 hours at 42°C. After hybridization, the slides were rinsed in a coupling jar containing 2 × SSC 0.1% SDS, followed by washing for 5 minutes in 1 × SSC, then for 5 minutes in 0.2 × SSC, and finally for 10 minutes in 0.05 × SSC. The slides were dried as described above. The final visualization is carried out using Axon 4000B fluorescence scanner. Griding and analysis of images was performed using Gene Pix 6.0 software package (Molecular Devices, Sunnyvale, Calif, USA). Specific genes were characterized according to fluorescence intensity of Cy3/Cy5 Dyes. The fluorescent intensities of Cy5 and Cy3 for each target spot were adjusted so that the mean Cy3/Cy5 ratio of housekeeping genes was equal to one, a design allowing more precise analysis of differentially expressed genes.

2.2.7. Statistics. Statistical analysis of microarrays was preformed by The Genomic Data Analysis Unit of Hadassah Medical School, Hebrew University of Jerusalem.

The quality assurance, calibration, data normalization (Lowess) [13] and Volcano plot for GPR files were performed by custom built package written in MATLAB R2007a. An additional statistical analysis and clustering were carried out using the Spotfire (Somerville, MA) and Partek (St. Louis, MO) software packages.

Gene annotations and specific pathways were fingered out using online free access programs such as: GO annotation (http://www.geneontology.org/), Onto-Express, Pathway-Express (Intelligent Systems and Bioinformatics Laboratory, Computer Science Department, Wayne State University).

2.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.3.1. Reverse-Transcriptase Reaction. Total RNA from specimens analyzed for *BCAR1,VIL2* and *DCN* expression was extracted using Tri-Reagent (Sigma) according to the manufacturer's guidelines. 0.5 μg total RNA was reverse-transcribed using the M-MLV Reverse Transcriptase (Promega) with incubation of 2 hours at 37°C, followed by 5 minutes at 95°C, and diluted to 1 : 5 with RNase-free water.

2.3.2. Semiquantitative RT-PCR. RT-PCR was performed on complementary DNA samples using a DNA thermal cycler (Eppendorf Mastercycler gradient, Eppendorf, Hamburg, Germany) with Reddymix PCR master mix (ABgene, Surrey, UK). Primer sequence was as follows:

BCAR1. sense 5′-GGG-CCA-CAG-GAC-ATC-TAT--GAT-3′, antisense 5′-GAG-GAA-CGT-CGT-AGA-CTG-CG-3′ (amplicon size, 318 base pairs [bp]). *VIL2.* sense 5′-GTT-TTC-CCC-AGT-TGT-AAT-AGT-GCC-3′, antisense 5′-TGC-CTT-TGC-AAA-GCT-TTT-ATT-TCA-3′ (amplicon size, 995 bp).

Conditions were as follows: *BCAR1*: 95°C for 3 minutes, denaturation at 95° for 15 seconds, annealing at 59° for 30 seconds, extension at 72° for 20 seconds, 34 cycles; *VIL2*: 95° for 3 minutes, denaturation at 95° for 15 seconds, annealing at 64° for 30 seconds, extension at 72° for 20 seconds, 33 cycles. The HT-1080 fibrosarcoma cell line served as a positive control in both reactions.

Products were separated on 1.5% agarose gels, isolated using the Invisorb Spin DNA extraction kit (Invitek GmbH, Berlin, Germany) and sequenced. Gels were photographed by the Kodak EDAS 290 system (Kodak, Rochester, NY, USA). Densitometer analysis of films was performed using a computerized image analysis (NIH IMAGE 1.63) program. *BCAR1* and *VIL2* mRNA levels were established by calculating the target molecule/28S ratio (all cases scored for band intensity compared to control). Expression intensity of 5% or less of control levels was interpreted as negative. Measurements were made at the linear phase of the reaction.

2.3.3. Quantitative RT-PCR (qRT-PCR). qRT-PCR was preformed using the Mx3000P QPCR System (Stratagene, Calif, USA). Oligonucleotide primers were designed in the Primer Express program (Applied Biosystems, Foster City, Calif, USA). Primer sequences for *DCN* were 5′-TCC-GCT-GAA-GAG-CTC-AGG-AAT-3′ for the forward primer, and 5′-CCT-TGA-GGA-ATG-CTG-GTG-ATA-TTG-3′ for the reverse primer. The primers for RPLPO normalizer gene were: 5′-CCA-ACT-ACT-TCC-TTA-AGA-TCA-TCC-AAC-TA-3′ for the forward primer and 5′-ACA-TGC-GGA-TCT-GCT-GCA-3′ for the reverse primer. One of the primers in each primer pair was designed in exon-exon boundaries region in order to minimize the DNA contamination noise. The specificity of primer binding was analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/) with Human genomic + transcript (Human G + T) database for highly similar sequences (megablast). The primer optimal concentration and the sensitivity, efficiency, and accuracy of qPCR were calibrated by amplifying serial geometric dilutions of pooled sample consisted from five primary tumor and five effusion cDNA samples. 0.1 μg of cDNA product from the Reverse Transcriptase reaction were amplified using DyNAmo SYBR Green qPCR Kit with ROX passive reference dye (Finnzymes Oy, Espoo, Finland) according to the manufacturer's instructions. Absence of primer-dimers and non-specific products was verified by single product peak in the qPCR dissociation curve. In addition, the PCR product was separated by gel electrophoresis and sequenced (Hebrew University facilities).

2.4. Immunohistochemistry. Formalin-fixed paraffin-embedded sections were available from 52 breast carcinoma

effusions (47 pleural, 4 peritoneal, 1 pericardial) from 51 female patients (one patient with 2 effusions) aged 33–86 (mean = 59) years with histologically verified breast cancer. In 27 cases, the primary carcinoma was additionally available for analysis.

Slides from the primary breast carcinoma specimens were available in our archives for 45 cases. These were diagnosed as infiltrating duct carcinoma (38), lobular carcinoma (5) or mixed duct and lobular carcinoma (2). In the remaining 6 cases, effusion specimens were submitted to our clinic from patients operated at other hospitals.

These 79 above-described specimens were manually immunostained for p130cas, phospho-Ezrin (p-Ezrin), and claudin-4. The monoclonal mouse p130cas antibody (clone CAS-14) was purchased from NeoMarkers (LabVision Corporation, Fremont, Calif, USA). A monoclonal mouse p-Ezrin antibody was purchased from BD Pharmingen (San Jose, Calif, USA). The rabbit polyclonal claudin-4 antibody was purchased from Zymed (San Francisco, Calif, USA). All slides underwent pretreatment in a microwave oven for 20 minutes (p-Ezrin and claudin-4 slides in Tris/EDTA buffer, pH = 9-9.1, $p130^{cas}$ slides in citrate buffer, pH = 6). Antibody dilutions were 1 : 200 for all antibodies. Visualization was achieved using the EnVision + peroxidase system (Dako A/S, Glostrup, Denmark).

Negative controls consisted of sections that underwent similar staining procedures with isotype-matched mouse antibody, normal goat IgG or non-relevant rabbit immunoglobulins according to the antibody host species. Positive controls consisted of a breast carcinoma biopsy that demonstrated immunoreactivity for the studied antigens in a pilot study.

Staining was considered positive only when localized to the cell membrane in a linear pattern for p-Ezrin and claudin-4, and when present in the cytoplasm for the p130cas reaction. Staining extent was scored on a scale of 0–4, as follows: 0 = no staining, 1 = staining of 1–5%, 2 = staining of 6–25%, 3 = staining of 26–75%, 4 = staining of 76–100% of cells. No specimen contained less than 100 tumor cells. Slides were scored by a surgical pathologist experienced in effusion cytology and breast pathology (BD).

2.5. Western Blotting. Frozen specimens were thawed and subsequently lysed in 1% NP-40, 20 mM Tris HCl (pH 7.5), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM phenyl-methylsulfonyl fluoride, 1 μg/mL Aprotinin, 2 μg/mL leupeptin, 1 mM sodium orthovanadate, and 0.1% SDS. 25 μg of a total protein from each sample were separated by electrophoresis through SDS-10% polyacrylamide gels under reducing conditions. After electrophoresis, proteins were transferred to Immobilon transfer membranes (Millipore, Bedford, Mass, USA). Membranes were blocked in 5% Non Fat Dry Milk (NFDM) in 0.1% Tween TBS (TBST) and incubated overnight at 4°C in 5% BSA TBST containing anti-p-Ezrin (Thr^{567}) rabbit mAb (Cell Signaling Technology Inc., Danvers, Mass, USA).

After incubation, membranes were washed and incubated for 1 hour with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) in TBST containing 5% BSA. Membranes were developed using the enhanced chemiluminescence kit (Pierce, Rockford, Ill, USA), according to manufacturer's specifications. Membranes were then washed, stripped in 0.2 M glycine, 0.1% SDS, and 1% Tween 20 (pH 2.2), blocked in TBST containing 5% NFDM, and incubated overnight at 4°C in 5% BSA in TBST containing a rabbit polyclonal anti-Ezrin Ab (Abcam, Cambridge, UK). Total Ezrin activity was normalized to β-actin activity measured using rabbit anti-β-actin polyclonal antibody (Cell Signaling Technology Inc.). Protein detection was preformed as described above. Protein lysate from MCF-7 breast carcinoma cells served as control.

Levels of phosphrylation of the TORC1 substrate p70S6K were analyzed using goat anti-p-$p70S6K^{Thr389}$ and rabbit anti-p70 S6K antibodies (Santa Cruz biotechnology, inc., Santa Cruz, Calif, USA) as described above. Secondary peroxidase-conjugated donkey anti-goat IgG (Santa Cruz biotechnology, inc.) was used for anti-p-$p70S6K^{Thr389}$ detection.

2.5.1. Quantification of Blotting Results. Gels were scanned by the KODAK EDAS 290 system. Densitometer analysis of films was performed using a computerized image analysis program (NIH IMAGE 1.63)

3. Statistical Analysis

IHC and IB results were analyzed using the SPSS-PC package, version 15.0 (Chicago, Ill, USA). Comparative analyses of tumor cell expression results in all effusions versus primary tumors were performed using the Mann-Whitney U test. The same test was applied for analysis of the relationship between protein expression in effusions and clinicopathologic parameters. The Wilcoxon Signed Ranks test was applied for patient-matched analysis in the 27 cases with effusion and primary tumor. Univariate analysis for disease-free survival (DFS) and overall survival (OS) for 44 patients with clinical data were executed using the Kaplan-Meier method and Log-rank test. For this analysis, expression categories were grouped as focal (≤25% of cells) or diffuse (>25% of cells).

4. Results

4.1. Breast Carcinoma Effusions and Primary Carcinomas Have Different Gene Expression Patterns. We have previously shown that effusions constitute a unique form of breast carcinoma metastasis with mRNA and protein expression patterns that differ from primary tumors and solid metastases [7–10]. In the present study, we compared the global expression profile of breast carcinoma cells in effusions with that of primary carcinomas.

Figure 1(a) shows volcano plot of global gene expression in effusions and primary tumors. Differences of 1–5 fold in gene expression with cut-off P-value <.05 were defined as significant. We identified 255 significantly down-regulated

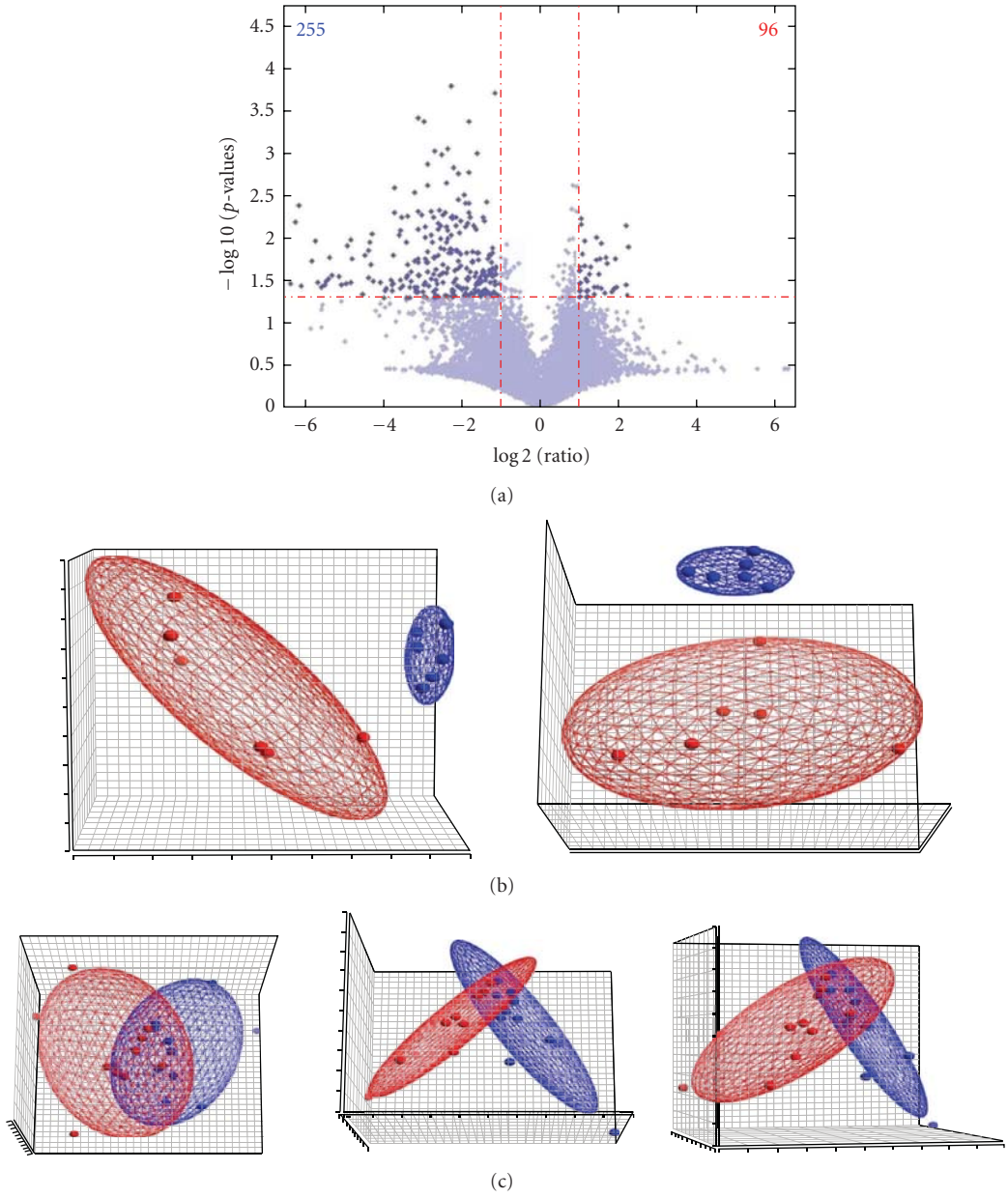


Figure 1: *Breast carcinoma effusion and primary carcinoma expression arrays.* (a)**:** Volcano plot of differentially expressed genes in malignant effusions in comparison to pooled primary tumors ($n = 6$). The x-axis indicates the differential expression profiles, plotting the fold-induction ratios in a log-2 scale. The y-axis indicates the statistical significance of the difference in expression (P-value, t-test) in a -log10 scale. Differentially expressed genes ($P < .05$) appear above the horizontal line. Numbers denote up-regulated (red) or down-regulated (blue) genes in effusions. (b)**:** Gene expression profiling of effusions (blue) and primary breast carcinomas (red) in three-dimensional space by Principal Component Analysis using 351 genes that showed significant up- or downregulation in effusions in comparison to the pooled primary tumor sample (two different view angles). (c): Gene expression profiling of 11 effusions (blue) and 11 primary carcinomas (red) in three-dimensional space by Principal Component Analysis (three different view angles).

and 96 significantly up-regulated genes in effusion samples (total = 351).

4.1.1. PCA Analysis. Principal Component Analysis (PCA) (Partek, St. Louis, Mon, USA) is a technique used to reduce multidimensional data sets to lower dimensions and to highlight their similarities and differences. PCA analysis of six effusion and primary tumor samples was performed using the set of 351 genes that were differentially expressed in effusions and primary carcinomas (Figure 1(b), supplementary Table 1, available at doi:10.1155/2010/969084.). Selected genes are shown in Table 1. The analysis showed that this gene set effectively separates tumors at these two anatomic sites. We additionally performed random PCA analysis of the gene expression pattern in all 11 effusions

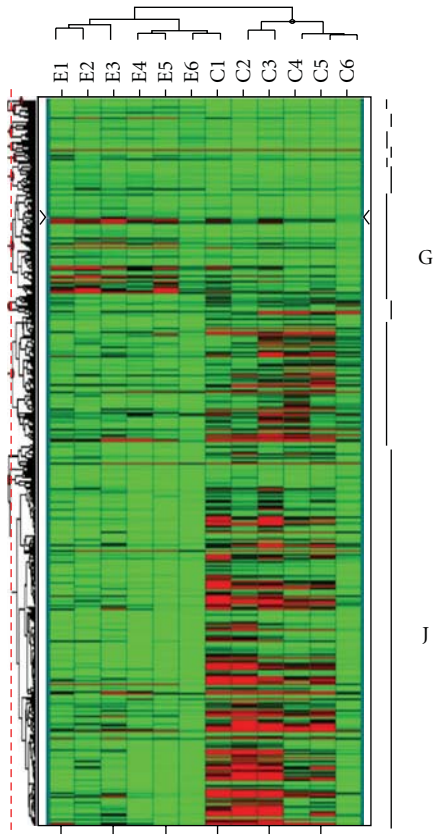


Figure 2: *Dendrogram*. A dendrogram of a supervised analysis of 386 genes (horizontal rows) across 6 effusion versus six primary tumor replicates. Each cell in the matrix represents the expression level of a single transcript in a single sample. The supervised hierarchical clustering analysis clearly distinguished between effusion and control expression pattern. Red or green color indicates high or low transcript levels relative to threshold based on overall gene expression values across each array slide, respectively, while black color indicates equal to median level of expression.

and 11 primary tumor pairs (Figure 1(c)). The analysis was performed using a set of 342 genes that showed trend of up- or downregulation in those patients. The difference between this gene number and the above-detailed 351 genes results from the fact that two different analyses were performed, the first being a pool versus individual specimen analysis, the second of individual case versus individual case. However, the pathways detected were identical. Three patterns were identified: (1) unique for primary tumors; (2) unique for effusions and (3) samples with overlapping gene expression.

4.2. Gene Ontology and Function. In order to understand the biological function of the genes that were up- or down-regulated in effusions, we used the GO annotation (http://www.geneontology.org/) and Pathway-Express (14, 15) programs.

We found multiple pathways involved in cell maintenance that are altered in effusions in comparison to primary tumors (Table 2). It can be seen that the pathways involved in focal adhesion, ECM-receptor interaction and regulation of the actin cytoskeleton are highly involved in phenotypic transformation of carcinoma cells in primary tumors to those in effusions. Some of the differentially-expressed genes were found to participate in the specific pathways listed above. Other differentially-expressed genes could not be classified as components of a specific pathway, but are of great clinical impact in breast carcinoma, for example, ERβ with a 3.23-fold downregulation in effusions and MTA3, an estrogen-sensitive gene involved in E-cadherin regulation, with a 2.42- fold downregulation in effusions.

4.3. Hierarchical Clustering of Gene Expression Profiles. The significantly altered genes in effusions (t-test/ ANOVA, P-value <.05) were selected for performing UPGMA hierarchical clustering (Figure 2). The unsupervised clustering analysis was performed using the Spotfire software. The differentially expressed genes were classified into 10 major clusters. Of these 10 clusters, cluster G included genes that were strongly up-regulated in effusions, while cluster J genes were strongly down-regulated in comparison to primary tumors. We found some clinically-relevant genes, such as *KRT8*, *CLDN4* and *VIL2* in cluster G, while cluster J included *CLDN19*, the ECM genes *COL1A1*, *COL22A1*, *COL5A2* and the *ITGA7* and *ITGA5* integrin genes.

4.4. Validation of Gene Array Results. Since cell motility and cell-ECM interactions may have a major effect on the metastatic potential of carcinoma cells in effusions, we focused on genes participating in regulation of the actin cytoskeleton, focal adhesion, and ECM-receptor interactions in validation of the array results. The genes focused on were the following: *DCN*, which encodes for the small cellular or pericellular matrix proteoglycan decorin [14] and was found to be significantly down-regulated in effusions; *VIL2*, encoding for ezrin, which controls the actin cytoskeleton dynamics; *BCAR*, encoding for the integrin signaling adaptor protein p130cas. In addition, Tuberous Sclerosis 1 (*TSC1*), also known as the tumor suppressor hamartin, the main inhibitor of the mTOR signaling pathway [15, 16], was one of the genes that were found to be down-regulated in effusions in comparison to primary tumors. Thus, we decided to analyze mTOR activity in effusions compared to solid primary tumors. Validation was by semiquantitative and quantitative RT-PCR, Western Blotting and immunohistochemistry, using an enlarged set of effusions and primary carcinomas.

4.4.1. DCN. *DCN* expression levels were analyzed in 29 effusions and 35 primary carcinomas using qRT-PCR. *DCN* levels were significantly higher in primary tumors ($P <$ .0001, Figure 3(a)), in agreement with the gene array results.

4.4.2. BCAR1/p130cas. Semiquantitative RT-PCR analysis of 35 primary tumors and of 29 effusions showed significantly higher up-regulation of *BCAR1* in effusions ($P <$ .0001, Figure 3(b)). Immunostaining of 52 effusions and 26 of the 27 primary carcinomas (one unsatisfactory reaction) for p130cas showed its presence in tumor cells in 50/52

Table 1: Selected genes identified by fold-change analysis using the MATLAB R2007a program that are differentially expressed in primary tumors versus effusions (complete list available in supplementary Table 1).

Genes more highly expressed in effusions		
Gene	Full name	Log2 change
CLDN4	Claudin-4	4.8544
KRT8	Keratin 8	3.9099
NTN4	Netrin-4	3.5957
VIL2	Villin 2	2.7965
FASN	Fatty acid synthase	2.7583
DLX3	Distal-less homeobox 3	2.3846
ZNF75C	ZNF75C	2.2346
TRAF4	TNF-receptor-associated factor 4	2.0682
NEDD4L	Neural precursor cell expressed, developmentally down-regulated	2.0061
NPC1L1	NPC1 (Niemann-Pick disease, type C1 gene)-like 1	1.9928
Genes more highly expressed in primary tumors		
CLDN19	Claudin-19	20.4327
HPR	Haptoglobin-related protein	11.7846
IGFBP7	Insulin-like growth factor binding protein 7	10.6376
ZFYVE20	Zinc finger FYVE domain containing 20	10.4542
HP	Haptoglobin	9.1384
GFRA1	GDNF family receptor alpha 1	8.6866
APOBEC2	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2	8.6565
STK39	Serine threonine kinase 39 (STE20/SPS1 homolog, yeast)	8.0239
SLC6A16	Solute carrier family 6, member 16	7.2568

effusions and 24/26 primary carcinomas (Figures 4(a) and 4(b)). Comparative analysis showed no significant difference in staining extent at these two anatomic sites ($P > .05$).

OS for the 44 patients with survival data ranged from 2–393 months (mean = 90 months), while DFS ranged from 0–336 months (mean = 55 months). In survival analysis, higher p130cas expression in effusions was associated with a trend for poor OS ($P = .062$) and DFS ($P = .098$; Figure 5).

4.4.3. VIL2/Ezrin. Semiquantitative RT-PCR analysis of 43 primary tumors and 25 effusions showed significantly higher *VIL2* expression in effusions ($P = .0021$, Figure 3(c)). Protein levels and phosphorylation extent of Ezrin were analyzed by Western blotting using phospho- and pan-specific antibodies. Pan-Ezrin protein level was significantly higher in effusions ($P = .004$, Figure 3(d)), whereas p-ezrin levels did not significantly differ. Although the fraction of phosphorylated protein did not significantly differ, the total amount of the protein was up-regulated in effusions ($P = .004$). Thus, the absolute phosphorylated ezrin levels were higher in effusions compared to primary tumors.

Immunostaining of 51 of the 52 effusions (one unsatisfactory reaction) and 27 primary carcinomas showed significantly higher p-Ezrin expression in effusions compared to primary carcinomas ($P < .001$ in analysis of all cases, as well as patient-matched specimens), as evidenced by score = 4 staining in 49/51 effusions and only 2/27 primary carcinomas (Figures 4(c)–4(e)). Ezrin was not analyzed for survival in view of the practically uniform score = 4 staining in effusions.

4.4.4. Claudin-4. Immunostaining of 52 effusions and 23 of the 27 primary carcinomas (4 unsatisfactory reactions) for claudin-4 showed its presence in tumor cells in 51/52 effusions and 20/23 primary carcinomas (Figures 4(f)–4(i)). However, staining extent was higher in effusions, a difference that was significant in analysis of all cases ($P = .002$), and showed a trend in matched specimen analysis ($P = .062$). Claudin-4 protein expression was unrelated to OS or DFS ($P > .05$).

4.4.5. mTOR Pathway Activity. TSC1 showed a 2.3-fold downregulation in effusions compared to primary tumors in the array analysis. Analysis of the phosphorylation level of the rapamycin sensitive mTOR Complex 1 (mTORC1) substrate p70S6K showed that in spite of the *TSC1* down-regulation, the levels of p70S6K phosphorylation were lower in effusions compared to primary tumors ($P = .003$, Figure 6).

5. Discussion

Breast carcinoma metastasis to the serosal cavities represents an advanced stage in tumor progression and is associated with extensive alterations at the molecular level, involving clinically established targets such as HER-2 and hormone receptors, as well as other cancer-associated molecules [7–10]. Despite the fact that breast carcinoma is one of the most extensively studied cancer forms, little effort has been directed towards understanding the biology of tumor cells in malignant effusions. The major aim of the present study

Table 2: Pathways involved in the differentially expressed genes (a): six individual effusion patients against the pooled sample of primary tumors; (b): 11 individual effusions against 11 individual primary tumors.

(a)

Pathway name	Number of genes in pathway	Number of differentially expressed genes in pathway	Percentage of differentially expressed genes in pathway	*P*-value
Cell adhesion molecules (CAMs)	132	10	7.576	1.36E-04
Regulation of actin cytoskeleton	208	11	5.288	0.001462
Focal adhesion	195	9	4.615	0.010329
Calcium signaling pathway	175	8	4.571	0.01545
ECM-receptor interaction	87	6	6.897	0.005234
Tight junction	119	4	3.361	0.175458
PPAR signaling pathway	70	3	4.286	0.123277
TGF-beta signaling pathway	84	1	1.19	0.780451
Wnt signaling pathway	149	1	0.671	0.935266
Apoptosis	84	1	1.19	0.792465
mTOR signaling pathway	47	1	2.128	0.584796
Gap junction	92	1	1.087	0.81801
MAPK signaling pathway	256	3	1.172	0.855694
Adherens junction	77	1	1.299	0.763346
Small cell lung cancer	86	1	1.163	0.800108
Cytokine-cytokine receptor interaction	256	1	0.391	0.990004

(b)

Pathway name	Number of genes in pathway	Number of differentially expressed genes in pathway	Percentage of differentially expressed genes in pathway	*P*-value
Cell adhesion molecules (CAMs)	132	9	6.818	4.22E-07
Regulation of actin cytoskeleton	208	4	1.923	0.061989
Focal adhesion	195	3	1.538	0.169683
ECM-receptor interaction	87	2	2.299	0.130698
Tight junction	119	1	0.84	0.580383
TGF-beta signaling pathway	84	5	5.952	3.19E-04
Wnt signaling pathway	149	3	2.013	0.09173
Apoptosis	84	1	1.19	0.46079
mTOR signaling pathway	47	1	2.128	0.291965
MAPK signaling pathway	256	3	1.172	0.288211
Adherens junction	77	1	1.299	0.432252
Small cell lung cancer	86	4	4.651	0.003658
Cytokine-cytokine receptor interaction	256	4	1.563	0.10534
Phosphatidylinositol signaling system	77	2	2.597	0.106703
Pancreatic cancer	73	3	4.11	0.016395
Colorectal cancer	85	3	3.529	0.023709
Cell cycle	114	3	2.632	0.050093
Jak-STAT signaling pathway	153	3	1.961	0.097521
Non-small cell lung cancer	53	2	3.774	0.057405
Endometrial cancer	52	2	3.846	0.055498
Basal cell carcinoma	56	2	3.571	0.059335
Renal cell carcinoma	69	2	2.899	0.09072

(b) Continued.

Pathway name	Number of genes in pathway	Number of differentially expressed genes in pathway	Percentage of differentially expressed genes in pathway	*P*-value
Melanoma	71	2	2.817	0.09521
ErbB signaling pathway	87	2	2.299	0.133163
Melanogenesis	102	2	1.961	0.166065
Thyroid cancer	31	1	3.226	0.203583
VEGF signaling pathway	70	1	1.429	0.397797
Prostate cancer	86	1	1.163	0.468679

was to characterize a general expression fingerprint that distinguishes effusions from primary tumors.

Our data show that 351 genes among 24,650 gene transcripts are significantly altered in effusions in comparison to primary carcinomas. Many of these genes are involved in ECM-receptor interaction, focal adhesion and regulation of the actin cytoskeleton pathways, which define the metastatic potential of carcinoma cells by enhancing their motility and leading to anoikis escape in the absence of ECM molecules.

PCA analysis of significantly altered genes distinguished clearly between primary tumors and effusions. The gene expression profile correlated with phenotypic change during the transition of breast carcinoma cells from the solid tumor to suspended cell clusters in pleural effusions. Carcinoma cells in effusions showed down-regulated ECM encoding molecules such as decorin, fibronectin, collagens I, XXII and V, concomitantly with the downregulation of the ECM-binding receptors, integrins $\alpha 5$ and $\alpha 7$. Thus, it appears as if the cells in effusions lose the requirement for interaction with matrix components, possibly by a compensatory signaling mechanism within the cells.

The second goal of the study was to highlight molecules with multiple functional influences on the metastatic potential of cells in effusions. One of these molecules is the cytoskeleton organizer ezrin. This protein provides a functional link between the plasma membrane and the actin cytoskeleton by interacting with the cytoplasmic domains of adhesion membrane proteins and regulation of cytoskeleton polymerization through the Rho pathway activation [17]. Moreover, ezrin promotes growth and survival via AKT/mTOR pathway activation in Ewing's sarcoma cell lines [18]. A recent study provided additional information regarding the role of ezrin in elevation of the metastatic potential of carcinoma cells, by showing that its downregulation of the cell-cell adhesion molecule E-cadherin, and suggesting that ezrin is associated indirectly with the E-cadherin/β-catenin complex by regulating Src activation [19]. Screening of a broad spectrum of human cancers, including breast, lung and prostate tumors showed high expression of ezrin in tumors of mesenchymal origin and in primary breast carcinomas. Moreover, ezrin expression was shown to be strongly associated with poor prognosis in breast carcinoma [20]. In agreement with the latter report, higher VIL2 mRNA expression in effusions was associated with poor disease-free survival in our cohort (data not shown). This finding requires further investigation, as it was obtained in analysis of only 17 effusions.

The up-regulation of ezrin in effusions was validated using RT-PCR, Western blotting and IHC. We found that the up-regulation of ezrin in effusions is associated with expression of the functionally active T567 phosphorylated ezrin [17, 18] at the plasma membrane. This gene is not only up-regulated at the mRNA and protein levels, but is also more active in effusions compared to solid tumors. Since the increase in ezrin activation may influence multiple metastasis-associated cell functions [17, 18], the therapeutic targeting of this protein may prove beneficial in effusion therapy.

Statistical analysis of the array results showed that the *TSC1* gene, encoding a protein involved in mTORC1 inhibition, is down-regulated in effusions in comparison to primary tumors. The mTOR pathway is highly involved in breast carcinoma pathogenesis and adjuvant therapy resistance [21]. Moreover, there is evidence that mTOR activation can lead to anchorage-independent growth of carcinoma cells [22], making it a potentially important factor for cell survival in effusions. Rapamycin analogues, such as temsirolimus (CCI-779) or everolimus (RAD-001) that target mTOR are now in different stages of clinical trials for anti-cancer therapy as a single agent [23] or as additive treatment having synergetic effect with ER- and HER2/neu-targeted therapy [24, 25]. Downregulation of the mTOR inhibitor *TSC1* in effusion may lead to subsequent activation of mTORC1 at this site of metastasis, suggesting that this signaling pathway may be altered along tumor progression in breast carcinoma.

Recent studies demonstrate that *TSC1* directly interacts with ezrin and through this interaction regulates focal adhesion complex formation and causes cytoskeletal remodeling [26, 27]. The loss of *TCS1* results in loss of focal adhesions, cell rounding and progressive detachment of cells from the substrate [27]. Since effusions are characterized as clusters of detached carcinoma cells, the parallel dysregulation of *TSC1* and ezrin expression may play a critical role in effusion formation. The relative phosphorylation extent of the mTORC1 substrate p70S6K [28, 29] was measured in order to analyze the effect of TSC1 downregulation on the mTORC1 activity. In spite of the downregulation of the mTORC1 inhibitor *TSC1*, the extent of p70S6K phosphorylation remains low in the effusions in comparison to primary tumors. The reason

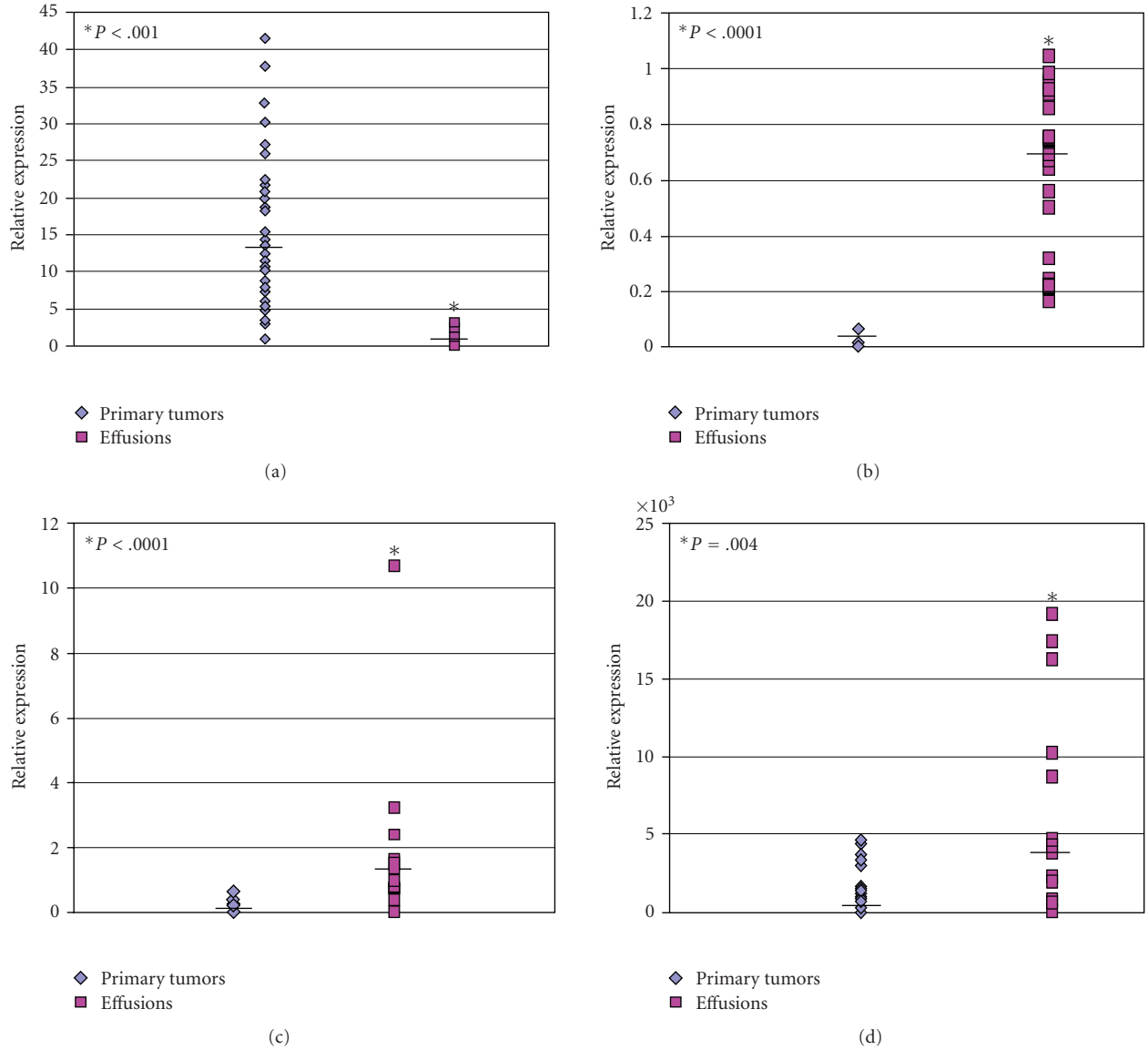


Figure 3: *Validation by PCR and Western blotting*. (a): *DCN* mRNA expression in effusions and solid primary tumors, showing higher expression in the latter specimen type ($P < .0001$). *DCN* levels were measured by qRT-PCR using specific primers for decorin A1, A2 and B isoformes. The pool sample consisting of cDNA from five primary tumors and five effusion samples served as a positive control. *DCN* mRNA expression levels were normalized to RPLPO reference gene expression levels. (b): *BCAR1* mRNA relative expression levels in primary tumors and effusions. Gene expression level was measured by semiquantitative RT-PCR and normalized to relative 28s expression. The MCF-7 breast carcinoma cell line served as positive control. *BCAR1* levels were significantly higher in effusions ($P < .0001$). (c): *VIL2* expression in effusions in comparison to primary carcinomas. *VIL2* mRNA levels were measured by RT-PCR with specific primers and normalized to relative 28s expression. Human HT1080 fibrosarcoma cell line served as a positive control. *VIL2* levels were significantly higher in effusions ($P < .0001$). (d): Total Ezrin protein expression in effusions in comparison to primary carcinomas. Ezrin protein levels were analyzed using specific antibodies and normalized to total beta-actin levels. Ezrin levels were significantly higher in effusions ($P = .004$).

for low mTORC1 activity in effusions should be investigated. One possible explanation is that other substrates of mTOR may be relevant and p70S6K may be a minor effector of this pathway in effusions.

Hormone receptor status and the relevance of adjuvant hormonal therapy at different stages of the disease are central in breast carcinoma research [30]. We have previously shown that ER is down-regulated in effusions in comparison to primary tumors [8]. In the present study we observed *BCAR1* gene up-regulation in effusions. High *BCAR1* expression was reported to be associated with a poor response to first-line tamoxifen therapy in patients with recurrent disease and with an increased rate of relapse [31]. Moreover, overexpression of *BCAR1* causes tamoxifen resistance in tamoxifen-sensitive breast carcinoma cells [32]. The up-regulation of p130cas in effusions can be a result of population enrichment by

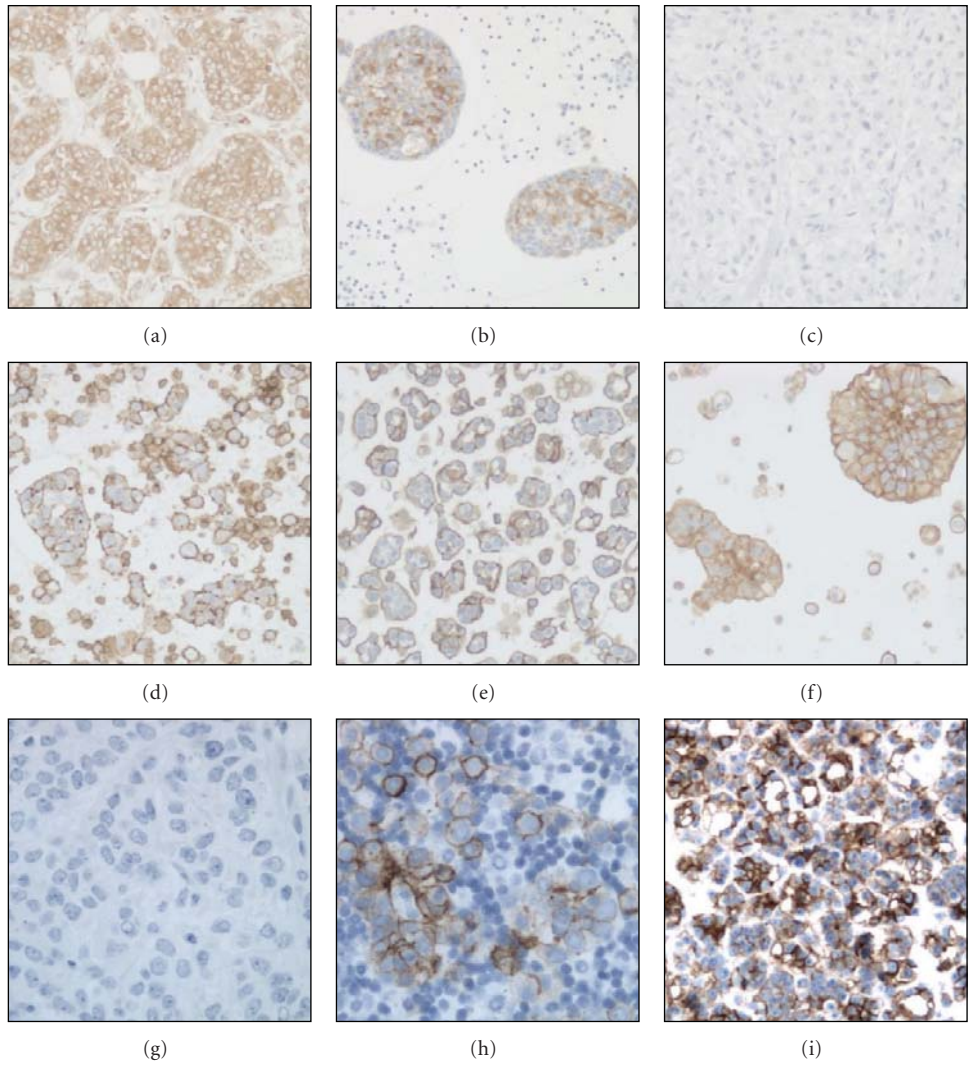


Figure 4: *Validation by immunohistochemistry*. (a)-(b), p130cas: Immunostaining for p130cas in a primary carcinoma (a) and pleural effusion (b) showing cytoplasmic staining in tumor cells. (c)–(f), p-Ezrin: Immunostaining for p-Ezrin in a primary carcinoma (c), pleural effusion from the same patient (d), and 2 additional effusions obtained from the pericardial (e) and pleural (f) cavity. Tumor cells in the primary carcinoma are p-Ezrin-negative, whereas cells in all three effusions are stained at the membrane. (g)–(i), claudin-4: immunostaining for Claudin-4 in a primary carcinoma (g), pleural effusion from the same patient (h), and an additional effusion obtained from the pericardial cavity (i, same specimen as in e). Tumor cells in the primary carcinoma are Claudin-4 -negative, whereas cells in the two effusions are stained at the membrane.

resistant cells due to tamoxifen treatment. Thus, *BCAR1* expression status in effusions must be taken under consideration while choosing therapeutic regimen in patients with breast carcinoma effusions. On the other hand, p130cas is a downstream effector of integrins [33]. The up-regulation of p130cas can lead to subsequent activation of Rac pathway and actin cytoskeleton rearrangements [34, 35]. This can elevate the metastatic potential of the cells in effusions by enhancing cell migration and leading to anoikis escape, as has been shown in in vitro systems [36, 37].

In contrast to the established importance of ER-α as a breast cancer marker, the prognostic and predictive relevance of ER-β remains unclear. Several previous reports have shown correlation between low ER-β expression and advanced disease stage and shorter survival in various tumors, including gynecological carcinomas [38–40]. In the present study we found downregulation of ER-β in effusions. Since breast carcinoma effusions constitute stage IV disease, this observation is concordant with the above-detailed publications. Low ER-β levels in effusions may contribute to tamoxifen resistance, as had been shown in ER-positive primary breast carcinomas [41]. Thus, the expression levels of ER-β may influence decisions regarding therapeutic regimens for patients with this form of metastatic disease.

Claudins are a family of tight junction (TJ)-specific integral membrane proteins, including more than 20 members to date. TJs, located between epithelial or endothelial cells, at the apical region of the adjacent lateral membranes,

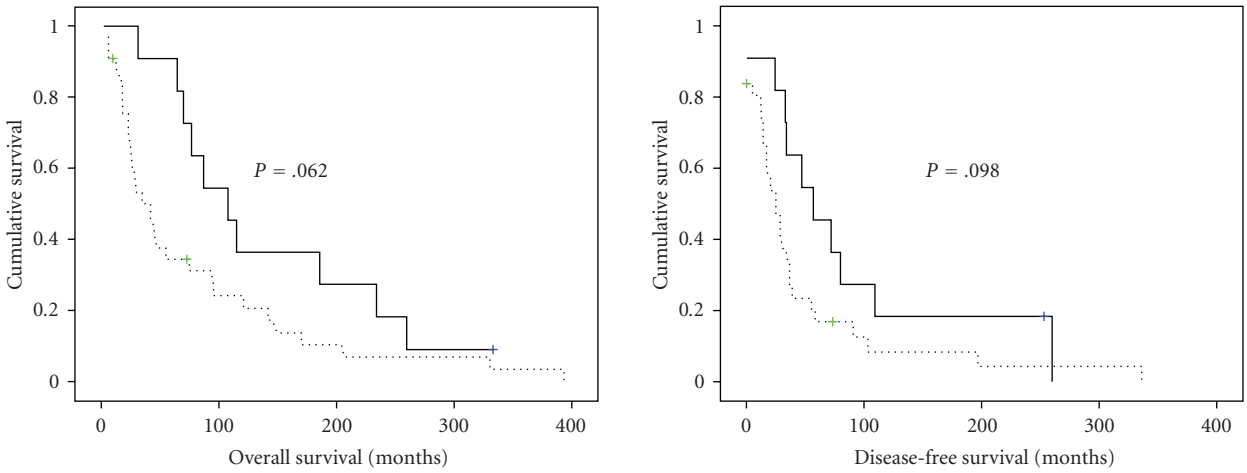


Figure 5: *The prognostic role of p130cas expression in breast carcinoma effusions.* (a): Kaplan-Meier survival curve showing the trend for association between p130cas expression and overall survival (OS) for 44 patients with breast carcinoma effusions. Patients with effusions with higher expression (>25% of tumor cells) ($n = 33$, dashed line) had a mean OS of 78 months versus 142 months for patients whose effusions showed low (≤25%) expression ($n = 11$, solid line; $P = .062$). (b): Kaplan-Meier survival curve showing the trend for association between p130cas expression and disease-free survival (DFS) for 42 patients with breast carcinoma effusions. Patients with effusions with higher expression (>25% of tumor cells) ($n = 31$, dashed line) had a mean DFS of 48 months versus 89 months for patients whose effusions showed low (≤25%) expression ($n = 11$, solid line; $P = .098$). DFS data were unavailable for 2 patients.

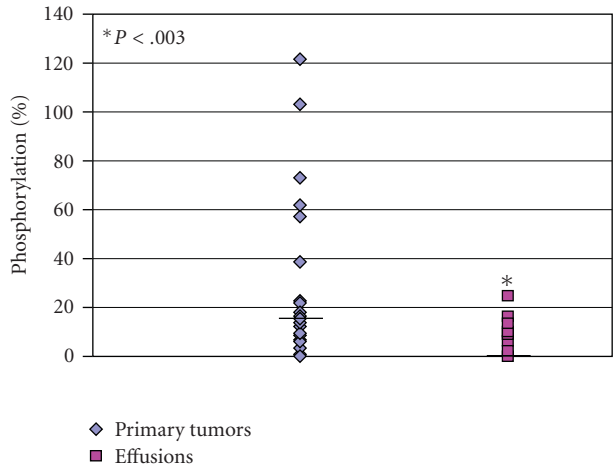


Figure 6: *Reduced p70S6K phosphorylation in breast carcinoma effusions compared to primary tumors.* mTOR activity measured by Western blot analysis of the mTORC1 substrate p70S6K phosphorylation extent in effusions ($n = 16$) and primary tumors ($n = 22$). Phosphorylated p70-S6K protein levels were analyzed using phospho-specific antibodies and normalized to total p70S6K levels. The 3T3-NIH cell line served as a positive control.

control the paracellular transport of solutes and maintain cell polarity by blocking the free diffusion of proteins and lipids between the apical and basolateral domains of the plasma membrane [42–44]. TJ filaments also contain occludin, the first TJ-specific integral membrane protein identified, yet it has been shown that claudins are essential and sufficient to form TJ strands [45]. The structure of claudins consists of intracellular amino and carboxy termini, four transmembrane domains, and two extracellular loops mediating interactions between claudins on adjacent cells [42–44]. The second extracellular loop serves as a binding site for *Clostridium perfringens* enterotoxin in claudin-3 and -4 [45]. The carboxy terminus of most claudins contains potential serine and/or thereonine phosphorylation sites and a PDZ-binding motif, to which the TJ cytoplasmic scaffolding proteins ZO-1, -2 and -3 bind [44].

We have recently shown that several claudin family members are upregulated in ovarian carcinoma effusions compared to corresponding primary carcinomas [46]. In the present study, we found upregulation of claudin-4 in breast carcinoma effusions compared to primary carcinomas, suggesting that members of this family are upregulated at this anatomic site in multiple epithelial malignancies. Our observations are in agreement with a recent study in which claudin-4 expression was shown to be associated with high grade and poor prognosis in breast carcinoma [46]. The previously discovered role of claudin-3 and claudin-4 in cell motility and increased MMP-2 activity [47] suggests that this may be yet another metastasis-promoting molecule in breast carcinoma effusions.

In conclusion, gene array analysis of breast carcinoma effusions and primary carcinomas showed differences in expression of multiple genes regulating cell motility, invasion and metastasis. The study of effusions and the way they differ from solid tumors will expand our knowledge regarding tumor progression in general, as well as regarding malignancies affecting this anatomic site in particular, and may have an impact on treatment modalities and prognostic models.

References

[1] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *CA: A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[2] F. H. hausheer and J. W. Yarbro, "Diagnosis and treatment of malignant pleural effusion," *Seminars in Oncology*, vol. 12, no. 1, pp. 54–75, 1985.

[3] E. Martinez-Moragón, J. Aparicio, J. Sanchis, R. Menéndez, M. Cruz Rogado, and F. Sanchis, "Malignant pleural effusion: prognostic factors for survival and response to chemical pleurodesis in a series of 120 cases," *Respiration*, vol. 65, no. 2, pp. 108–113, 1998.

[4] J. D. Wilkes, P. Fidias, L. Vaickus, and R. P. Perez, "Malignancy-related pericardial effusion: 127 cases from the Roswell Park Cancer Institute," *Cancer*, vol. 76, no. 8, pp. 1377–1387, 1995.

[5] M. Dieterich, S. N. Goodman, R. R. Rojas-Corona, A. B. Emralino, D. Jimenez-Joseph, and M. E. Sherman, "Multivariate analysis of prognostic features in malignant pleural effusions from breast cancer patients," *Acta Cytologica*, vol. 38, no. 6, pp. 945–952, 1994.

[6] A. K. Banerjee, I. Willetts, J. F. R. Robertson, and R. W. Blamey, "Pleural effusion in breast cancer: a review of the Nottingham experience," *European Journal of Surgical Oncology*, vol. 20, no. 1, pp. 33–36, 1994.

[7] B. Davidson, R. Reich, P. Lazarovici, V. A. Flørenes, S. Nielsen, and J. M. Nesland, "Altered expression and activation of the nerve growth factor receptors TrkA and p75 provide the first evidence of tumor progression to effusion in breast carcinoma," *Breast Cancer Research and Treatment*, vol. 83, no. 2, pp. 119–128, 2004.

[8] B. Davidson, S. Konstantinovsky, S. Nielsen, et al., "Altered expression of metastasis-associated and regulatory molecules in effusions from breast cancer patients: a novel model for tumor progression," *Clinical Cancer Research*, vol. 10, no. 21, pp. 7335–7346, 2004.

[9] B. Davidson, S. Konstantinovsky, L. Kleinberg, et al., "The mitogen-activated protein kinases (MAPK) p38 and JNK are markers of tumor progression in breast carcinoma," *Gynecologic Oncology*, vol. 102, no. 3, pp. 453–461, 2006.

[10] L. Kleinberg, V. A. Flørenes, J. M. Nesland, and B. Davidson, "Survivin, a member of the inhibitors of apoptosis family, is down-regulated in breast carcinoma effusions," *American Journal of Clinical Pathology*, vol. 128, no. 3, pp. 389–397, 2007.

[11] V. N. Dupont, D. Gentien, M. Oberkampf, Y. De Rycke, and N. Blin, "A gene expression signature associated with metastatic cells in effusions of breast carcinoma patients," *International Journal of Cancer*, vol. 121, no. 5, pp. 1036–1046, 2007.

[12] B. Davidson, S. Nielsen, J. Christensen, et al., "The role of desmin and N-cadherin in effusion cytology: a comparative study using established markers of mesothelial and epithelial cells," *American Journal of Surgical Pathology*, vol. 25, no. 11, pp. 1405–1412, 2001.

[13] Y. H. Yang, S. Dudoit, P. Luu, et al., "Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation," *Nucleic Acids Research*, vol. 30, no. 4, article e15, 2002.

[14] C. C. Reed, A. Waterhouse, S. Kirby, et al., "Decorin prevents metastatic spreading of breast cancer," *Oncogene*, vol. 24, no. 6, pp. 1104–1110, 2005.

[15] P.-J. Aspuria, T. Sato, and F. Tamanoi, "The TSC/Rheb/TOR signaling pathway in fission yeast and mammalian cells: temperature sensitive and constitutive active mutants of TOR," *Cell Cycle*, vol. 6, no. 14, pp. 1692–1695, 2007.

[16] P. T. Bhaskar and N. Hay, "The two TORCs and Akt," *Developmental Cell*, vol. 12, no. 4, pp. 487–502, 2007.

[17] A. Gautreau, D. Louvard, and M. Arpin, "ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling," *Current Opinion in Cell Biology*, vol. 14, no. 1, pp. 104–109, 2002.

[18] K. Krishnan, B. Bruce, S. Hewitt, D. Thomas, C. Khanna, and L. J. Helman, "Ezrin mediates growth and survival in Ewing's sarcoma through the AKT/mTOR, but not the MAPK, signaling pathway," *Clinical and Experimental Metastasis*, vol. 23, no. 3-4, pp. 227–236, 2006.

[19] Q. Li, M. Wu, H. Wang, et al., "Ezrin silencing by small hairpin RNA reverses metastatic behaviors of human breast cancer cells," *Cancer Letters*, vol. 261, no. 1, pp. 55–63, 2008.

[20] B. Bruce, G. Khanna, L. Ren, et al., "Expression of the cytoskeleton linker protein ezrin in human cancers," *Clinical and Experimental Metastasis*, vol. 24, no. 2, pp. 69–78, 2007.

[21] A. S. Clark, K. West, S. Streicher, and P. A. Dennis, "Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells," *Molecular Cancer Therapeutics*, vol. 1, no. 9, pp. 707–717, 2002.

[22] B. Bedogni, S. M. Welford, D. S. Cassarino, B. J. Nickoloff, A. J. Giaccia, and M. B. Powell, "The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation," *Cancer Cell*, vol. 8, no. 6, pp. 443–454, 2005.

[23] K. K. L. Chan, N. Wei, S. S. Liu, L. Xiao-Yun, A. N. Cheung, and H. Y. S. Ngan, "Estrogen receptor subtypes in ovarian cancer: a clinical correlation," *Obstetrics and Gynecology*, vol. 111, no. 1, pp. 144–151, 2008.

[24] W. H. Dragowska, M. Verreault, D. T. T. Yapp, et al., "Decreased levels of hypoxic cells in gefitinib treated ER+HER-2 overexpressing MCF-7 breast cancer tumors are associated with hyperactivation of the mTOR pathway: therapeutic implications for combination therapy with rapamycin," *Breast Cancer Research and Treatment*, vol. 106, no. 3, pp. 319–331, 2007.

[25] J. Zhou, J. Wulfkuhle, H. Zhang, et al., "Activation of the PTEN/mTOR/$STAT_3$ pathway in breast cancer stem-like cells is required for viability and maintenance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 41, pp. 16158–16163, 2007.

[26] M. Rosner, M. Hanneder, N. Siegel, A. Valli, and M. Hengstschläger, "The tuberous sclerosis gene products hamartin and tuberin are multifunctional proteins with a wide spectrum of interacting partners," *Mutation Research*, vol. 658, no. 3, pp. 234–246, 2008.

[27] R. F. Lamb, C. Roy, T. J. Diefenbach, et al., "The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho," *Nature Cell Biology*, vol. 2, no. 5, pp. 281–287, 2000.

[28] Y. Mamane, E. Petroulakis, O. LeBacquer, and N. Sonenberg, "mTOR, translation initiation and cancer," *Oncogene*, vol. 25, no. 48, pp. 6416–6422, 2006.

[29] K. Jastrzebski, K. M. Hannan, E. B. Tchoubrieva, R. D. Hannan, and R. B. Pearson, "Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function," *Growth Factors*, vol. 25, no. 4, pp. 209–226, 2007.

[30] J. C. Boughey, R. J. Gonzalez, E. Bonner, and H. M. Kuerer, "Current treatment and clinical trial developments for ductal carcinoma in situ of the breast," *Oncologist*, vol. 12, pp. 1276–1287, 2007.

[31] L. C. J. Dorssers, S. van der Flier, A. Brinkman, et al., "Tamoxifen resistance in breast cancer elucidating mechanisms," *Drugs*, vol. 61, no. 12, pp. 1721–1733, 2001.

[32] T. van Agthoven, J. Veldscholte, M. Smid, et al., "Functional identification of genes causing estrogen independence of human breast cancer cells," *Breast Cancer Research and Treatment*, vol. 114, no. 1, pp. 23–30, 2009.

[33] B. D. Cox, M. Natarajan, M. R. Stettner, and C. L. Gladson, "New concepts regarding focal adhesion kinase promotion of cell migration and proliferation," *Journal of Cellular Biochemistry*, vol. 99, no. 1, pp. 35–52, 2006.

[34] H. Honda, H. Oda, T. Nakamoto, et al., "Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas," *Nature Genetics*, vol. 19, no. 4, pp. 361–365, 1998.

[35] S. J. Pratt, H. Epple, M. Ward, Y. Feng, V. M. Braga, and G. D. Longmore, "The LIM protein Ajuba influences p130Cas localization and Rac1 activity during cell migration," *Journal of Cell Biology*, vol. 168, no. 5, pp. 813–824, 2005.

[36] M. A. Sanders and M. D. Basson, "p130cas but not paxillin is essential for Caco-2 intestinal epithelial cell spreading and migration on collagen IV," *Journal of Biological Chemistry*, vol. 280, no. 25, pp. 23516–23522, 2005.

[37] S. Daouti, W.-H. Li, H. Qian, et al., "A selective phosphatase of regenerating liver phosphatase inhibitor suppresses tumor cell anchorage-independent growth by a novel mechanism involving p130Cas cleavage," *Cancer Research*, vol. 68, no. 4, pp. 1162–1169, 2008.

[38] K. K. L. Chan, N. Wei, S. S. Liu, L. Xiao-Yun, A. N. Cheung, and H. Y. S. Ngan, "Estrogen receptor subtypes in ovarian cancer: a clinical correlation," *Obstetrics and Gynecology*, vol. 111, no. 1, pp. 144–151, 2008.

[39] J. S. Park, W. H. Jung, J. K. Kim, et al., "Estrogen receptor α, estrogen receptor β, and progesterone receptor as possible prognostic factor in radically resected gallbladder carcinoma," *Journal of Surgical Research*, vol. 152, no. 1, pp. 104–110, 2009.

[40] H. Sugiura, T. Toyama, Y. Hara, et al., "Expression of estrogen receptor β wild-type and its variant ER βcx/β2 is correlated with better prognosis in breast cancer," *Japanese Journal of Clinical Oncology*, vol. 37, no. 11, pp. 820–828, 2007.

[41] M. Esslimani-Sahla, J. Simony-Lafontaine, A. Kramar, et al., "Estrogen receptor β (ERβ) level but not its ERβ cx variant helps to predict tamoxifen resistance in breast cancer," *Clinical Cancer Research*, vol. 10, no. 17, pp. 5769–5776, 2004.

[42] S. Tsukita, M. Furuse, and M. Itoh, "Multifunctional strands in tight junctions," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 4, pp. 285–293, 2001.

[43] L. González-Mariscal, A. Betanzos, P. Nava, and B. E. Jaramillo, "Tight junction proteins," *Progress in Biophysics and Molecular Biology*, vol. 81, no. 1, pp. 1–44, 2003.

[44] C. M. Van Itallie and J. M. Anderson, "Claudins and epithelial paracellular transport," *Annual Review of Physiology*, vol. 68, pp. 403–429, 2006.

[45] M. Furuse, H. Sasaki, K. Fujimoto, and S. Tsukita, "A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occluding in fibroblasts," *The Journal of Cell Biology*, vol. 143, pp. 391–401, 1998.

[46] L. Kleinberg, A. Holth, C. G. Trope, R. Reich, and B. Davidson, "Claudin upregulation in ovarian carcinoma effusions is associated with poor survival," *Human Pathology*, vol. 39, no. 5, pp. 747–757, 2008.

[47] F. Lanigan, E. McKiernan, D. J. Brennan, et al., "Increased claudin-4 expression is associated with poor prognosis and high tumour grade in breast cancer," *International Journal of Cancer*, vol. 124, no. 9, pp. 2088–2097, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 646340, 8 pages
doi:10.1155/2010/646340

Research Article

Tumor Spreading to the Contralateral Ovary in Bilateral Ovarian Carcinoma Is a Late Event in Clonal Evolution

Francesca Micci,[1] Lisbeth Haugom,[1] Terje Ahlquist,[2,3] Vera M. Abeler,[4] Claes G. Trope,[5] Ragnhild A. Lothe,[2,3] and Sverre Heim[1,6]

[1] *Department of Medical Genetics, The Norwegian Radium Hospital, Oslo University Hospital, 0310 Oslo, Norway*
[2] *Department of Cancer Prevention, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital, 0310 Oslo, Norway*
[3] *Centre for Cancer Biomedicine, University of Oslo, 0310 Oslo, Norway*
[4] *Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, 0310 Oslo, Norway*
[5] *Department of Gynecology, The Norwegian Radium Hospital, Oslo University Hospital, 0310 Oslo, Norway*
[6] *Faculty of Medicine, University of Oslo, 0310 Oslo, Norway*

Correspondence should be addressed to Francesca Micci, francesca.micci@labmed.uio.no

Received 25 May 2009; Accepted 25 June 2009

Academic Editor: Ben Davidson

Cancer of the ovary is bilateral in 25%. Cytogenetic analysis could determine whether the disease in bilateral cases is metastatic or two separately occurring primary tumors, but karyotypic information comparing the two cancerous ovaries is limited to a single report with 11 informative cases. We present a series of 32 bilateral ovarian carcinoma cases, analyzed by karyotyping and high-resolution CGH. Our karyotypic findings showed that spreading to the contralateral ovary had occurred in bilateral ovarian cancer cases and that it was a late event in the clonal evolution of the tumors. This was confirmed by the large number of similar changes detected by HR-CGH in the different lesions from the same patient. The chromosomal bands most frequently involved in structural rearrangements were 19p13 ($n = 12$) and 19q13 ($n = 11$). The chromosomal bands most frequently gained by both tumorous ovaries were 5p14 (70%), 8q23-24 (65%), 1q23-24 (57%), and 12p12 (48%), whereas the most frequently lost bands were 17p11 (78%), 17p13 (74%), 17p12 (70%), 22q13 (61%), 8p21 and 19q13 (52%), and 8p22-23 (48%). This is the first time that 5p14 is seen gained at such a high frequency in cancer of the ovary; possibly oncogene(s) involved in bilateral ovarian carcinogenesis or tumor progression may reside in this band.

1. Introduction

Cancer of the ovary represents 30% of all malignancies of the female genital organs [1]. The most common ovarian neoplasms, typically occurring in women of reproductive age and beyond, originate from the ovarian surface epithelium and belong to one of three major types: serous, mucinous, and endometrioid tumors. These neoplasms range from the clearly benign (80%) to highly malignant carcinomas, with tumors of borderline malignancy in between.

Bilateral carcinomas of the ovary vary in frequency depending on which tumor type is involved but can be found in roughly 25% of all ovarian cancer cases [2]. The question of whether bilateral ovarian carcinomas are the result of metastatic spreading from one ovary harboring the primary tumor to the contralateral ovary, as opposed to the alternative, simultaneous occurrence of two independent primary tumors, was addressed nearly two decades ago by Pejovic et al. [3]. In a chromosome banding analysis they found no clear-cut difference in the karyotypic pattern between the tumors of the two ovaries in each woman but considerable differences from case to case. Hence, metastatic spreading from one side to the other must have been the pathogenetic mechanism, although the side carrying the primary tumor could not be identified. No similar later studies have been undertaken to confirm or falsify the findings and conclusions then made.

We present a series of 32 bilateral ovarian carcinoma cases, in six also including cancerous lesions in the omentum or peritoneum, analyzed primarily by karyotyping and high-resolution comparative genomic hybridization (HR-CGH) but also tested for microsatellite instability. Because the findings in the two or three samples from each woman were largely similar, we conclude that bilateral ovarian cancer occurs by a metastatic process and that spreading to the contralateral ovary mostly is a late event in the clonal evolution of these cancers.

2. Materials and Methods

2.1. Tumors. The examined material consists of 70 fresh samples from ovarian carcinomas surgically removed at The Norwegian Radium Hospital from 1999 to 2004 (see Table 1 in Supplementary Material available online at doi:10.1155/2010/646340). The tumors were part of a consecutive series of 248 ovarian tumors cultured and karyotyped by us (of which 203 were carcinomas; unpublished data). The 70 tumors came from altogether 32 patients with bilateral ovarian cancer. From all patients we had tumor material from both ovaries, and from six patients we also had samples from a metastasis to the omentum (three patients) or peritoneal cavity (three patients). Neoadjuvant therapy had been given to four patients before surgery (cases 5, 9, 22, and 24); otherwise, no preoperative chemotherapy or irradiation had been given. The tumors were classified as serous papillary adenocarcinoma (22 cases; Supplementary Table 1), endometrioid carcinoma (cases 23, 29, and 31), adenocarcinoma NOS (case 24), carcinosarcoma (case 21), and mucinous adenocarcinoma (case 26), and four cases showed a mixed histology and were classified as endometrioid and serous papillary (cases 3, 6, and 27) and clear cell and serous papillary carcinoma (case 28). The tumors also showed different patterns of differentiation (well, moderately, and poorly differentiated; Supplementary Table 1).

2.2. Cell Culturing and Karyotyping. The tumor samples were manually minced and disaggregated with Collagen II (Worthington, Freehold, NJ, USA) until a suitable suspension of cells and cell clumps was obtained. After 6-7 days of culturing in a selective medium [4], colchicine was added and the cultures harvested according to Mandahl [5]. The chromosomes of the dividing cells were then G-banded and a karyotype established according to the recommendations of the ISCN [6].

2.3. High-Resolution Comparative Genomic Hybridization (HR-CGH). DNA was isolated by the phenol-chloroform method as previously described [7]. CGH [8] was performed according to our modifications of standard procedures [9]. Chromosomes were karyotyped based on their inverted DAPI appearance and the relative hybridization signal intensity was determined along each chromosome. An average of 10–15 metaphases was analyzed. A negative (normal versus normal) and a positive (a cell line with known copy number changes) controls were included in the experiments. For the scoring of CGH results, we adopted the use of dynamic standard reference intervals (D-SRI). A D-SRI represents a "normal" ratio profile that takes into account the amount of variation detected in negative controls for each chromosome band. This provides a more objective and sensitive scoring criterion than fixed thresholds [10–12] and, consequently, a higher resolution. The D-SRI used was generated with data from 10 normal versus normal hybridizations (totalling 110 cells). This interval was automatically scaled onto each sample profile, and aberrations were scored whenever the case profile and the standard reference profile at 99% confidence intervals did not overlap. The description of the CGH copy number changes was based on the recommendation of the ISCN [6].

2.4. Microsatellite Instability Status. The tumor's microsatellite instability (MSI) status was determined in all samples using a consensus panel of five microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250) [13]. A tumor was considered to be MSI-high if two or more of the five markers exhibited novel alleles compared to normal DNA, MSI-low if only one marker deviated from the normal pattern, and microsatellite stable (MSS) if none of the tumor genotypes showed an aberrant pattern. Control DNA corresponding to the individual tumors was not available from the patients and therefore single allele changes, that is, the presence of two different alleles, can reflect a heterozygous constitutional genotype or a homozygous genotype with a novel tumor-specific allele. Thus, dinucleotide markers were not scored when such a pattern appeared in the tumors. The MSI status was assessed according to Wu et al. [14]. Allelic sizes were determined using GeneMapper 3.7 software (Applied Biosystems, Foster City, CA, USA), and the results were independently scored by two investigators. A second round of analyses was always performed, confirming the findings.

3. Results

The cell culturing and subsequent G-banding cytogenetic analysis gave informative results in 58 samples (Supplementary Table 1), 39 of which showed an abnormal karyotype whereas 19 were normal. The remaining 12 samples were culture failures and therefore could not be examined using this technique. The abnormal karyotypes were complex; that is, more than four abnormalities were present in all informative cases but one, case 21. This case was the only carcinosarcoma of our series; the tumor of the right ovary (case 21b) showed a der(16)t(1;16)(q21;q22) as the sole abnormality whereas a complex karyotype was seen in the tumor of the left ovary (case 21a), but with a similar der(16)t(1;16)(q21;q22) as one of many changes. Mostly many more aberrations than four were seen, several of which could not be completely identified. The modal chromosome number was hypodiploid in six cases, diploid in three, hyperdiploid in three, hypotriploid in one case, hypertriploid in 19, neartetraploid in one, hyperpentaploid in three cases,

and a mix of hyperdiploid and hypertetraploid clones in one (case 5a). For 14 cases, we obtained abnormal karyotypes from either both ovaries or from one ovarian tumor and a metastasis in the omentum/peritoneum, but in six of these cases, the technical quality was not sufficiently good to be able to compare the karyotypic data from different lesions in a reliable way. For eight patients, however, it was possible to compare the abnormal karyotypes, and in one additional patient both ovarian tumors and the peritoneal metastasis yielded informative results and could be compared (cases 8a, 8b, and 8c in Supplementary Table 1). In all these nine cases, more or less extensive karyotypic similarities between the samples taken from the different tumor lesions were found, with the number of identical aberrations ranging from one to eight per karyotype. However, because in seven out of these nine cases the karyotypic description was incomplete due to the complexity of the rearrangements, it is possible that more common abnormalities were present. In cases 19a and 19b, the karyotypic description was identical for the two tumorous ovaries (Figure 1).

The chromosomes seen to be most frequently involved in structural rearrangements by G-banding analysis were, in order of falling frequency, chromosomes 19, 1, 11, and 16 (Figure 2). The bands most frequently rearranged were 19p13 (involved in 12 rearrangements), 19q13 (11 rearrangements), 1q21, 16q22, and 19q10-11 (six rearrangements), 11p15, 12p13, and 15p11 (five rearrangements each), and 1p36 and 16q24 (four rearrangements each). The chromosomes most frequently involved in numerical aberrations were the X chromosome (in eight tumors) and chromosomes 8 and 14, involved in six tumors each.

The HR-CGH gave informative results on 60 samples showing genomic imbalances in 56 of them. From seven lesions there was no DNA available for analysis. No informative results were obtained in cases 13b, 22b, and 32b because of poor quality of the hybridization signal, despite running the experiments twice. In six cases, the G-banding karyotype matched the imbalances detected by CGH well. However, because the G-banding analysis often showed an incomplete karyotype with marker chromosomes and additional material of unknown origin sitting on known chromosomes, the CGH analysis allowed the identification of more imbalances. In 17 cases, a normal karyotype was detected by G-banding analysis whereas the CGH experiments showed genomic imbalances in the tumor samples. We gained information also on the 10 cases that were culture failures, finding imbalances in seven of them. Gains were more frequent than losses as seen by HR-CGH, and high-level amplification was found in 23 lesions. The major copy number changes were gains of or from chromosome arms 1p, 1q, 2p, 3q, 5p, 8q, 11q, 12p, and 20q and losses of or from Xp, 4q, 5q, 6q, 8p, 13q, 16q, 17p, 17q, 18q, 19q, and 22q. More specifically, the most frequently gained bands were, in order of decreasing frequency, 5p14 and 8q23 (39%), 2p23 (38%), 1q24, 3q25, and 3q27q28 (36%), 1q21, and 3q22 (34%), 2p13, 3q13, and 8q21 (32%), 1p31 (30%), 20q13 (29%), and 11q22 and 12p12 (25%). The most frequently lost bands were 17p11 and 17p13 (45%), 17p12 (43%), 16q23 and 22q13 (38%), 8p21 and 17q21 (34%), 8p22-23 (32%), Xp21 and 6q25 (30%), 4q34, and 18q22 (29%), 13q14 and 19q13 (27%), and 5q13-14 (25%) (Figure 3(a)). A comparison of the imbalances scored for the tumors in the two ovaries and/or the omentum/peritoneum showed that the bands most often gained by both ovarian tumors were 5p14 (70% of the 23 cases or 46 samples showing informative results), 8q23-24 (65%), 1q23-24 (57%), 12p12 (48%), 2q23 and 3q22 (43%), and 2p23, 3q13-21, 3q24-28, and 11q14 (39%). The most often lost bands were 17p11 (78%), 17p13 (74%), 17p12 (70%), 22q13 (61%), 8p21 and 19q13 (52%), 8p22-23 (48%), 16q22-23, 17q12-21, and 18q22 (43%), and 4q31, 4q33q34, 11p15, and Xp21 (39% each; Figure 3(b)).

The samples showed from one (samples 4a and 4b) to 58 (sample 11a) copy number alterations with an average number of copy alterations (ANCA) index of 37.9. Amplifications were most often scored on chromosome arms 8q (eight tumors) and 3q and 12p (four tumors each).

As ovarian cancer can be part of the hereditary nonpolyposis colon cancer (HNPCC) spectrum, characterized by microsatellite instability (MSI); we tested for this in the present series. Fifty-nine tumors gave informative results. Fifty-six of the tumors were classified as microsatellite stable (MSS) as none of the tumor genotypes showed an aberrant pattern; these were MSS tumors. Three cases (9a, 9c, and 16b) were scored as MSI-low (MSI-L; Supplementary Table 1). In four cases, the MSI status could not be determined in spite of running the experiments twice. The remaining seven samples were not analyzed as there was no DNA available.

4. Discussion

Cytogenetic studies of bilateral ovarian cancer are limited to the one by Pejovic et al. [3] who karyotyped tumors from both ovaries in 15 patients. Because the baseline karyotypes in each tumor pair were identical in the 11 patients from whom informative results were obtained, the conclusion was that the second tumor always arose by spreading of a monoclonal process from the first one. However, since the clonal evolution of the neoplastic cells in the two locations was similar, one could not determine which tumor was primary and which was metastatic.

We report a series of 32 patients with bilateral ovarian cancer analyzed by karyotyping, HR-CGH, and a microsatellite instability assay. As in the study by Pejovic et al. [3], considerable similarity was observed between the left-sided and right-sided tumors. From one to eight common aberrations were seen by karyotyping in the nine patients from whom informative results for both ovaries/omentum/peritoneum were obtained, averaging 3.22 common aberrations per patient. Because the karyotypic descriptions were incomplete, more common aberrations may have remained hidden among the markers. Indeed, the HR-CGH analysis revealed from one (cases 4 and 31) to 43 (case 1) genomic imbalances common to both or all lesions in the 23 patients from whom informative results for both ovaries/omentum/peritoneum were obtained, giving an average of 25.5 common aberrations per case or patient. Our findings therefore confirm the conclusions of Pejovic et

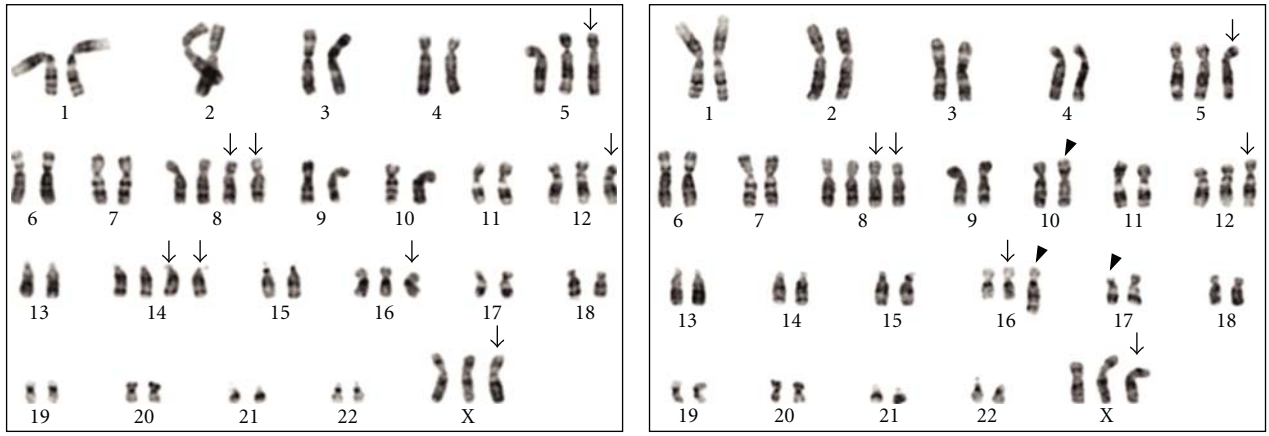


Figure 1: Tumor karyotypes from case 19. The two cancerous ovaries showed an identical karyotype with two related clones: (a) 54,XX,+X,+5,+8,+8,+12,+14,+14,+16, and (b) 52,XX,+X,+5,+8,+8,inv(10)(p12q22),+12,+der(16)t(14;16)(q13;q22),del(17)(p12). Arrows point to numerical changes, arrowheads to structural rearrangements.

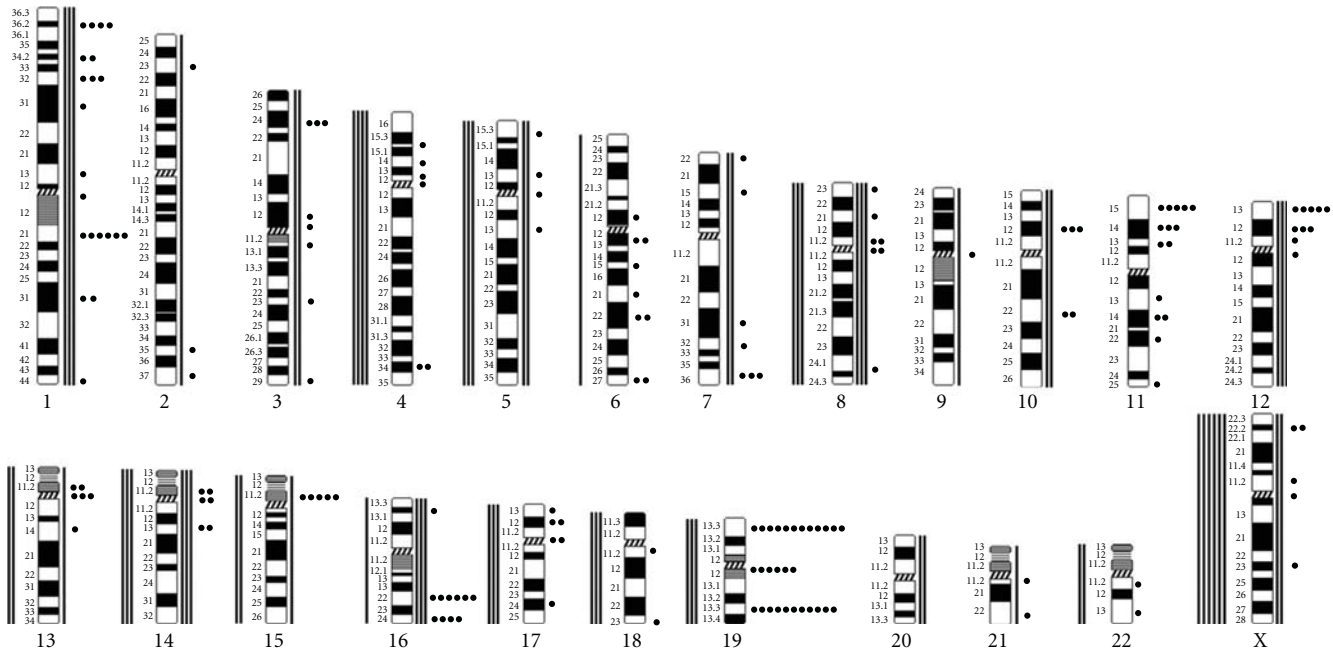


Figure 2: Breakpoint positions (circles to the right) and numerical changes (lines; losses to the left and gains to the right) detected in the chromosome aberrations of 32 cases of bilateral ovarian cancer.

al. [3] that bilateral ovarian cancer occurs via a metastatic mechanism, but the addition of CGH data allowed us to expand on this assessment of the pathogenetic connection between macroscopically discrete tumor lesions: the fact that so many aberrations are common to the tumors in both sides indicates that spreading to the contralateral ovary is a late event in the clonal evolution of the neoplastic parenchyma cells. The aberrations unique to each tumor lesion, on the other hand, in all likelihood arose after the metastasis was set up, in the other-sided ovary or in the omentum/peritoneum. The data are too sparse to conclude with certainty whether the latter metastases differ in any significant way from the tumors situated in the ovaries themselves when it comes to acquired genomic aberrations, but this does not appear to be the case.

In the present study, the chromosomes most frequently involved in numerical aberrations were the X chromosome and chromosomes 8 and 14. More precisely, the X chromosome was lost in six tumors and gained in two, whereas chromosomes 8 and 14 were involved in numerical aberrations in six tumors each, equally often in gains and losses. These numerical aberrations are well known in ovarian carcinomas [15]. The mechanisms behind their occurrence are unknown as are their pathogenetic effects.

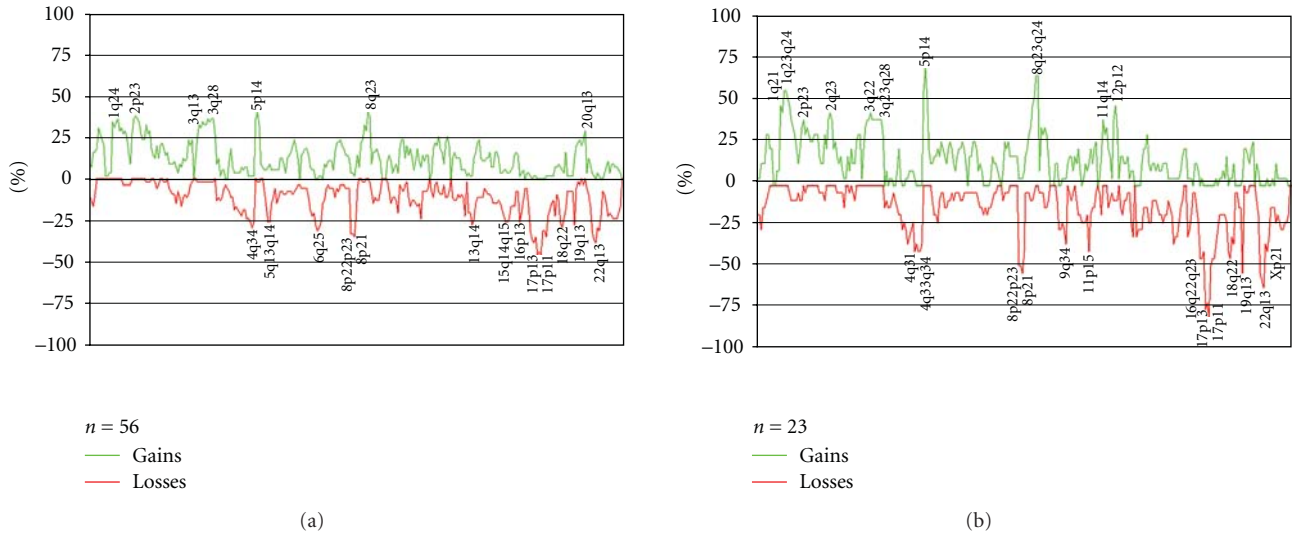


FIGURE 3: (a) All genomic imbalances detected by HR-CGH in 56 tumor lesions from altogether 32 cases of bilateral ovarian cancer (including six metastases to the omentum/peritoneum). (b) Genomic imbalances detected by HR-CGH in both tumorous ovaries from the 23 cases yielding informative results. The data from the latter subset are likely to reflect the earliest genomic changes, since they are present in both tumor lesions. Some of the imbalances in the former and larger group may have been acquired after spreading, since they also include findings in only one tumor per case.

The chromosome most frequently involved in structural rearrangements was chromosome 19; more specifically, 19p13 was involved in 12 rearrangements in eight tumors, whereas 19q13 was involved in 11 rearrangements in eight tumors, and the centromeric region of chromosome 19 was involved in six rearrangements in three cases. Alterations of 19p13 particularly, but also 19q13, in the form of added extra material are known to be among the most frequent cytogenetic aberrations in ovarian carcinomas [4, 16–18]. Sometimes the 19p+ or 19q+ markers look alike [16, 19], but the origin of the additional material could only rarely be identified [4]. The rearrangements of chromosome 19 have never been seen as the only chromosomal aberration in ovarian carcinoma, and so it seems likely that they are progressional rather than primary anomalies. Their detection here in many cases indicates that they show no frequency-difference between unilateral and bilateral ovarian carcinomas.

Rearrangements of chromosome 1 were also detected quite often in the present series, involving mostly 1q21 (six rearrangements) and 1p36 (four rearrangements). Similar aberrations were also previously reported. Pejovic et al. [16, 19] found frequent deletions of the distal half of 1q and various abnormalities resulting in loss of 1p34-36. Again, no difference between the current series of bilateral carcinomas and ovarian carcinomas in general is discernible.

Another hot-spot of chromosomal rearrangements in our series was 11p14-15 (eight rearrangements in total, five mapped to 11p15 and three to 11p14). Often the rearrangements are described as an add(11)(p14-15) which, in addition to the added material, may well also lead to loss of 11p-material distal to the breakpoint. Similar changes have been reported by other investigators [16, 20, 21]. Also chromosomal bands 16q22 and 16q24 were repeatedly rearranged in the present series, in six and four cases, respectively. The Mitelman database of chromosome aberrations in cancer reports such changes in five and 14 ovarian carcinomas, respectively [22]. As for the other above-mentioned changes, neither the genes involved nor anything else about the mechanism of their contribution to tumorigenesis is known.

The karyotypic features of the only bilateral carcinosarcoma analyzed in the present series deserve special mention. The tumor of the left ovary showed two apparently unrelated clones with many abnormalities in an incomplete karyotypic description. The right-sided tumor had a 46,XX,der(16)t(1;16)(q21;q22) as the sole abnormality in all analyzed cells, an aberration that was shared also by the contralateral tumor. In contrast to what appears to be the general rule in the other bilateral tumors, therefore, spreading to the left side seems to have taken place early in clonal evolution in this case. Unfortunately, no DNA was available to perform CGH on the tumor from the right side of this case to compare the genomic imbalances of the two lesions. Carcinosarcomas or malignant mixed mesodermal tumors comprise less than 1% of ovarian neoplasms [23]. They are microscopically characterized by a mixture of malignant epithelial and stromal element, similar to what is observed in corresponding uterine tumors [23]. There are altogether ten such tumors reported with karyotypic aberrations [24–30]. Rearrangement of chromosome 1 seems to be the most frequent cytogenetic change in carcinosarcomas of the ovary as well as of the uterus [9].

The bands most often seen by HR-CGH to be gained in both tumorous ovaries were 5p14 (70% of the 23 cases or 46 samples), 8q23-24 (65%), 1q23-24 (57%), 12p12

(48%), 2q23 and 3q22 (43%), and 2p23, 3q13-21, 3q24-28, and 11q14 (39%). The most often lost bands were 17p11 (78%), 17p13 (74%), 17p12 (70%), 22q13 (61%), 8p21 and 19q13 (52%), 16q22-23, 17q12-21, and 18q22 (43%), and 4q31, 4q33-34, 11p15, and Xp21 (39%). This picture of imbalances by and large tallies well with what has been reported before in unilateral ovarian carcinomas. The most common imbalances detected in ovarian carcinomas by chromosome-based CGH have been gains of or from chromosome arms 1q, 3q, 8q, 12p, and 20q and losses of or from 4p, 4q, 8p, 13q, 16q, 18q, and Xp [31–39]. Interestingly, the chromosomal band most frequently gained in our series of bilateral ovarian carcinomas was 5p14, being gained in 70% of the cases. This is the first time that the short arm of chromosome 5 is seen to be gained so often in ovarian carcinomas. The two only previous reports in which the same band was found gained showed a frequency of 17.6% (three out of 17 sampled analyzed; [40]) and 41.1% (six out of 13 samples analyzed; [41]). In addition, a CGH profile of 543 cases of ovarian carcinomas (summarized in http://www.progenetix.net/) shows a profile for 5p14 with 14.4% gains, 3.9% losses, and 1.3% amplifications. An explanation for the higher frequency of gain of 5p14 observed in our series could be found in the fact that our data are based on high-resolution CGH, a method that is known to be more sensitive in the detection of imbalances compared to normal chromosomal CGH [12]. The most exciting possibility, of course, would be that the observed gain has to do with a tumor's propensity to spread to the contralateral side, but confirmation in independent studies is needed before we ascribe much credibility to this explanation. Regardless of what it might mean, the direct pathogenetic significance of this specific gain remains unknown; possibly oncogene(s) located in 5p14 may be active in ovarian carcinogenesis and/or tumor progression and spreading.

The most frequent losses seen in the present series were from 17p, more precisely 17p11 in 78% of the samples, 17p13 in 74%, and 17p12 in 70%. Much interest has focused on the loss of genetic information from the short arm of chromosome 17, where losses seem to occur especially at 17p13.1 [42, 43] as well as at an even more distal locus in 17p13.3 [42, 44]. Two possible target tumor suppressor genes have been mapped to 17p13.3, *OVCA1* and *OVCA2* [45]. The target of the more proximal (17p13.1) 17p change could be *TP53*. Indeed, loss at 17p is the most common genetic alteration thus far detected in ovarian cancer, with mutation rates as high as 50% in advanced stage carcinomas [46]. However, we did not perform any further, detailed analysis to see if *TP53* or possibly other gene(s) were directly involved in the losses detected in this series.

DNA microsatellite instability reflects an altered pattern of short tandem repeat sequences (microsatellites) in dividing cells and has been described in HNPCC as well as other tumor types. Ovarian cancer, although most often sporadic, can occur together with HNPCC as part of the Lynch cancer family syndrome [47]. Several studies have suggested an association between MSI and certain histological types of ovarian carcinoma. However, in most of these studies, different kinds of microsatellite markers were used, and the presence of MSI was declared based on the demonstration of instability at only one locus [48–52]. In studies where the NCI markers and criteria were used, Sood et al. [53] found MSI-H in 12% of invasive carcinomas, whereas Gras et al. [54] reported that MSI-H was limited to endometrioid and clear cell carcinomas (12.5%). Liu et al. [55] and Cai et al. [56] found that 20% of endometrioid carcinomas were MSI-H. In our series of bilateral ovarian carcinomas, where 22 of 32 were serous papillary tumors, all cases but one (tumors 9a and 9c; these tumors were serous papillary carcinomas and MSI-L) were MSS. This is in agreement with previous studies finding that most of the MSI were scored in tumors of the clear cell and endometrioid subtype of ovarian cancer.

References

[1] F. A. Tavassoli and P. Devilee, "Tumors of the breast and female genital organs," in *World Health Organization Classification of Tumors*, IARC Press, Lyon, France, 2003.

[2] R. S. Cotran, V. Kumar, and T. Collins, *Robbins Pathologic Basis of Disease*, W.B. Sauders, Philadelphia, Pa, USA, 1999.

[3] T. Pejovic, S. Heim, N. Mandahl, et al., "Bilateral ovarian carcinoma: cytogenetic evidence of unicentric origin," *International Journal of Cancer*, vol. 47, no. 3, pp. 358–361, 1991.

[4] F. Micci, J. Weimer, L. Haugom, et al., "Reverse painting of microdissected chromosome 19 markers in ovarian carcinoma identifies a complex rearrangement map," *Genes Chromosomes and Cancer*, vol. 48, no. 2, pp. 184–193, 2009.

[5] N. Mandahl, "Methods in solid tumors," in *Human Cytogenetics—A Practical Approach, vol II, Malignancy and Acquired Abnormalities*, D. E. Rooney and B. H. Czepulkovski, Eds., pp. 155–187, IRL Press, Oxford, UK, 1992.

[6] ISCN, *An International System for Human Cytogenetic Nomenclature*, Karger, Basel, Switzerland, 2005.

[7] P. Brandal, B. Bjerkehagen, and S. Heim, "Molecular cytogenetic characterization of tenosynovial giant cell tumors," *Neoplasia*, vol. 6, no. 5, pp. 578–583, 2004.

[8] A. Kallioniemi, O.-P. Kallioniemi, D. Sudar, et al., "Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors," *Science*, vol. 258, no. 5083, pp. 818–821, 1992.

[9] F. Micci, M. R. Teixeira, L. Haugom, G. Kristensen, V. H. Abeler, and S. Heim, "Genomic aberrations in carcinomas of the uterine corpus," *Genes Chromosomes and Cancer*, vol. 40, no. 3, pp. 229–246, 2004.

[10] M. Kirchhoff, T. Gerdes, H. Rose, J. Maahr, A. M. Ottesen, and C. Lundsteen, "Detection of chromosomal gains and losses in comparative genomic hybridization analysis based on standard reference intervals," *Cytometry*, vol. 31, no. 3, pp. 163–173, 1998.

[11] M. Kirchhoff, T. Gerdes, J. Maahr, et al., "Deletions below 10 megabasepairs are detected in comparative genomic hybridization by standard reference intervals," *Genes Chromosomes and Cancer*, vol. 25, no. 4, pp. 410–413, 1999.

[12] F. R. Ribeiro, C. B. Diep, C. Jeronimo, et al., "Statistical dissection of genetic pathways involved in prostate carcinogenesis," *Genes Chromosomes and Cancer*, vol. 45, no. 2, pp. 154–163, 2006.

[13] C. R. Boland, S. N. Thibodeau, S. R. Hamilton, et al., "A national cancer institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of

microsatellite instability in colorectal cancer," *Cancer Research*, vol. 58, no. 22, pp. 5248–5257, 1998.

[14] Q. Wu, R. A. Lothe, T. Ahlquist, et al., "DNA methylation profiling of ovarian carcinomas and their in vitro models identifies HOXA9, HOXB5, SCGB3A1, and CRABP1 as novel targets," *Molecular Cancer*, vol. 6, no. 45, 2007.

[15] F. Micci and S. Heim, "Tumors of the female genital organs," in *Cancer Cytogenetics*, S. Heim and F. Mitelman, Eds., Wiley-Blackwell, NJ, USA, 3rd edition, 2009.

[16] T. Pejovic, S. Heim, N. Mandahl, et al., "Chromosome aberrations in 35 primary ovarian carcinomas," *Genes Chromosomes and Cancer*, vol. 4, no. 1, pp. 58–68, 1992.

[17] R. B. Jenkins, D. Bartelt Jr., P. Stalboerger, et al., "Cytogenetic studies of epithelial ovarian carcinoma," *Cancer Genetics and Cytogenetics*, vol. 71, no. 1, pp. 76–86, 1993.

[18] F. H. Thompson, J. Emerson, D. Alberts, et al., "Clonal chromosome abnormalities in 54 cases of ovarian carcinoma," *Cancer Genetics and Cytogenetics*, vol. 73, no. 1, pp. 33–45, 1994.

[19] T. Pejovic, S. Heim, N. Mandahl, et al., "Consistent occurrence of a 19p+ marker chromosome and loss of 11p material in ovarian seropapillary cystadenocarcinomas," *Genes Chromosomes and Cancer*, vol. 1, no. 2, pp. 167–171, 1989.

[20] A. D. Panani and C. Roussos, "Non-random structural chromosomal changes in ovarian cancer: i(5p) a novel recurrent abnormality," *Cancer Letters*, vol. 235, no. 1, pp. 130–135, 2006.

[21] A. D. Panani, "Preferential involvement of chromosome 11 as add(11)(p15) in ovarian cancer: is it a common cytogenetic abnormality in cancer?" *Cancer Letters*, vol. 258, no. 2, pp. 262–267, 2007.

[22] F. Mitelman, B. Johansson, and F. Mertens, "Mitelman database of chromosome aberrations in cancer," 2009, http://cgap.nci.nih.gov/Chromosomes/Mitelman.

[23] R. J. Kurman, *Blaustein's Pathology and of the Female Genital Tract*, Springer, New York, NY, USA, 2002.

[24] N. B. Atkin and M. C. Baker, "Chromosome 1 in 26 carcinomas of the cervix uteri. Structural and numerical changes," *Cancer*, vol. 44, no. 2, pp. 604–613, 1979.

[25] N. B. Atkin and V. J. Pickthall, "Chromosomes 1 in 14 ovarian cancers. Heterochromatin variants and structural changes," *Human Genetics*, vol. 38, no. 1, pp. 25–33, 1977.

[26] J. A. Fletcher, H. P. Kozakewich, F. A. Hoffer, et al., "Diagnostic relevance of clonal cytogenetic aberrations in malignant soft-tissue tumors," *The New England Journal of Medicine*, vol. 324, no. 7, pp. 436–443, 1991.

[27] X.-Y. Guan, C. B. Cargile, S. L. Anzick, et al., "Chromosome microdissection identifies cryptic sites of DNA sequence amplification in human ovarian carcinoma," *Cancer Research*, vol. 55, no. 15, pp. 3380–3385, 1995.

[28] T. Pejovic, S. Heim, C. Orndal, et al., "Simple numerical chromosome aberrations in well-differentiated malignant epithelial tumors," *Cancer Genetics and Cytogenetics*, vol. 49, no. 1, pp. 95–101, 1990.

[29] T. Pejovic, P. Alm, S. C. Iosif, F. Mitelman, and S. Heim, "Cytogenetic findings in four malignant mixed mesodermal tumors of the ovary," *Cancer Genetics and Cytogenetics*, vol. 88, no. 1, pp. 53–56, 1996.

[30] K. Tanaka, C. R. Boice, and J. R. Testa, "Chromosome aberrations in nine patients with ovarian cancer," *Cancer Genetics and Cytogenetics*, vol. 43, no. 1, pp. 1–14, 1989.

[31] H. Iwabuchi, M. Sakamoto, H. Sakunaga, et al., "Genetic analysis of benign, low-grade, and high-grade ovarian tumors," *Cancer Research*, vol. 55, no. 24, pp. 6172–6180, 1995.

[32] N. Arnold, L. Hagele, L. Walz, et al., "Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer," *Genes Chromosomes and Cancer*, vol. 16, no. 1, pp. 46–54, 1996.

[33] G. Sonoda, J. Palazzo, S. du Manoir, et al., "Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q 13 in human ovarian carcinomas," *Genes Chromosomes and Cancer*, vol. 20, no. 4, pp. 320–328, 1997.

[34] J. Tapper, L. Sarantaus, P. Vahteristo, et al., "Genetic changes in inherited and sporadic ovarian carcinomas by comparative genomic hybridization: extensive similarity except for a difference at chromosome 2q24-q32," *Cancer Research*, vol. 58, no. 13, pp. 2715–2719, 1998.

[35] S. Suzuki, D. H. Moore, D. G. Ginzinger, et al., "An approach to analysis of large-scale correlations between genome changes and clinical endpoints in ovarian cancer," *Cancer Research*, vol. 60, no. 19, pp. 5382–5385, 2000.

[36] M. Kiechle, A. Jacobsen, U. Schwarz-Boeger, J. Hedderich, J. Pfisterer, and N. Arnold, "Comparative genomic hybridization detects genetic imbalances in primary ovarian carcinomas as correlated with grade of differentiation," *Cancer*, vol. 91, no. 3, pp. 534–540, 2001.

[37] J. S. T. Sham, T. C.-M. Tang, Y. Fang, et al., "Recurrent chromosome alterations in primary ovarian carcinoma in Chinese women," *Cancer Genetics and Cytogenetics*, vol. 133, no. 1, pp. 39–44, 2002.

[38] O. Israeli, W. H. Gotlieb, E. Friedman, et al., "Familial vs sporadic ovarian tumors: characteristic genomic alterations analyzed by CGH," *Gynecologic Oncology*, vol. 90, no. 3, pp. 629–636, 2003.

[39] K. Partheen, K. Levan, L. Osterberg, K. Helou, and G. Horvath, "Analysis of cytogenetic alterations in stage III serous ovarian adenocarcinoma reveals a heterogeneous group regarding survival, surgical outcome, and substage," *Genes Chromosomes and Cancer*, vol. 40, no. 4, pp. 342–348, 2004.

[40] K. Helou, H. Padilla-Nash, D. Wangsa, et al., "Comparative genome hybridization reveals specific genomic imbalances during the genesis from benign through borderline to malignant ovarian tumors," *Cancer Genetics and Cytogenetics*, vol. 170, no. 1, pp. 1–8, 2006.

[41] H. Blegen, N. Einhorn, K. Sjovall, et al., "Prognostic significance of cell cycle proteins and genomic instability in borderline, early and advanced stage ovarian carcinomas," *International Journal of Gynecological Cancer*, vol. 10, no. 6, pp. 477–487, 2000.

[42] A. K. Godwin, L. Vanderveer, D. C. Schultz, et al., "A common region of deletion on chromosome 17q in both sporadic and familial epithelial ovarian tumors distal to BRCA1," *American Journal of Human Genetics*, vol. 55, no. 4, pp. 666–677, 1994.

[43] G. Saretzki, U. Hoffmann, P. Rohlke, et al., "Identification of allelic losses in benign, borderline, and invasive epithelial ovarian tumors and correlation with clinical outcome," *Cancer*, vol. 80, no. 7, pp. 1241–1249, 1997.

[44] N. J. Phillips, M. R. Ziegler, and L. L. Deaven, "A cDNA from the ovarian cancer critical region of deletion on chromosome 17p13.3," *Cancer Letters*, vol. 102, no. 1-2, pp. 85–90, 1996.

[45] D. C. Schultz, L. Vanderveer, D. B. Berman, T. C. Hamilton, A. J. Wong, and A. K. Godwin, "Identification of two candidate tumor suppressor genes on chromosome 17p13.3," *Cancer Research*, vol. 56, no. 9, pp. 1997–2002, 1996.

[46] M. Schuijer and E. M. J. J. Berns, "TP53 and ovarian cancer," *Human Mutation*, vol. 21, no. 3, pp. 285–291, 2003.

[47] I. I. Arzimanoglou, T. Lallas, M. Osborne, H. Barber, and F. Gilbert, "Microsatellite instability differences between familial and sporadic ovarian cancers," *Carcinogenesis*, vol. 17, no. 9, pp. 1799–1804, 1996.

[48] M. Fujita, T. Enomoto, K. Yoshino, et al., "Microsatellite instability and alterations in the hMSH2 gene in human ovarian cancer," *International Journal of Cancer*, vol. 64, no. 6, pp. 361–366, 1995.

[49] M. Ohwada, M. Suzuki, Y. Saga, and I. Sato, "DNA replication errors are frequent in mucinous cystadenocarcinoma of the ovary," *Cancer Genetics and Cytogenetics*, vol. 117, no. 1, pp. 61–65, 2000.

[50] B. L. King, M.-L. Carcangiu, D. Carter, et al., "Microsatellite instability in ovarian neoplasms," *British Journal of Cancer*, vol. 72, no. 2, pp. 376–382, 1995.

[51] J. Tangir, N. S. Loughridge, R. S. Berkowitz, et al., "Frequent microsatellite instability in epithelial borderline ovarian tumors," *Cancer Research*, vol. 56, no. 11, pp. 2501–2505, 1996.

[52] C. J. Haas, J. Diebold, A. Hirschmann, H. Rohrbach, S. Schmid, and U. Lohrs, "Microsatellite analysis in serous tumors of the ovary," *International Journal of Gynecological Pathology*, vol. 18, no. 2, pp. 158–162, 1999.

[53] A. K. Sood, R. Holmes, M. J. C. Hendrix, and R. E. Buller, "Application of the National Cancer Institute international criteria for determination of microsatellite instability in ovarian cancer," *Cancer Research*, vol. 61, no. 11, pp. 4371–4374, 2001.

[54] E. Gras, L. Catasus, R. Argelles, et al., "Microsatellite instability, MLH-1 promoter hypermethylation, and frameshift mutations at coding mononucleotide repeat microsatellites in ovarian tumors," *Cancer*, vol. 92, no. 11, pp. 2829–2836, 2001.

[55] J. Liu, C. T. Albarracin, K.-H. Chang, et al., "Microsatellite instability and expression of hMLH1 and hMSH2 proteins in ovarian endometrioid cancer," *Modern Pathology*, vol. 17, no. 1, pp. 75–80, 2004.

[56] K. Q. Cai, C. Albarracin, D. Rosen, et al., "Microsatellite instability and alteration of the expression of hMLH1 and hMSH2 in ovarian clear cell carcinoma," *Human Pathology*, vol. 35, no. 5, pp. 552–559, 2004.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 139087, 9 pages
doi:10.1155/2010/139087

Research Article

The Synergistic Effect of Conditional *Pten* Loss and Oncogenic *K-ras* Mutation on Endometrial Cancer Development Occurs via Decreased Progesterone Receptor Action

Tae Hoon Kim,[1] Jinrong Wang,[1] Kevin Y. Lee,[2] Heather L. Franco,[1] Russell R. Broaddus,[3] John P. Lydon,[1] Jae-Wook Jeong,[1] and Francesco J. DeMayo[1]

[1] *Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA*
[2] *Joslin Diabetes Center, Harvard Medical School, Boston, MA 02215, USA*
[3] *Department of Pathology, University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, USA*

Correspondence should be addressed to Francesco J. DeMayo, fdemayo@bcm.tmc.edu

Received 27 May 2009; Accepted 10 August 2009

Academic Editor: Gudrun Pohl

Endometrial cancer is the most common gynecological cancer. Estrogen-dependent endometrioid carcinoma is the most common type of endometrial cancer, and alterations in the expression of *PTEN* and *K-ras* have been associated with this disease. To study the roles of *Pten* and *K-ras* in endometrial cancer, we generated *Pten* ablation and oncogenic *K-ras* mutation in progesterone receptor positive cells ($PR^{cre/+}Pten^{f/f}K\text{-}ras^{G12D}$). Double mutant mice dramatically accelerated the development of endometrial cancer compared to a single mutation of either gene. Histological analysis showed that all of the 1-month old double mutant female mice developed endometrial cancer with myometrial invasion. The expression of PR was downregulated in double mutant mice compared to a single mutation of either gene which resulted in decreased suppression of estrogen signaling. Therefore, these results suggest a synergistic effect of dysregulation of the *Pten* and *K-ras* signaling pathways during endometrial tumorigenesis.

1. Introduction

Endometrial cancer is the most common type of gynecological cancer in the United States with approximately 40 100 cases diagnosed and about 7470 deaths from the disease each year [1]. Therefore, there is great interest in identifying novel ways to treat and prevent this disease. Estrogen-dependent endometrioid carcinoma is the most common type of endometrial cancer. An increased incidence of endometrial cancer has been found in association with prolonged, unopposed estrogen (E2) exposure [2, 3] and alterations in the expression of the tumor suppressor gene, *PTEN*, as well as the oncogenes, β-catenin and Ras [4, 5]. Currently, progesterone (P4) therapy is used as a means to prevent the development of endometrial cancer associated with unopposed E2 as a means to block E2 actions [6].

The uterus consists of heterogeneous cell types that undergo dynamic changes in response to the ovarian steroid hormones E2 and P4 to support embryo development and implantation. E2 stimulates the proliferation of epithelial cells in the mouse uterus [7, 8]. In contrast, P4 is inhibitory to this E2-mediated proliferation of the luminal and glandular epithelial cells. However, P4, alone or in conjunction with E2, leads to uterine stromal cell proliferation [8–10]. The ability of the ovarian steroid hormones to regulate uterine cell proliferation depends upon the ability of hormonal stimulation to regulate growth factor communication networks between the uterine stroma and epithelium. For instance, P4 attenuates E2-stimulated uterine epithelial cell proliferation by regulating uterine stromal cell gene expression [11]. An imbalance caused by increased E2 action and/or decreased P4 action can result in abnormal endometrial proliferation and endometrial adenocarcinoma. Therefore, elucidating the molecular mechanisms by which the steroid hormones control uterine physiology is important to understanding the pathology of these diseases.

Two common mutations that occur in endometrial cancer are in the tumor suppressor *Pten* and the oncogene *K-ras*

[4, 5]. *K-ras* encodes a guanine nucleotide binding protein of 21 kDa that has a central role in the regulation of cell growth and differentiation by transducing signals from activated transmembrane receptors. It has been shown to be mutated in 10%–30% of endometrial cancers [4]. These mutations have been found in all grades of endometrioid carcinoma and have been reported in complex atypical hyperplasia, suggesting a relatively early role for *K-ras* mutations in endometrial tumorigenesis [12]. *PTEN* (phosphatase and tensin homologue deleted from chromosome 10) is one of the most frequently mutated tumor suppressor genes in human cancers [13, 14]. *PTEN* is completely lost or mutated in >50% of primary endometrioid endometrial cancer [15] and in at least 20% of endometrial hyperplasias, the precancerous lesions of the endometrium [15, 16]. Thus, loss of *PTEN* is a very early event in the multistep process leading to endometrioid endometrial cancer [16, 17]. Phosphoinositide 3-kinases (PI3K) regulates a number of cellular functions through the activation of Akt [18]. *PTEN* acts as a negative regulator of PI3K signaling [19]. Previously, loss of *Pten* (either as a heterozygote or by uterine specific ablation) has been shown to induce endometrial cancer in mice highlighting its important role in cancer development [20, 21]. This mutation and subsequent Akt activation resulted in the activation of ERα-dependent pathways that play an important role in the tumorigenesis of endometrial cancer [21]. Interestingly, the *PTEN*/PI3K/AKT signaling pathway can also be activated by E2 suggesting a complex interaction between these two signaling pathways [22].

In this study, we utilized conditional *Pten* ablation and oncogenic *K-ras* mutation in the uteri of mice to demonstrate a synergistic effect of dysregulation of the *Pten* and *K-ras* signaling pathways during endometrial tumorigenesis. *Pten* ablation and oncogenic *K-ras* mutation dramatically accelerated the development of endometrial cancer compared to single mutation of either gene. Thus, these results demonstrate the importance of *Pten* and *K-ras* regulation in the tumorigenesis of endometrial cancer.

2. Materials and Methods

2.1. Animals. Mice were maintained in the designated animal care facility at Baylor College of Medicine according to the institutional guidelines for the care and use of laboratory animals. $PR^{Cre/+}$ mice were previously generated [23]. The $Pten^{f/f}$ were acquired from Dr. Hong Wu (University of California, Los Angeles, Los Angeles, CA) [24]. The lox-stop-Lox $Kras^{G12D}$ mice were acquired from Dr. Tyler Jacks (MIT, Cambridge, MA) [25]. Mice of various genotypes were sacrificed at 2 and 4 weeks of age. At the time of dissection, uterine tissues were placed in the appropriate fixative or flash frozen and stored at −80°C. For the genotyping of the oncogenic *K-ras* mutation, total RNA was isolated from uterus according to, Qiagen minieasy kit protocol. cDNA was generated from RNA samples by reverse transcription, followed by PCR amplification using primers *K-ras*1S, 5′-GCCATTTCGGACCCGGAGCGA and *K-ras*1A, 5′-CCTACCAGGACCATAGGCACATC. RNA expression of wild-type *K-ras* and mutant *K-ras* G12D was determined by digestion of 5 μL of the reverse transcription-PCR products with HindIII for 1 hour at 37°C. The restriction products were resolved in a 2% agarose gel. The mutant *K-ras* G12D allele contains a HindIII restriction site engineered in exon 1, which is absent in the wild-type allele. Therefore, digestion of the 488-bp products generates 300-bp and 148-bp restriction fragments in the mutant but not in the wild-type PCR product.

2.2. Western Blot Analysis. Samples containing 15 μg of proteins were applied to SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were blocked overnight with 0.5% casein (wt/vol) in PBS with 0.1% Tween 20 (vol/vol) (Sigma-Aldrich, St. Louis, MO) and probed with anti-Akt(Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-Akt (Cell Signaling Technology, Inc., Danvers, MA), anti-PR (DAKO Corp., Capinteria, CA), or anti-ERα (DAKO Corp., Capinteria, CA) antibodies. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody and treatment with ECL reagents. To control for loading, the membrane was stripped and probed with anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and developed again.

2.3. Immunohistochemistry and TUNEL Assay. Uterine sections from paraffin-embedded tissue were cut at 5 μm and mounted on silane-coated slides, deparafinized, and rehydrated in a graded alcohol series. Sections were preincubated with 10% normal serum in PBS (pH 7.5) and then incubated with anti-PR antibody (DAKO Corp., Capinteria, CA) or anti-ERα (DAKO Corp., Capinteria, CA) in 10% normal serum in PBS (pH 7.5). On the following day, sections were washed in PBS and incubated with a secondary antibody (5 μL/mL; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Immunoreacitivity was detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The TUNEL assay was performed according to manufacturer's instructions using the Roche In Situ Cell Death Detection Kit, Fluorescein (Roche, Boulder, CO).

2.4. RNA Isolation and Quantitative Real-Time RT-PCR. Total RNA was extracted from uterine tissues using the Qiagen RNeasy total RNA isolation kit (Valencia, CA). Quantitative real-time RT-PCR analysis was conducted on isolated RNA. Expression levels of *Ltf, Clca3,* and *C3* were measured by real-time RT-PCR TaqMan analysis (Applied Biosystems, Foster City, CA). cDNA was made from 1 μg of total RNA using random hexamers and M-MLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). RT-PCR was performed using RT-PCR Universal Master Mix reagent (Applied Biosystems, Foster City, CA). All real-time RT-PCR results were normalized against 18S RNA using ABI rRNA control reagents. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple range test with the Instat package from GraphPad (San Diego, CA, USA).

3. Results

3.1. Generation of Pten Ablation and Oncogenic K-Ras Mutation in the Murine Uterus. Homozygous $Pten^{-/-}$ mouse embryos die around E8.5 and heterozygous $Pten^{+/-}$ mice develop numerous pathologies and have decreased longevity [21, 26] making our ability to investigate the role of *Pten* in the mouse uterus severely limited. Likewise, constitutive activation of a *K-ras* mutation results in embryonic lethality [27]. In order to achieve ablation of *Pten* and activation of *K-ras* in the uterus, mice with floxed *Pten* ($Pten^{f/f}$) [24] and mice with loxP-Stop-loxP-$Kras^{G12D/+}$ (LSL-$K\text{-}ras^{G12D/+}$) [25] were bred to the PR^{Cre} mouse [23]. Using this mouse with Cre recombinase inserted into the progesterone receptor (PR) locus, floxed genes are edited in PR expressing cells including all compartments of the mouse uterus. This model was previously used to ablate *Pten* in the uterus resulting in endometrial adenocarcinoma [20]. Therefore, in order to effectively investigate the effects of the *Pten* and *K-ras* signaling pathways in endometrial cancer, mice with uterine *Pten* ablation ($PR^{cre/+}Pten^{f/f}$; $Pten^{d/d}$) and oncogenic *K-ras* mutation ($K\text{-}ras^{G12D}$) were generated and mated to generate double mutant mice ($PR^{cre/+}Pten^{f/f}K\text{-}ras^{G12D}$; $Pten^{d/d}K\text{-}ras^{G12D}$) [23–25]. Ablation of *Pten* in the $Pten^{d/d}$ mice was assayed by Western blot and immunohistochemical analysis (Figures 1(a) and 1(b)). *PTEN* protein was expressed in the endometrium of $Pten^{f/f}$ mice. However, the level of *PTEN* protein was significantly decreased in the uteri of $Pten^{d/d}$ mice demonstrating efficient ablation of *Pten*. To confirm the oncogenic *K-ras* mutation in the uterus, the PCR products encompassing the *K-ras* mutation were digested with HindIII (see Section 2). The mutant *K-ras* G12D allele contains a HindIII restriction site engineered in exon 1, which is absent in the wild-type allele. Therefore, digestion of the 488-bp products generates 300-bp and 148-bp restriction fragments in the mutant but not in the wild-type PCR product. Analysis of the HindIII digestion revealed the presence of the 300-bp and 148-bp fragments in the $K\text{-}ras^{G12D}$ but not in the wild type uteri (Figure 1(c)). These results suggest that PR-cre efficiently generated uterine *Pten* ablation and oncogenic *K-ras* mutation.

3.2. Development of Vaginal Papillomas in Mice with the Oncogenic K-ras Mutation in PR-Expressing Cells. Introduction of the oncogenic *K-ras* mutation in all PR-positive cells resulted in the development of vaginal papillomas. Lesions were present at the vaginal opening of $K\text{-}ras^{G12D}$ mice as early as 2 months of age but were never observed in control mice (Figure 2(a)). Examination of the histology of these lesions revealed an abnormal vaginal architecture in the $K\text{-}ras^{G12D}$ mice compared to controls (Figure 2(b)). The vaginal epithelium exhibited increased keratinization accompanied by disorganization of the vaginal lumen. In addition, there was a decrease in the vaginal stroma. Interestingly, these lesions remained benign, never developing into a cancerous lesion. $K\text{-}ras^{G12D}$ mice did not show any pathological phenotype in the uterus. Proliferation was not affected in the *K-ras* mutant mice; however, there was increased apoptosis in the $K\text{-}ras^{G12D}$ uteri compared to controls (Figure 2(c)).

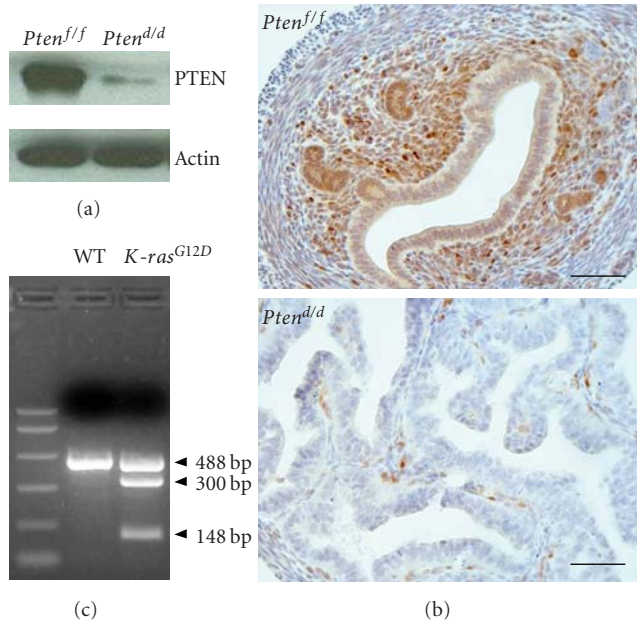


Figure 1: Analysis of conditionally ablated *Pten* and oncogenic *K-ras* mutation in the murine uterus. (a) Western blot analysis of *PTEN* in whole uterine extracts from $Pten^{f/f}$ and $Pten^{d/d}$ mice. (b) Immunohistochemical analysis for *PTEN* in $Pten^{f/f}$ and $Pten^{d/d}$mice uteri. Four-week-old $Pten^{f/f}$ and $Pten^{d/d}$ mice were used for Western blot analysis and immunohistochemical analysis. These experiments demonstrate that *Pten* is conditionally ablated in the uteri of these mice. Scale bar: 50 μm. (c) PR-cre mediated recombination of $K\text{-}ras^{G12D}$ in uterus. The mutant *K-ras G12D* allele contains a HindIII restriction site engineered in exon 1, which is absent in the wild-type allele. Therefore, digestion of the 488-bp products generates 300-bp and 148-bp restriction fragments in the mutant but not in the wild-type PCR product.

3.3. Development of Endometrial Cancer in Mice with Pten Ablation and the Oncogenic K-ras Mutation in PR-Expressing Cells. To investigate the impact of *Pten* and *K-ras* signaling on endometrial cancer development and progression, control, $Pten^{d/d}$, $K\text{-}ras^{G12D}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ mice were sacrificed, excised uteri were weighed, and morphology was examined at gross and histological levels. $Pten^{d/d}K\text{-}ras^{G12D}$mice showed a significant increase in uterine weight at 2 weeks of age compared to control, $Pten^{d/d}$, and $K\text{-}ras^{G12D}$ mice (Figures 3(a) and 3(b)). $Pten^{d/d}$ and $Pten^{d/d}K\text{-}ras^{G12D}$ mice showed a significant increase in uterine wet weight compared to control mice at 4 weeks of age. The uterine weight of $Pten^{d/d}$ $K\text{-}ras^{G12D}$ mice was significantly increased compared to other mice including $Pten^{d/d}$ mice at 4 weeks of age (Figures 3(a) and 3(b)). The uterine weight of $K\text{-}ras^{G12D}$mice did not change compared to control mice at either timepoint.

Histological analysis of the uteri showed an increase in the number of endometrial glands and in the gland/stroma ratio in the uteri of 2-week-old $Pten^{d/d}$ and $Pten^{d/d}K\text{-}ras^{G12D}$ mice (Figure 4); however, the myometrium was not enlarged. These histological changes demonstrate that the uteri of 2 week old $Pten^{d/d}$ and $Pten^{d/d}K\text{-}ras^{G12D}$mice

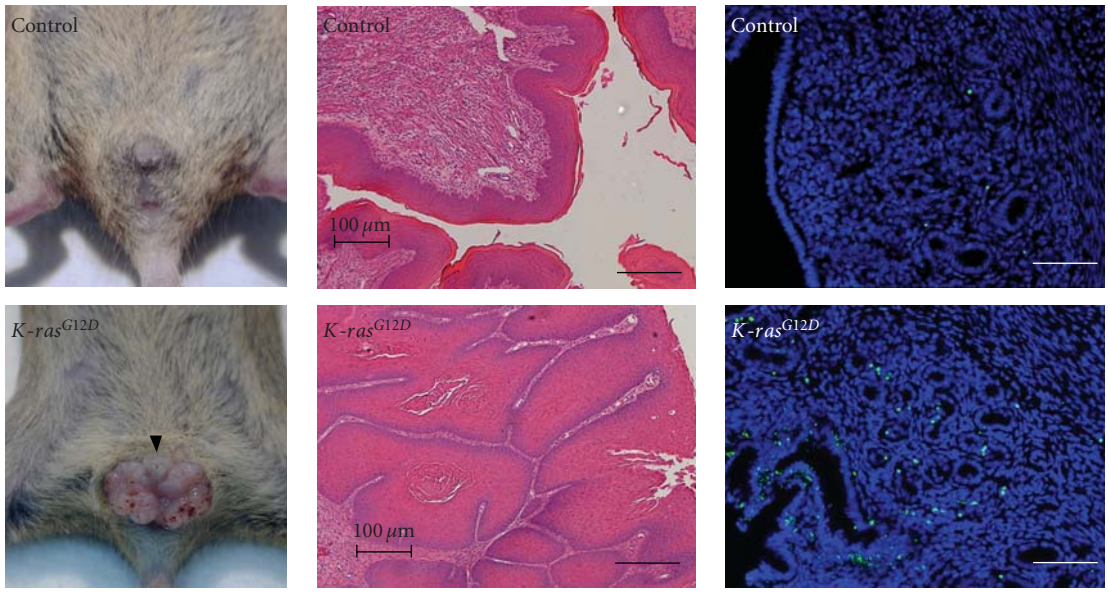


Figure 2: Development of vaginal papiloma and increased apoptosis in *K-ras*^{G12D} mice. (a) The development of vaginal papiloma. Control and *K-ras*^{G12D} mice at 2 months of age. (b) H&E staining of vagina of control and *K-ras*^{G12D} mice. The histology of these lesions revealed an abnormal vaginal architecture in the *K-ras*^{G12D} mice compared to controls. (c) TUNEL assay in the uterus of control and *K-ras*^{G12D} mice at 2 months of age. The number of apoptotic cells was significantly increased in epithelial cells of *K-ras*^{G12D} uteri compared to controls. Scale bar: 100 μm.

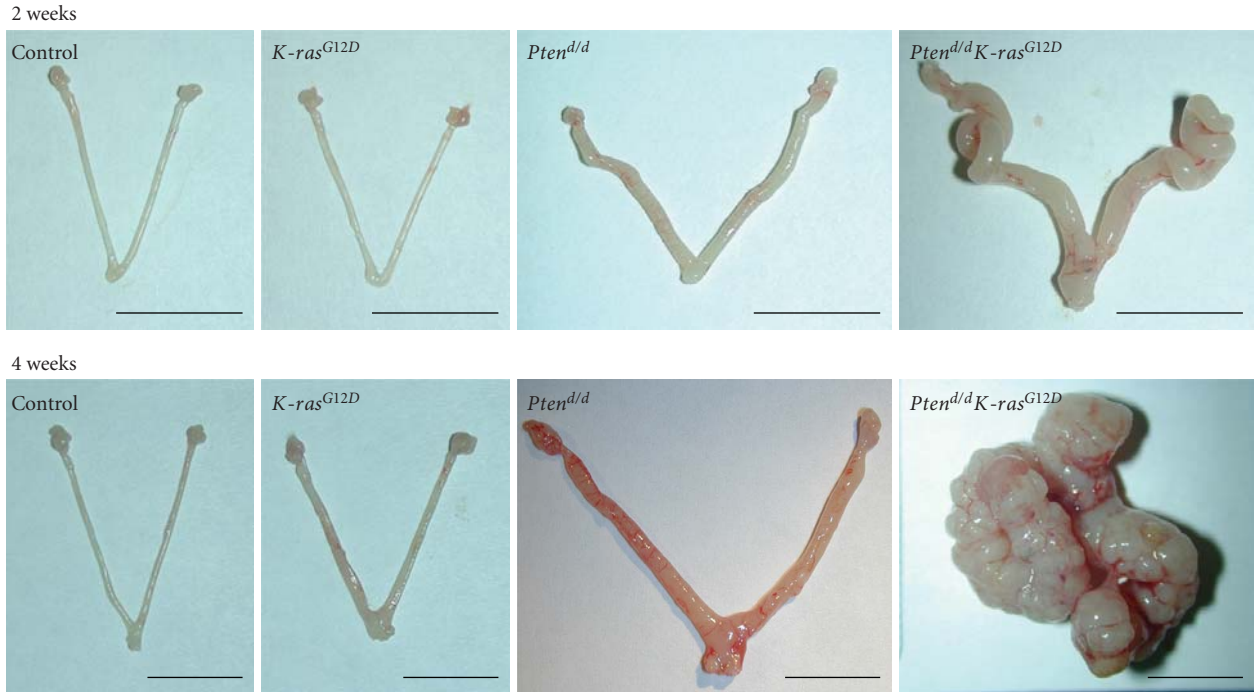
display endometrial hyperplasia, a predisposing factor to endometrial adenocarcinoma in humans. Thus, even though the uterine weight of the *Pten*$^{d/d}$*K-ras*^{G12D} mice was increased compared to the *Pten*$^{d/d}$ mice, the uteri of *Pten*$^{d/d}$ and *Pten*$^{d/d}$*K-ras*^{G12D} mice exhibit a similar hyperplastic phenotype at 2 weeks of age.

Interestingly, all of the *Pten*$^{d/d}$*K-ras*^{G12D} mice developed invasive endometrioid-type endometrial adenocarcinoma by 4 weeks of age. The neoplastic endometrial glands in the *Pten*$^{d/d}$*K-ras*^{G12D}mice invaded through the uterine muscle wall and invaded adjacent structures such as the colon, pancreas, and skeletal muscle (Figure 3(a)). The *Pten*$^{d/d}$ mice displayed endometrial hyperplasia at 4 weeks of age (Figure 4). Although the *Pten*$^{d/d}$ mice have been previously shown to develop endometrial hyperplasia and endometrial cancer [20], they did not develop invasive endometrial cancer within 4 weeks of age. Thus, histological analysis showed that *Pten* ablation in conjunction with the oncogenic *K-ras* mutation dramatically accelerated the development of endometrial cancer compared to single ablation of either gene (Figure 4). These results suggest that *Pten* ablation in addition to the oncogenic *K-ras* mutation dramatically accelerated the development of endometrial cancer compared to single mutation of either gene.

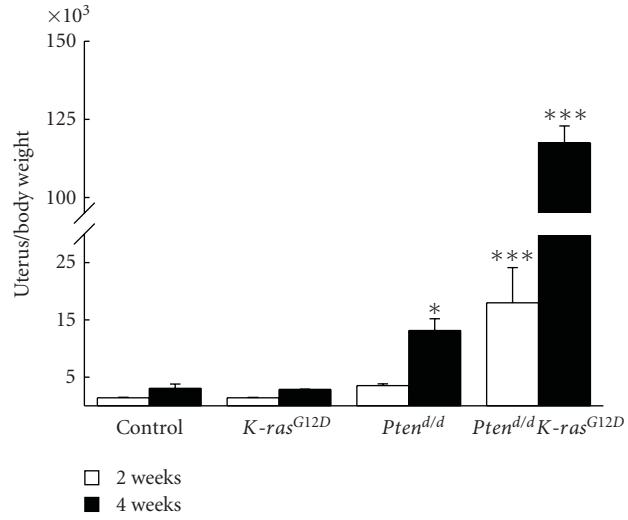
3.4. Down-regulation of P4 Signaling in Mice with Pten Ablation and the Oncogenic K-ras Mutation. Ablation of *Pten* resulted in increased activation of AKT as expected (Figure 5(a)). To determine if the hyperplastic phenotype observed was due to altered ovarian steroid hormone signaling, we examined the expression of ERα and PR in control, *K-ras*^{G12D}, *Pten*$^{d/d}$, and *Pten*$^{d/d}$*K-ras*^{G12D} uteri at 2 weeks of age using Western blot analysis. The expression of ERα was decreased in *Pten*$^{d/d}$ and *Pten*$^{d/d}$*K-ras*^{G12D} uteri compared to control and *K-ras*^{G12D} uteri (Figure 5(a)). Interestingly, the level of PR, both the PR-A and PR-B isoforms, was decreased only in the *Pten*$^{d/d}$ *K-ras*^{G12D} uteri compared to control, *K-ras*^{G12D}, and *Pten*$^{d/d}$ uteri at 2 weeks of age (Figure 5(a)).

To analyze the spatial expression of ERα and PR, we performed immunohistochemical analysis in control, *K-ras*^{G12D}, *Pten*$^{d/d}$, and *Pten*$^{d/d}$*K-ras*^{G12D} uteri at 2 weeks of age. The expression of ERα was decreased in the endometrial stroma of *Pten*$^{d/d}$ *K-ras*^{G12D} compared to *Pten*$^{d/d}$ mice. However, the level of ERα was increased in the epithelium of *Pten*$^{d/d}$*K-ras*^{G12D} compared to *Pten*$^{d/d}$ mice. These immunohistochemical results suggest that the decreased level of ERαin the whole uterus was due primarily to decreased ERα expression in the endometrial stroma (Figure 5(b)). The spatial expression of PR was altered in the *Pten*$^{d/d}$ uteri compared to control and *K-ras*^{G12D} mice. Instead of uniform expression throughout the endometrial epithelium, PR was localized to the distal regions of the epithelium in the *Pten*$^{d/d}$ uteri (Figure 5(c)). However, this strong PR expression was almost lost in the *Pten*$^{d/d}$*K-ras*^{G12D} mice.

We next investigated the molecular impact of *Pten* ablation and the oncogenic *K-ras* mutation on the expression of ER target genes (*Ltf, Clca3,* and *C3*) in control, *K-ras*^{G12D}, *Pten*$^{d/d}$, and *Pten*$^{d/d}$*K-ras*^{G12D} uteri at 2 weeks of age. *Ltf* and *C3* were increased in the *Pten*$^{d/d}$ mice as compared to control and *K-ras*^{G12D} mice (Figure 6). *Ltf, Clca3,* and *C3*



(a)



(b)

Figure 3: Development of endometrial cancer in mice with *Pten* ablation and oncogenic *K-ras* mutation. (a) Gross anatomy of control, $K\text{-}ras^{G12D}$, $Pten^{d/d}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ uteri at 2 and 4 weeks of age. Scale bar: 1 cm (b) The ratio of uterine weight to body weight in control, $K\text{-}ras^{G12D}$, $Pten^{d/d}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ mice at 2 and 4 weeks of age. Uterine weight was determined for females 2 and 4 weeks old. Increased uterine weight was observed for $Pten^{d/d}K\text{-}ras^{G12D}$ mice after 2 weeks of age. *, $P < .05$; ***, $P < .001$, one-way ANOVA followed by Tukey's post hoc multiple range test.

were significantly increased in the $Pten^{d/d}K\text{-}ras^{G12D}$ mice as compared to $Pten^{d/d}$ mice. Since P4 attenuates E2 regulation of proliferation and gene expression by regulating the expression of a yet to be identified paracrine signal from the stromal cells to the epithelial cells, the regulation of the expression of ERα and PR in the endometrial stroma and epithelium by *Pten* ablation and the oncogenic *K-ras* mutation is critical for the expression of ER target genes and the tumorigenesis of endometrial cancer.

4. Discussion

Endometrial cancer is the most common gynecological cancer and has been shown to be associated with mutations

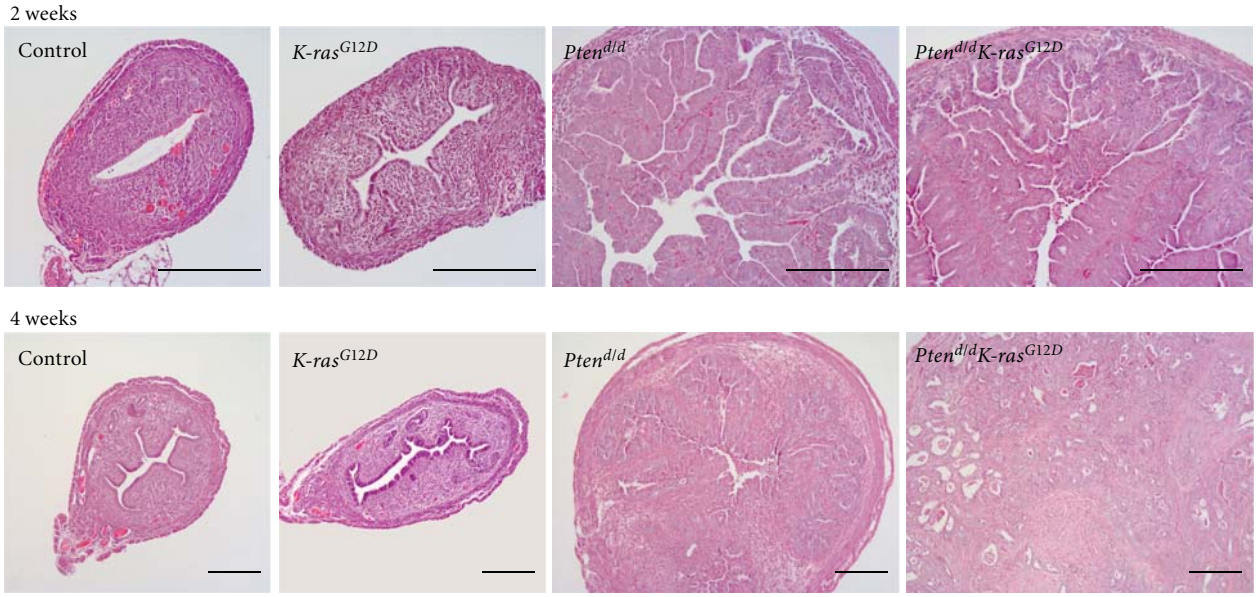


Figure 4: Histology of uteri from mice with *Pten* ablation and oncogenic *K-ras* mutation. H&E staining of control, $K\text{-}ras^{G12D}$, $Pten^{d/d}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ mice at 2 and 4 weeks of age. Endometrial cancer was induced in the uteri of $Pten^{d/d}K\text{-}ras^{G12D}$ mice, but not in other mice at 4 weeks of ages. Scale bar: 200 μm.

in the tumor suppressor *Pten* and the oncogene *K-ras* among others [4]. Previous mouse model studies in the skin, ovary, and lung suggest that these mutations exert cooperative or antagonistic effects on tumorigenesis depending upon their interactions with tissue-specific factors [28–30]. Dinulescu et al. generated *Pten* loss and oncogenic *K-ras* mutations in ovarian surface epithelium using adenoviral vector delivery of Cre recombinase [28]. These mice developed endometriosis and endometrioid ovarian adenocarcinoma. *K-ras* mutations occur in a very small percentage of human cases of ovarian cancer but the mutation is important for the development of ovarian cancer [31]. However, whether *Pten* loss and oncogenic *K-ras* mutations interact to promote or inhibit the development of endometrial cancer has not yet been defined. In order to study the role of *Pten* and *K-ras* in the development of endometrial cancer, we generated mice in which *Pten* was ablated and *K-ras* was activated in the reproductive tract using the PR^{Cre} mouse model [23–25].

PTEN is completely lost or mutated in >50% of primary endometrioid endometrial cancer [15] and in at least 20% of endometrial hyperplasias, the precancerous lesions of the endometrium [15, 16]. Thus, loss of *PTEN* is a very early event in the multistep process leading to endometrioid endometrial cancer. $Pten^{+/-}$ and mice with *Pten* conditionally ablated in the uterus ($Pten^{d/d}$) develop endometrioid endometrial adenocarcinoma [20, 32]. This mutation and subsequent Akt activation results in the activation of ERα-dependent pathways that play an important role in the tumorigenesis of endometrial cancer [21]. Introduction of the oncogenic *K-ras* mutation into the $Pten^{d/d}$ mice accelerated the tumorigenesis of endometrial cancer as compared to *Pten* ablation. The neoplastic endometrial glands in the double mutant mice invaded through the uterine muscle wall and invaded adjacent structures such as the colon, pancreas, and skeletal muscle.

The *K-ras* mutation alone was not sufficient enough to exert a pathological phenotype in the uterus even though mutations in *K-ras* have been consistently identified in 10%–30% of endometrial cancers [4]. Interestingly, $K\text{-}ras^{G12D}$ mice exhibited abnormal vaginal architecture due to increased keratinization in the vaginal epithelium resulting in vaginal papilloma. The development of vaginal papillomas confounds its impact on the tumorigenesis of endometrial cancer. Thus, we have only examined these mice up to 3 months of age without evidence of any hyperplasia or pathological phenotype in the uterus. To determine why endometrial cancer failed to develop in the $K\text{-}ras^{G12D}$ uteri, we examined proliferation and apoptosis in these mice. Proliferation was normal; however, apoptosis was significantly increased in the epithelial cells of $K\text{-}ras^{G12D}$ uteri compared to control mice as shown by the TUNEL assay (Figure 2(c)) and immunohistochemistry of cleaved caspase 3 (data not shown). These results suggest that activation in *K-ras* is not sufficient for the development of hyperplasia or endometrial cancer due to increased apoptosis of the endometrial epithelial cells.

Mutations in the β-catenin gene have been found in approximately 15%–20% of endometrioid carcinomas, with nuclear accumulation of the protein found in 38% of cases [33]. Subsets of endometrial carcinomas, especially those with nuclear translocation of β-catenin [34], are associated with squamous morule differentiation. Some of the tumors in $Pten^{d/d}$ mice exhibited squamous differentiation, which can also be observed in human endometrial cancers while we did not observed squamous differentiation in the tumors from $Pten^{d/d}K\text{-}ras^{G12D}$ mice (data not shown).

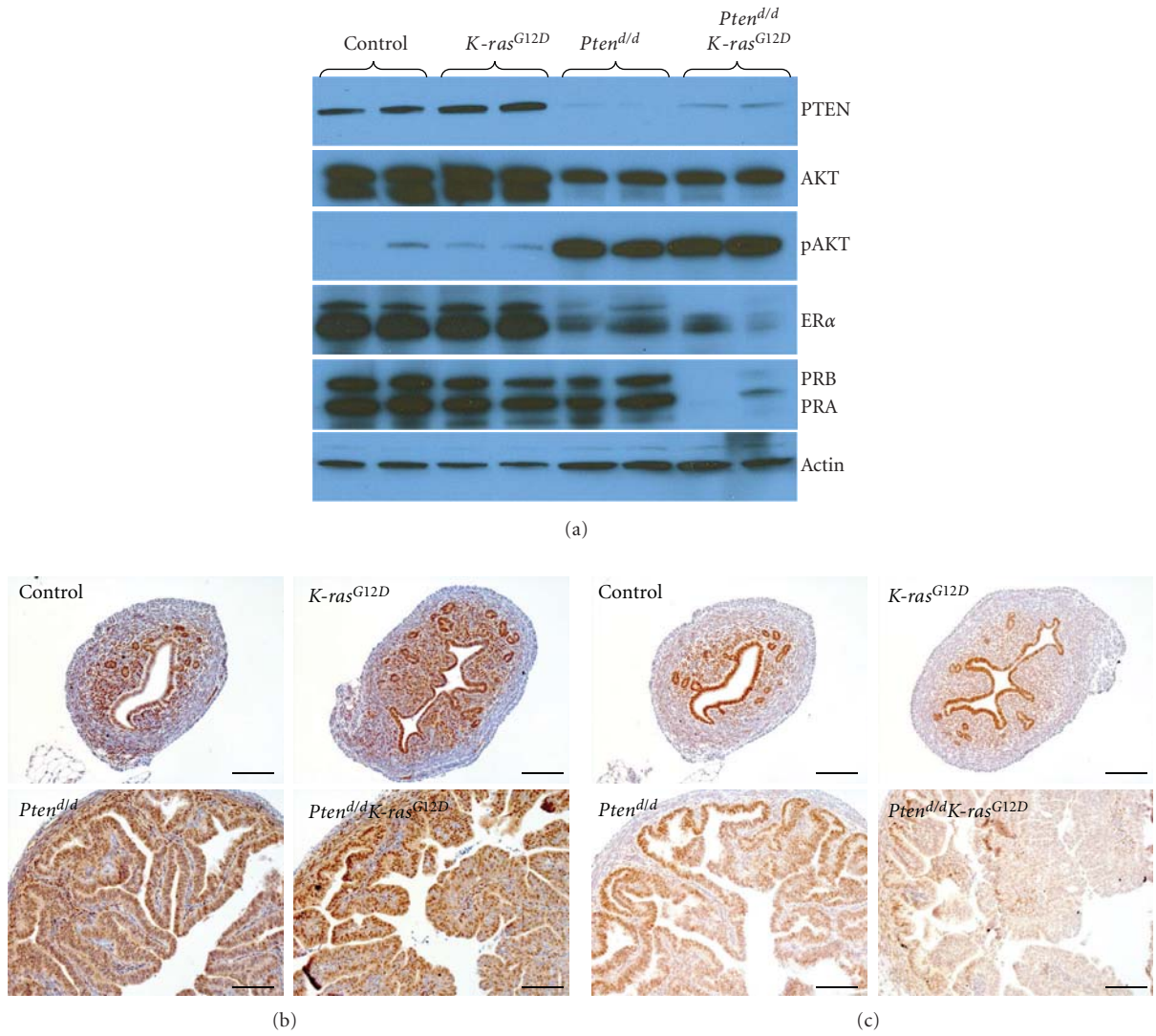


Figure 5: The decrease of PR in the 2-week-old $Pten^{d/d}K\text{-}ras^{G12D}$ mice. (a) Western blot analysis of *PTEN*, AKT, phosphor-AKT, ERα, and PR in 2 week old control, $K\text{-}ras^{G12D}$, $Pten^{d/d}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ mice. (b)-(c) Immunohistochemical analysis of ERα (b) and PR (c) in uteri of control, $K\text{-}ras^{G12D}$, $Pten^{d/d}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ mice. Immunohistochemical analysis of PR shows that it is decreased in the $Pten^{d/d}K\text{-}ras^{G12D}$ uteri compared to other mice. Scale bar: 100 μm.

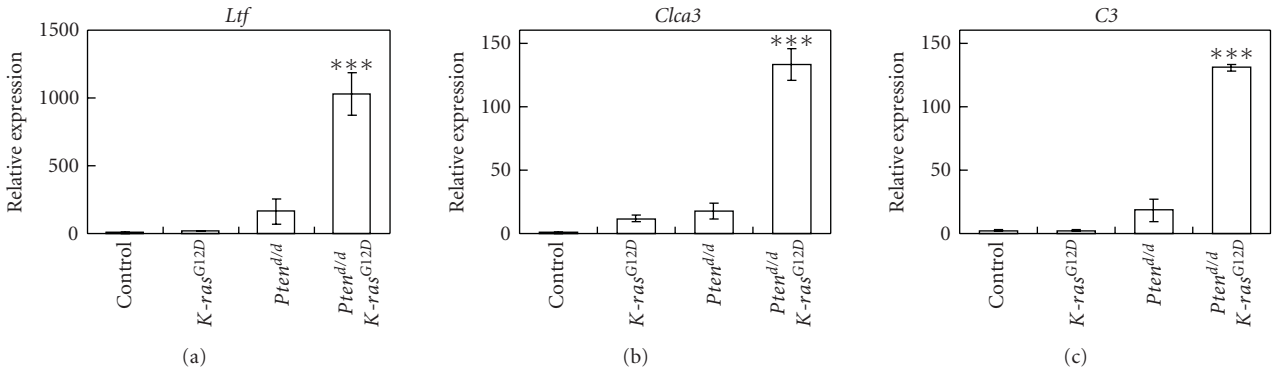


Figure 6: The increase of ERα signaling in the 2-week-old $Pten^{d/d}K\text{-}ras^{G12D}$ mice. Real-time RT-PCR analysis of *Ltf, Clca3,* and *C3* was performed in uteri of control, $K\text{-}ras^{G12D}$, $Pten^{d/d}$, and $Pten^{d/d}K\text{-}ras^{G12D}$ mice at 2 weeks of age. The results represent the mean ± SEM of three independent RNA sets. ***, $P < .001$, one-way ANOVA followed by Tukey's post hoc multiple range test.

Nuclear translocation of β-catenin was not observed in the *Pten*$^{d/d}$ mice and *Pten*$^{d/d}$*K-ras*^{G12D} mice (data not shown). It indicates that the histopathology of the endometrial cancer lesions in the mice is very similar to human endometrial cancer but does not totally resemble human endometrial cancer which is mainly composed of glandular components with squamous differentiation.

ER and PR are usually found in high concentration in endometrial hyperplasia and endometrioid carcinomas of low grade and stage. However, the level of ER and PR diminishes with increases in stage and grade [35, 36]. The level of total ERα was decreased in *Pten*$^{d/d}$ and *Pten*$^{d/d}$*K-ras*^{G12D} uteri compared to control and *K-ras*^{G12D} uteri using Western analysis (Figure 5(a)). The amount of epithelial cells in *Pten*$^{d/d}$ and *Pten*$^{d/d}$*K-ras*^{G12D} uteri is much higher than control and *K-ras*^{G12D} mice because of the hyperplasia phenotype. The amount of epithelial cells was different in the 4 different groups of the Western analysis because we have not purified epithelial cells and normalized to epithelial marker proteins. Therefore, we performed immunohistochemical analysis to determine these possible compartmental differences. The results demonstrated that the expression of ERα was decreased in the endometrial stroma but was increased in the endometrial epithelium of *Pten*$^{d/d}$*K-ras*^{G12D} compared to *Pten*$^{d/d}$ mice. These results support that epithelial ERα is important for the tumorigenesis of endometrial cancer [4].

E2 induces cell proliferation in the luminal and glandular epithelium. In the uterine luminal epithelium, E2 inhibits GSK3β action by the stimulation of a protein kinase B-(AKT)-mediated inhibitory phosphorylation of Ser9. AKT is in turn regulated through activation of phosphoinositide 3-kinase [37]. P4 inhibits this pathway by blocking AKT phosphorylation and, thus, the inactivation of GSK3β with the resultant loss of nuclear cyclin D1 [37]. PR status is considered an independent prognostic factor for endometrial cancer patients [38, 39]. PR exists as two isoforms, PR-A and PR-B, and reduced expression of either one or both of the PR isoforms has been observed in a great majority of endometrial cancers, compared with hyperplastic or normal endometrium [40]. Loss of PR in human endometrioid endometrial carcinoma results in more aggressive biological characteristics which play important roles in the prognosis and recurrence of the disease [40–42]. We observed reduced expression of both PR isoforms in *Pten*$^{d/d}$*K-ras*^{G12D} uteri, but not in *Pten*$^{d/d}$ or other control uteri. Thus, this loss of PR in the double mutant mice may be the reason for the accelerated tumorigenesis of the *Pten*$^{d/d}$*K-ras*^{G12D} mice as compared to other mice including the *Pten*$^{d/d}$ mice.

Our results demonstrate that the synergistic effect of conditional *Pten* loss and oncogenic *K-ras* mutation on endometrial cancer development occurs via decreased expression of PR. This study has established an endometrial cancer mouse model which replicates common characteristics of the human disease. Using this mouse model, further studies can be undertaken to investigate the genetic and molecular events involved in the transition from normal to hyperplastic/neoplastic endometrium. In summary, these results greatly contribute to the understanding of the molecular mechanism of tumorigenesis and to the development of therapeutic approaches for endometrial cancer.

Acknowledgments

The authors thank Jinghua Li and Jie Yang for technical assistance. They thank Dr. Hong Wu for floxed *Pten* mice and Dr. Tyler Jacks for *K-ras*^{G12D} mice. The anti-PR antibody was kindly provided by Dr. Dean Edwards. This work was supported by the NIH Grant R01HD042311 and U54HD0077495 (to F. J. D.), SPORE in Uterine Cancer NIH P50CA098258 (to R. R. B.), NIH Grant R01HD057873 (to J. W. J.), and NIH Grant R01-CA77530 and the Susan G. Komen Award BCTR0503763 (to J. P. L).

References

[1] A. Jemal, R. Siegel, E. Ward, et al., "Cancer statistics, 2008," *CA: Cancer Journal for Clinicians*, vol. 58, no. 2, pp. 71–96, 2008.

[2] S. S. Jick, A. M. Walker, and H. Jick, "Estrogens, progesterone, and endometrial cancer," *Epidemiology*, vol. 4, no. 1, pp. 20–24, 1993.

[3] H. K. Ziel and W. D. Finkle, "Increased risk of endometrial carcinoma among users of conjugated estrogens," *New England Journal of Medicine*, vol. 293, no. 23, pp. 1167–1170, 1975.

[4] A. Di Cristofano and L. H. Ellenson, "Endometrial carcinoma," *Annual Review of Pathology*, vol. 2, pp. 57–85, 2007.

[5] J. L. Hecht and G. L. Mutter, "Molecular and pathologic aspects of endometrial carcinogenesis," *Journal of Clinical Oncology*, vol. 24, no. 29, pp. 4783–4791, 2006.

[6] S. S. Jick, "Combined estrogen and progesterone use and endometrial cancer," *Epidemiology*, vol. 4, no. 4, p. 384, 1993.

[7] L. Martin, C. A. Finn, and G. Trinder, "Hypertrophy and hyperplasia in the mouse uterus after oestrogen treatment: an autoradiographic study," *Journal of Endocrinology*, vol. 56, no. 1, pp. 133–144, 1973.

[8] Y. M. Huet-Hudson, G. K. Andrews, and S. K. Dey, "Cell type-specific localization of c-myc protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period," *Endocrinology*, vol. 125, no. 3, pp. 1683–1690, 1989.

[9] B. C. Paria, Y. M. Huet-Hudson, and S. K. Dey, "Blastocyst's state of activity determines the 'window' of implantation in the receptive mouse uterus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 21, pp. 10159–10162, 1993.

[10] L. Martin, R. M. Das, and C. A. Finn, "The inhibition by progesterone of uterine epithelial proliferation in the mouse," *Journal of Endocrinology*, vol. 57, no. 3, pp. 549–554, 1973.

[11] T. Kurita, K.-J. Lee, P. S. Cooke, J. P. Lydon, and G. R. Cunha, "Paracrine regulation of epithelial progesterone receptor and lactoferrin by progesterone in the mouse uterus," *Biology of Reproduction*, vol. 62, no. 4, pp. 831–838, 2000.

[12] M. Esteller, A. Garcia, J. M. Martinez-Palones, J. Xercavins, and J. Reventos, "The clinicopathological significance of K-RAS point mutation and gene amplification in endometrial cancer," *European Journal of Cancer*, vol. 33, no. 10, pp. 1572–1577, 1997.

[13] P. A. Steck, M. A. Pershouse, S. A. Jasser, et al., "Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers," *Nature Genetics*, vol. 15, no. 4, pp. 356–362, 1997.

[14] D.-M. Li and H. Sun, "TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β," *Cancer Research*, vol. 57, no. 11, pp. 2124–2129, 1997.

[15] H. Sun, T. Enomoto, M. Fujita, et al., "Mutational analysis of the PTEN Gene in endometrial carcinoma and hyperplasia," *American Journal of Clinical Pathology*, vol. 115, no. 1, pp. 32–38, 2001.

[16] R. L. Levine, C. B. Cargile, M. S. Blazes, B. van Rees, R. J. Kurman, and L. H. Ellenson, "PTEN mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma," *Cancer Research*, vol. 58, no. 15, pp. 3254–3258, 1998.

[17] G. L. Maxwell, J. I. Risinger, C. Gumbs, et al., "Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias," *Cancer Research*, vol. 58, no. 12, pp. 2500–2503, 1998.

[18] I. Vivanco and C. L. Sawyers, "The phosphatidylinositol 3-kinase-AKT pathway in human cancer," *Nature Reviews Cancer*, vol. 2, no. 7, pp. 489–501, 2002.

[19] B.-H. Jiang and L.-Z. Liu, "PI3K/PTEN signaling in tumorigenesis and angiogenesis," *Biochimica et Biophysica Acta*, vol. 1784, no. 1, pp. 150–158, 2008.

[20] T. Daikoku, Y. Hirota, S. Tranguch, et al., "Conditional loss of uterine Pten unfailingly and rapidly induces endometrial cancer in mice," *Cancer Research*, vol. 68, no. 14, pp. 5619–5627, 2008.

[21] A. Vilgelm, Z. Lian, H. Wang, et al., "Akt-mediated phosphorylation and activation of estrogen receptor α is required for endometrial neoplastic transformation in Pten+/− mice," *Cancer Research*, vol. 66, no. 7, pp. 3375–3380, 2006.

[22] K. L. Chambliss, I. S. Yuhanna, R. G. W. Anderson, M. E. Mendelsohn, and P. W. Shaul, "Erβ has nongenomic action in caveolae," *Molecular Endocrinology*, vol. 16, no. 5, pp. 938–946, 2002.

[23] S. M. Soyal, A. Mukherjee, K. Y. S. Lee, et al., "Cre-mediated recombination in cell lineages that express the progesterone receptor," *Genesis*, vol. 41, no. 2, pp. 58–66, 2005.

[24] R. Lesche, M. Groszer, J. Gao, et al., "Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene," *Genesis*, vol. 32, no. 2, pp. 148–149, 2002.

[25] L. Johnson, K. Mercer, D. Greenbaum, et al., "Somatic activation of the K-ras oncogene causes early onset lung cancer in mice," *Nature*, vol. 410, no. 6832, pp. 1111–1116, 2001.

[26] A. Di Cristofano, B. Pesce, C. Cordon-Cardo, and P. P. Pandolfi, "Pten is essential for embryonic development and tumour suppression," *Nature Genetics*, vol. 19, no. 4, pp. 348–355, 1998.

[27] D. A. Tuveson, A. T. Shaw, N. A. Willis, et al., "Endogenous oncogenic K-rasG12D stimulates proliferation and widespread neoplastic and developmental defects," *Cancer Cell*, vol. 5, no. 4, pp. 375–387, 2004.

[28] D. M. Dinulescu, T. A. Ince, B. J. Quade, S. A. Shafer, D. Crowley, and T. Jacks, "Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer," *Nature Medicine*, vol. 11, no. 1, pp. 63–70, 2005.

[29] K. Iwanaga, Y. Yang, M. G. Raso, et al., "Pten inactivation accelerates oncogenic K-ras-initiated tumorigenesis in a mouse model of lung cancer," *Cancer Research*, vol. 68, no. 4, pp. 1119–1127, 2008.

[30] J.-H. Mao, M. D. To, J. Perez-Losada, D. Wu, R. Del Rosario, and A. Balmain, "Mutually exclusive mutations of the Pten and ras pathways in skin tumor progression," *Genes and Development*, vol. 18, no. 15, pp. 1800–1805, 2004.

[31] R. J. Kurman and I.-M. Shih, "Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications," *International Journal of Gynecological Pathology*, vol. 27, no. 2, pp. 151–160, 2008.

[32] Z. Lian, P. De Luca, and A. Di Cristofano, "Gene expression analysis reveals a signature of estrogen receptor activation upon loss of Pten in a mouse model of endometrial cancer," *Journal of Cellular Physiology*, vol. 208, no. 2, pp. 255–266, 2006.

[33] T. Fukuchi, M. Sakamoto, H. Tsuda, K. Maruyama, S. Nozawa, and S. Hirohashi, "β-catenin mutation in carcinoma of the uterine endometrium," *Cancer Research*, vol. 58, no. 16, pp. 3526–3528, 1998.

[34] M. Saegusa, M. Hashimura, T. Kuwata, M. Hamano, and I. Okayasu, "Upregulation of TCF4 expression as a transcriptional target of β-catenin/p300 complexes during transdifferentiation of endometrial carcinoma cells," *Laboratory Investigation*, vol. 85, no. 6, pp. 768–779, 2005.

[35] G. Emons, G. Fleckenstein, B. Hinney, A. Huschmand, and W. Heyl, "Hormonal interactions in endometrial cancer," *Endocrine-Related Cancer*, vol. 7, no. 4, pp. 227–242, 2000.

[36] W. Kleine, T. Maier, H. Geyer, and A. Pfleiderer, "Estrogen and progesterone receptors in endometrial cancer and their prognostic relevance," *Gynecologic Oncology*, vol. 38, no. 1, pp. 59–65, 1990.

[37] B. Chen, H. Pan, L. Zhu, Y. Deng, and J. W. Pollard, "Progesterone inhibits the estrogen-induced phosphoinositide 3-kinase → AKT → GSK-3β → cyclin D1 → pRB pathway to block uterine epithelial cell proliferation," *Molecular Endocrinology*, vol. 19, no. 8, pp. 1978–1990, 2005.

[38] S. K. Mohsin, H. Weiss, T. Havighurst, et al., "Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study," *Modern Pathology*, vol. 17, no. 12, pp. 1545–1554, 2004.

[39] P. G. Rose, "Endometrial carcinoma," *New England Journal of Medicine*, vol. 335, no. 9, pp. 640–649, 1996.

[40] R. L. Arnett-Mansfield, A. deFazio, G. V. Wain, et al., "Relative expression of progesterone receptors A and B in endometrioid cancers of the endometrium," *Cancer Research*, vol. 61, no. 11, pp. 4576–4582, 2001.

[41] K. Fukuda, M. Mori, M. Uchiyama, K. Iwai, T. Iwasaka, and H. Sugimori, "Prognostic significance of progesterone receptor immunohistochemistry in endometrial carcinoma," *Gynecologic Oncology*, vol. 69, no. 3, pp. 220–225, 1998.

[42] K. Ito, H. Utsunomiya, N. Yaegashi, and H. Sasano, "Biological roles of estrogen and progesterone in human endometrial carcinoma—new developments in potential endocrine therapy for endometrial cancer," *Endocrine Journal*, vol. 54, no. 5, pp. 667–679, 2007.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 364508, 6 pages
doi:10.1155/2010/364508

Research Article

Lack of a Y-Chromosomal Complement in the Majority of Gestational Trophoblastic Neoplasms

Kai Lee Yap,[1,2] Michael J. Hafez,[1] Tsui-Lien Mao,[3] Robert J. Kurman,[1,4] Kathleen M. Murphy,[1] and Ie-Ming Shih[1,2,4]

[1] *Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA*
[2] *Pathobiology Graduate Program, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA*
[3] *Department of Pathology, National Taiwan University Hospital, Taipei 100, Taiwan*
[4] *Department of Gynecology, Obstetrics and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA*

Correspondence should be addressed to Ie-Ming Shih, shihie@yahoo.com

Received 16 December 2009; Accepted 4 January 2010

Academic Editor: Tian-Li Wang

Gestational trophoblastic neoplasms (GTNs) are a rare group of neoplastic diseases composed of choriocarcinomas, placental site trophoblastic tumors (PSTTs) and epithelioid trophoblastic tumors (ETTs). Since these tumors are derivatives of fetal trophoblastic tissue, approximately 50% of GTN cases are expected to originate from a male conceptus and carry a Y-chromosomal complement according to a balanced sex ratio. To investigate this hypothesis, we carried out a comprehensive analysis by genotyping a relatively large sample size of 51 GTN cases using three independent sex chromosome genetic markers; Amelogenin, Protein Kinase and Zinc Finger have X and Y homologues that are distinguishable by their PCR product size. We found that all cases contained the X-chromosomal complement while only five (10%) of 51 tumors harbored the Y-chromosomal complement. Specifically, Y-chromosomal signals were detected in one (5%) of 19 choriocarcinomas, one (7%) of 15 PSTTs and three (18%) of 17 ETTs. The histopathological features of those with a Y-chromosome were similar to those without. Our results demonstrate the presence of a Y-chromosomal complement in GTNs, albeit a low 10% of cases. This shortfall of Y-chromosomal complements in GTNs may reinforce the notion that the majority of GTNs are derived from previous molar gestations.

1. Introduction

Gestational trophoblastic neoplasms (GTNs) represent a unique group of human tumors that develop as a semiallograft from fetus-derived tissues [1]. GTNs were originally considered a homogeneous group of diseases arising from the neoplastic transformation of trophoblastic cells. However, recent clinicopathological studies have provided evidence that there are at least three distinctive types of GTNs including the most common type, choriocarcinomas, and the less common placental site trophoblastic tumors (PSTTs) and epithelioid trophoblastic tumors (ETTs). GTNs have been proposed to develop from trophoblastic stem cells, presumably the cytotrophoblastic cells, and the patterns of differentiation in GTNs recapitulate the early stages of placental development [1]. According to this view, choriocarcinomas are composed of variable amounts of neoplastic cytotrophoblasts, syncytiotrophoblasts, and extravillous (intermediate) trophoblasts, resembling the previllous blastocyst which is composed of a similar mixture of trophoblastic subpopulations. In contrast, the neoplastic cytotrophoblasts in PSTTs differentiate mainly into extravillous (intermediate) trophoblastic cells; whereas the neoplastic cytotrophoblasts in ETTs differentiates into chorionic-type extravillous (intermediate) trophoblastic cells. According to this model, choriocarcinomas are the most primitive trophoblastic tumors, whereas PSTTs and ETTs are relatively more differentiated.

Clinically, choriocarcinoma is a highly malignant epithelial tumor arising from the trophoblasts of any type of gestational event, most often a complete hydatidiform mole [2]. Patients are in their reproductive age and present with abnormal vaginal bleeding and occasionally signs of distant metastasis. Microscopically, it predominantly consists of a biphasic proliferation of mononucleate trophoblasts

and syncytiotrophoblasts, accompanied by prominent hemorrhage and necrosis [3]. With the advent of modern chemotherapy, the overall survival for patients with choriocarcinomas currently approaches 100% [4] although some patients develop nonoperable and chemoresistant recurrent disease. As compared to choriocarcinomas, PSTTs and ETTs are rare [5–7]. Like choriocarcinomas, PSTTs and ETTs occur in women of reproductive age and present with either amenorrhea or abnormal bleeding [5, 7, 8]. Despite deep myometrial invasion, most cases of PSTTs and ETTs are successfully treated [2] but approximately 10–15% are clinically malignant and have a fatal outcome. Histologically, PSTTs are characterized by masses or sheets of intermediate trophoblastic cells resembling implantation site intermediate trophoblasts, while ETTs are characterized by chorionic-type intermediate trophoblasts of the normal implantation site and placenta. In addition to distinct morphological features, both PSTTs and ETTs are characterized by unique gene expression patterns, suggesting that the molecular pathogeneses of PSTTs and ETTs are dissimilar [9].

Hydatidiform moles [10, 11] are precursor lesions of numerous cases of GTNs. Previous clinicopathological and molecular studies have provided fundamental insight into the pathogenesis of hydatidiform moles but the molecular and cellular basis for the development of GTNs remain poorly understood. A similar number of GTN cases with and without a Y-chromosome are expected if sex chromosomes play no role in the development of GTNs. On the contrary, more than 85% of patients with PSTTs were found by history records or genetic analysis to have had a female antecedent gestation. Moreover, a recent study using the Amelogenin assay demonstrated the presence of a X-chromosome and absence of a Y in a small series of PSTTs [12], raising the possibility that a Y-chromosomal complement may be preferentially deleted in PSTTs. In this paper we describe our findings in a larger number of PSTTs, as well as other types of GTNs including choriocarcinomas and ETTs. In addition, unlike previous studies using a single marker, we examined a total of three genetic markers including the commonly used amelogenin. These genes have X and Y homologues that can be distinguished by their polymerase chain reaction (PCR) product sizes using specific primer pairs, to detect the presence of a Y allele.

2. Methods

2.1. Tissue Specimens. Paraffin tissues from a total of 51 GTNs were retrieved from the archival files in the Department of Pathology at the Johns Hopkins Hospital, USA. Most of the specimens were consultation cases sent to two of the authors (R. J. Kurman and I. M. Shih). Hematoxylin and eosin stained sections from tissue specimens were reviewed and the diagnosis of specific types of GTNs were confirmed by an expert gynecologic pathologist (I. M. Shih). The specimens included 15 PSTTs, 17 ETTs, and 19 choriocarcinomas. All the specimens were anonymized and thus clinical information was not obtained. Tissues collection was conducted in compliance with institutional review board regulations. The tumor areas on paraffin sections were carefully dissected from the surrounding normal (maternal) tissues on hematoxylin-stained tissue sections. Genomic DNA was prepared by using the Formapure kit (Agencourt, Cambridge, MA). One representative tissue block was selected for DNA extraction except for five cases in which the DNA was purified from two separate tissue blocks.

Table 1: The primer sequences used to amplify the *PRK* and *ZF* loci.

Primer Name	Sequence
PRK-F	5′ FAM-TTTTGTTTCTTTCTGTCCATACTTAAAG 3′
PRK-R	5′ TCCCAAACCACTCAACTG 3′
ZF-F	5′ FAM-TGTGCATAACTTTGTTCCTGATG 3′
ZF-R	5′ AGCACTTGCTCAGGAATGATG 3′

2.2. Genotyping Using Sex Chromosome-Specific Genetic Markers. The presence of either a X or a Y-chromosome in GTNs was determined by the analysis of three genes that have X and Y-chromosomal homologues distinguishable by their PCR product size with specific primer sets; *Amelogenin* X and Y (AMELX and AMELY), *Protein Kinase* X and Y (*PRKX* and *PRKY*), and *Zinc Finger* X and Y (*ZFX* and *ZFY*). The amelogenin gene has X and Y homologues located on Xp22.1–22.3 (*AMELX*) and Yp11.2 (*AMELY*), which are differentiated using a primer pair that amplifies a region of intron 1 which spans a 6-base pair deletion in *AMELX* as compared to *AMELY*. The Amelogenin analysis was performed using the commercially available AmpFlSTR Profiler kit (Applied Biosystems, Foster City, CA). Thermal cycling conditions and capillary electrophoresis were carried out according to the manufacturer's instructions. Briefly, the PCR conditions were 95°C for 11 minutes followed by 28 cycles of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute, followed by a final extension at 60°C for 45 minutes. After amplification, capillary electrophoresis was carried out using 1 μl of multiplex PCR product, mixed with 9 μl of deionized formamide/GeneScan 500 (ROX) size standard (Applied Biosystems). Samples were then denatured at 95°C for 2 minutes before analysis on the ABI3100 Genetic Analyzer (Applied Biosystems).

Although the Amelogenin-based sex chromosome assay has been frequently used in basic research and forensic medicine, a false negative result for the detection of a Y-chromosome has been documented when tumors of high genomic instability are analyzed [13]. Therefore, we analyzed two additional genes with X and Y homologues. The *PRK* gene has its X and Y homologues located on Xp22.3 (*PRKX*) and Yp11.2 (*PRKY*) respectively. The *PRKY* gene is located approximately 0.35 Mb centromeric to *AMELY*. To differentiate *PRKX* and *PRKY*, we designed a PCR reaction to amplify exon 8 of the *PRKX* and *PRKY* genes, using a primer set that spans a three-base pair deletion (Table 1) [14]. The *PRKY* amplification product is three bases shorter than the *PRKX* product. The *ZF* gene has X and Y homologues located at Xp22.1 (*ZFX*) and Yp11.2 (*ZFY*), respectively.

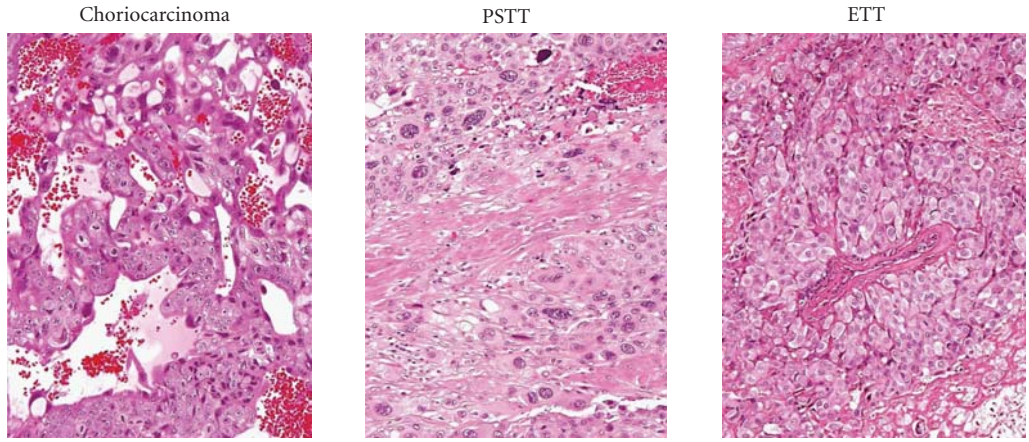


Figure 1: Histological features of gestational trophoblastic neoplasms. Choriocarcinoma is characterized by biphasic growth pattern composed of syncytiotrophoblast and mononucleate trophoblastic cells, forming vasculogenic mimicry. Placental site trophoblastic tumor (PSTT) is composed of confluent masses of neoplastic intermediate (extravillous) trophoblastic cells, infiltrating within smooth muscle cells. Epithelioid trophoblastic tumor (ETT) contains neoplastic chorionic-type intermediate (extravillous) trophoblastic cells surrounding an artery.

ZFY is located approximately 3.9 Mb telomeric to *AMELY*. To differentiate *ZFX* and *ZFY*, we designed a PCR reaction to amplify exon 3 of the *ZFX* and *ZFY* genes, with the primer set also spanning a 3-base pair deletion. In this case, the *ZFX* product is 3 bases shorter than the *ZFY* product. PCR amplifications were carried out and the primer sequences were listed in Table 1. Reactions were thermal cycled using the touchdown protocol: 1 cycle of 95°C for 2 minutes, 3 cycles each of 94°C for 30 seconds, 64°C, 61°C, or 58°C for 30 seconds, and 70°C for 30 seconds. This was followed by 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 70°C for 30 seconds, and 1 cycle of 70°C for 5 minutes. Products were analyzed by capillary electrophoresis as described above.

3. Results and Discussion

A total of 51 GTNs were histologically reviewed, including 19 choriocarcinomas, 17 ETTs, and 15 PSTTs (Table 2). Of these samples, ETT17 contained a small area of choriocarcinoma and PSTT15 contained a focal ETT component. Representative histologic features of the GTNs are illustrated in Figure 1. All cases yielded informative results in at least one of the gene markers utilized for the sex chromosome genotyping. We found that all informative cases contained a X-chromosomal complement while only five (10%, 95% CI: 18.2%–1.8%) of 51 tumors harbored a Y chromosome complement (Table 2). Specifically, Y-chromosomal signals were detected in one (5%) of 19 choriocarcinomas, one (7%) of 15 PSTTs, and three (18%) of 17 ETTs (Table 2). Figure 2 illustrates the genotypes in representative specimens. Of note, the genotypes were identical in genomic DNA obtained from different tissue blocks of the same case. For those specimens with a Y-chromosome, all three gene markers revealed consistent outcomes, although the relative abundance of a Y gene versus an X gene varied. For example, PSTT5 showed a small Y peak in both *amelogenin* and *ZF* loci that could make the Y assignment equivocal (Figure 2). However, by analyzing *PRKY*, we clearly detected a robust *PRKY* peak from the same specimen. Similarly, ETT12 contained a relatively small amelogenin Y peak but had significantly large peaks at both *PRKY* and *ZFY*. These findings indicate the variable efficiency of primers that amplify the different Y loci of the three genes on formalin-fixed paraffin tissues and underscore the importance to include additional markers to assess the presence of Y-chromosomal elements. The histopathological features in those tumors with a Y-chromosome were indistinguishable from those without a Y-chromosome. The percentage of cases showing Y peaks is listed in Table 3.

The lack of a Y-chromosomal complement in the majority of GTNs is intriguing and several theories can account for this phenomenon. The most likely cause of the phenomenon is that Y-chromosomal deletions have no functional effects on tumor progression [15]. In this case, the absence of Y chromosome in GTNs may simply reflect the fact that many GTNs develop from complete hydatidiform moles of which approximately 90% contain a karyotype of 46,XX due to fertilization of an "empty" ovum (without nucleus) by a single haploid (23X) sperm followed by haploid genome duplication [10, 11]. Thus, the GTNs that develop from complete hydatidiform moles retain the same sex chromosome assignment as their precursors and do not harbor a Y-chromosome. While 90% of complete hydatidiform moles arise from monospermy, approximately 10% are due to fertilization of an empty ovum with two sperm. Half of these cases that arise from dispermy would be expected to carry a Y-chromosome. Thus it could be predicted that approximately 5% of complete hydatidiform

Table 2: The sex chromosome assignment in all the GTN samples.

Case	Diagnosis	AME	PRK	ZF	Y peak
CC1	CC	XX	XX	XX	no
CC2	CC	XX	NA	XX	no
CC3	CC	XX	XX	XX	no
CC4	CC	XX	XX	XX	no
CC5	CC	XX	NA	XX	no
CC6	CC	NA	NA	XX	no
CC7	CC	XX	NA	NA	no
CC8	CC	XX	NA	XX	no
CC9	CC	XX	XX	XX	no
CC10	CC	XX	XX	XX	no
CC11	CC	XY	NA	NA	**yes**
CC12	CC	XX	XX	XX	no
CC13	CC	XX	XX	XX	no
CC14	CC	NA	NA	XX	no
CC15	CC	NA	XX	NA	no
CC16	CC	XX	NA	XX	no
CC17	CC	XX	XX	XX	no
CC18	CC	XX	XX	NA	no
CC19	CC	XX	XX	XX	no
ETT1	ETT	XX	XX	XX	no
ETT2	ETT	XX	XX	XX	no
ETT3	ETT	XX	NA	XX	no
ETT4	ETT	XX	XX	XX	no
ETT5	ETT	XX	XX	XX	no
ETT6	ETT	XX	XX	XX	no
ETT7	ETT	NA	XX	XX	no
ETT8	ETT	XX	XX	XX	no
ETT9	ETT	XX	XX	XX	no
ETT10	ETT	XX	XX	XX	no
ETT11	ETT	XX	XX	NA	no
ETT12	ETT	XY*	XY	XY	**yes**
ETT13	ETT	XX	XX	XX	no
ETT14	ETT	XY	XY	XY	**yes**
ETT15	ETT	XX	NA	XX	no
ETT16	ETT	XX	XX	XX	no
ETT17	ETT + CC	XY*	XY*	XY*	**yes**
PSTT1	PSTT	XX	XX	XX	no
PSTT2	PSTT	NA	NA	XX	no
PSTT3	PSTT	XX	XX	XX	no
PSTT4	PSTT	XX	XX	XX	no
PSTT5	PSTT	XY*	XY	XY*	**yes**
PSTT6	PSTT	NA	NA	XX	no
PSTT7	PSTT	NA	XX	XX	no
PSTT8	PSTT	XX	NA	XX	no
PSTT9	PSTT	XX	NA	XX	no
PSTT10	PSTT	NA	XX	XX	no
PSTT11	PSTT	NA	NA	XX	no
PSTT12	PSTT	XX	XX	XX	no
PSTT13	PSTT	XX	XX	NA	no
PSTT14	PSTT	XX	XX	XX	no
PSTT15	PSTT + ETT	XX	XX	XX	no

CC: CHORIOCARCINOMA, ETT: EPITHELIOID TROPHOBLASTIC TUMOR, PSTT: PLACENTAL SITE TROPHOBLASTIC TUMOR.

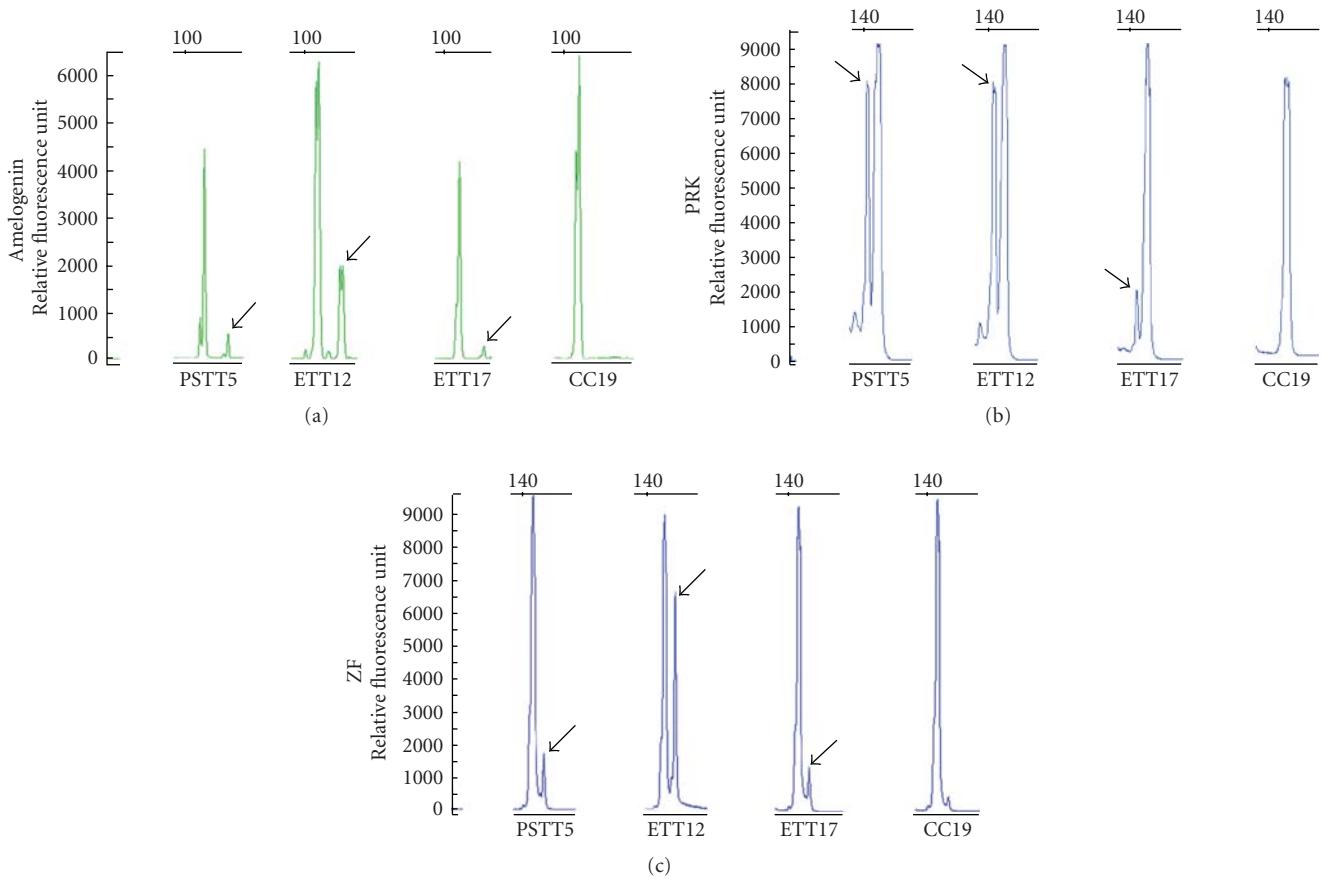


Figure 2: Genotypes in representative trophoblastic tumor specimens. The presence of either the X or Y-chromosome in GTNs was determined by the analysis of three genes that have X and Y homologues distinguishable by their PCR product size; *Amelogenin* X and Y (*AMELX* and *AMELY*), *Protein Kinase* X and Y (*PRKX* and *PRKY*), and *Zinc Finger* X and Y (*ZFX* and *ZFY*). Arrows denote the Y chromosomal peaks.

Table 3: Summary of percentage of tumor cases positive for a Y allele for at least one marker.

	Choriocarcinoma	ETT	PSTT
Total case no.	19	17	15
With Y peaks	1	3	1
% with Y peaks	5.3%	17.6%	6.7%
CI (95%)	15.4%–0%	35.7%–0%	19.4%–0%

ETT: epithelioid trophoblastic tumor; PSTT: placental site trophoblastic tumor; CI: confidence interval.

moles, and their resulting choriocarcinomas, would carry a Y-chromosome, which is exactly the percentage we obtained in this study.

Although the above represents our favorite view, other interpretations should also be indicated. It is possible that Y-chromosome deletions have a functional implication in the development of GTNs. In addition to GTNs developing from trophoblastic cells of a female conceptus, it can be speculated that GTNs arising from trophoblastic cells of a male conceptus will undergo clonal selection of trophoblastic cells with a deleted Y-chromosome due to their underlying genomic instability. In both scenarios, it is assumed that the presence of a Y-chromosome is not compatible with tumor initiation, possibly due to potential growth-inhibitory effects conferred by the products of genes located in the Y-chromosome. In support of this notion is the observation of a small but unambiguous Y peak of *AMELY*, *PRKY* and *ZFY* in the carefully dissected ETT17 (Figure 2). Also, previous reports have demonstrated Y-chromosome loss in several types of human cancer including prostate carcinoma, renal cell carcinoma, acute promyelocytic leukemia, and head and neck squamous carcinoma [15–18].

Lastly, the lack of Y-chromosome detection in other studies may be the result of micro-deletions in the Y-chromosomal regions analyzed, yielding a false negative result. This technical pitfall has been well documented in solid tumors when the amelogenin-based assay was applied [13]. To overcome this problem, in this study we have included two additional gene markers, *PRK* and *ZF*, along with the standard amelogenin test. Similar to the *Amelogenin (AMEL)* locus, the *PRK*, and *ZF* genes have X and Y homologues located on Xp (*PRKX* and *ZFX*) and Yp (*ZFX* and *ZFY*). The *PRKY* and *ZFY* are located 3.9 Mb telomeric to *AMELY* and 0.35 Mb centromeric to *AMELY*,

respectively. The failure to detect any of the three genes of the Y chromosome derives a more definitive conclusion and suggests that the absence of Y-chromosome is not likely due to somatic micro-deletions or microsatellite instability of the Y-chromosome-associated loci in GTNs.

Among 51 GTNs analyzed, we detected Y alleles in five tumors based on the presence of Y peaks in at least one of the *AMELY*, *PRKY* and *ZFY* loci. Among these five tumors was a PSTT. This finding is in contrast to a previous report demonstrating that none of 13 PSTTs harbored the *AMELY* [12]. The discrepancy is likely explained by the larger sample size and the additional Y markers employed in this study. The conclusion from the current study is also different from our previous report showing that approximately half of PSTTs and ETTs contained the sex-determining region Y (SRY) on Y chromosome [19]. In that study, a high cycle number of PCR amplification was used in order to detect a limited source of genomic DNA from paraffin tissues, raising the possibility of nonspecific amplification from contaminants. Thus, we believe that the results from the current study are more definitive in determining the sex chromosome assignment of GTNs.

In conclusion, this study provides a comprehensive analysis of sex chromosome distributions in all types of GTNs using three independent gene markers with differing PCR product lengths in the X and Y-chromosomes when specific primer pairs are used. Our results, based on a relatively large number of cases, clearly demonstrate the presence of a distinct but low Y-chromosomal complement in choriocarcinomas, PSTTs, and ETTs, that contributes to an overall figure of approximately 10%. It is most likely that the shortfall of Y chromosomal complements in GTNs may simply be due to the genetic basis of their precursor lesions, complete hydatidiform moles in which the majority of cases had the genotype of XX [20]. In conclusion, our results suggest that the majority of GTNs are preceded by antecedent complete molar pregnancy, many of which may be under recognized as the early complete moles usually lack the characteristic histopathological features.

References

[1] I.-M. Shih, "Gestational trophoblastic neoplasia-pathogenesis and potential therapeutic targets," *Lancet Oncology*, vol. 8, no. 7, pp. 642–650, 2007.

[2] I.-M. Shih and R. J. Kurman, "The pathology of intermediate trophoblastic tumors and tumor-like lesions," *International Journal of Gynecological Pathology*, vol. 20, no. 1, pp. 31–47, 2001.

[3] T.-L. Mao, R. J. Kurman, C.-C. Huang, M.-C. Lin, and I.-M. Shih, "Immunohistochemistry of choriocarcinoma: an aid in differential diagnosis and in elucidating pathogenesis," *American Journal of Surgical Pathology*, vol. 31, no. 11, pp. 1726–1732, 2007.

[4] A. Ilancheran, "Optimal treatment in gestational trophoblastic disease," *Annals of the Academy of Medicine Singapore*, vol. 27, no. 5, pp. 698–704, 1998.

[5] A. Hassadia, A. Gillespie, J. Tidy, et al., "Placental site trophoblastic tumour: clinical features and management," *Gynecologic Oncology*, vol. 99, no. 3, pp. 603–607, 2005.

[6] A. J. Papadopoulos, M. Foskett, M. J. Seckl, et al., "Twenty-five years' clinical experience with placental site trophoblastic tumors," *Journal of Reproductive Medicine for the Obstetrician and Gynecologist*, vol. 47, no. 6, pp. 460–464, 2002.

[7] I.-M. Shih and R. J. Kurman, "Epithelioid trophoblastic tumor: a neoplasm distinct from choriocarcinoma and placental site trophoblastic tumor simulating carcinoma," *American Journal of Surgical Pathology*, vol. 22, no. 11, pp. 1393–1403, 1998.

[8] R. J. Kurman, R. E. Scully, and H. J. Norris, "Trophoblastic pseudotumor of the uterus. An exaggerated form of 'syncytial endometritis' simulating a malignant tumor," *Cancer*, vol. 38, no. 3, pp. 1214–1226, 1976.

[9] I.-M. Shih, "Trophogram, an immunohistochemistry-based algorithmic approach, in the differential diagnosis of trophoblastic tumors and tumorlike lesions," *Annals of Diagnostic Pathology*, vol. 11, no. 3, pp. 228–234, 2007.

[10] H. W. Li, S. W. Tsao, and A. N. Y. Cheung, "Current understandings of the molecular genetics of gestational trophoblastic diseases," *Placenta*, vol. 23, no. 1, pp. 20–31, 2002.

[11] E. L. Garner, D. P. Goldstein, C. M. Feltmate, and R. S. Berkowitz, "Gestational trophoblastic disease," *Clinical Obstetrics and Gynecology*, vol. 50, no. 1, pp. 112–122, 2007.

[12] P. Hui, H. L. Wang, P. Chu, et al., "Absence of Y chromosome in human placental site trophoblastic tumor," *Modern Pathology*, vol. 20, no. 10, pp. 1055–1060, 2007.

[13] H. Vauhkonen, M. Hedman, M. Vauhkonen, P. Sipponen, and A. Sajantila, "Typing of XY (male) genotype from malignant neoplastic tissue by the amelogenin-based sex test," *Journal of Forensic Sciences*, vol. 49, no. 2, pp. 222–226, 2004.

[14] K. M. Murphy, J. S. Cohen, A. Goodrich, P. P. Long, and C. A. Griffin, "Constitutional duplication of a region of chromosome Yp encoding AMELY, PRKY, and TBL1Y: implications for sex chromosome analysis and bone marrow engraftment analysis," *Journal of Molecular Diagnostics*, vol. 9, no. 3, pp. 408–413, 2007.

[15] N. O. Bianchi, "Y chromosome structural and functional changes in human malignant diseases," *Mutation Research*, vol. 682, no. 1, pp. 21–27, 2009.

[16] Y. Wu, Y. Xue, and J. Pan, "Y-chromosome loss in acute promyelocytic leukemia," *Cancer Genetics and Cytogenetics*, vol. 157, no. 1, pp. 90–91, 2005.

[17] M. Kujawski, M. Jarmuz, M. Rydzanicz, et al., "Frequent chromosome Y loss in primary, second primary and metastatic squamous cell carcinomas of the head and neck region," *Cancer Letters*, vol. 208, no. 1, pp. 95–101, 2004.

[18] L. Bottarelli, C. Azzoni, F. Necchi, et al., "Sex chromosome alterations associate with tumor progression in sporadic colorectal carcinomas," *Clinical Cancer Research*, vol. 13, no. 15, pp. 4365–4370, 2007.

[19] R. J. Oldt III, R. J. Kurman, and I.-M. Shih, "Molecular genetic analysis of placental site trophoblastic tumors and epithelioid trophoblastic tumors confirms their trophoblastic origin," *American Journal of Pathology*, vol. 161, no. 3, pp. 1033–1037, 2002.

[20] R. A. Pattillo, S. Sasaki, K. P. Katayama, M. Roesler, and R. F. Mattingly, "Genesis of 46,XY hydatidiform mole," *American Journal of Obstetrics and Gynecology*, vol. 141, no. 1, pp. 104–105, 1981.