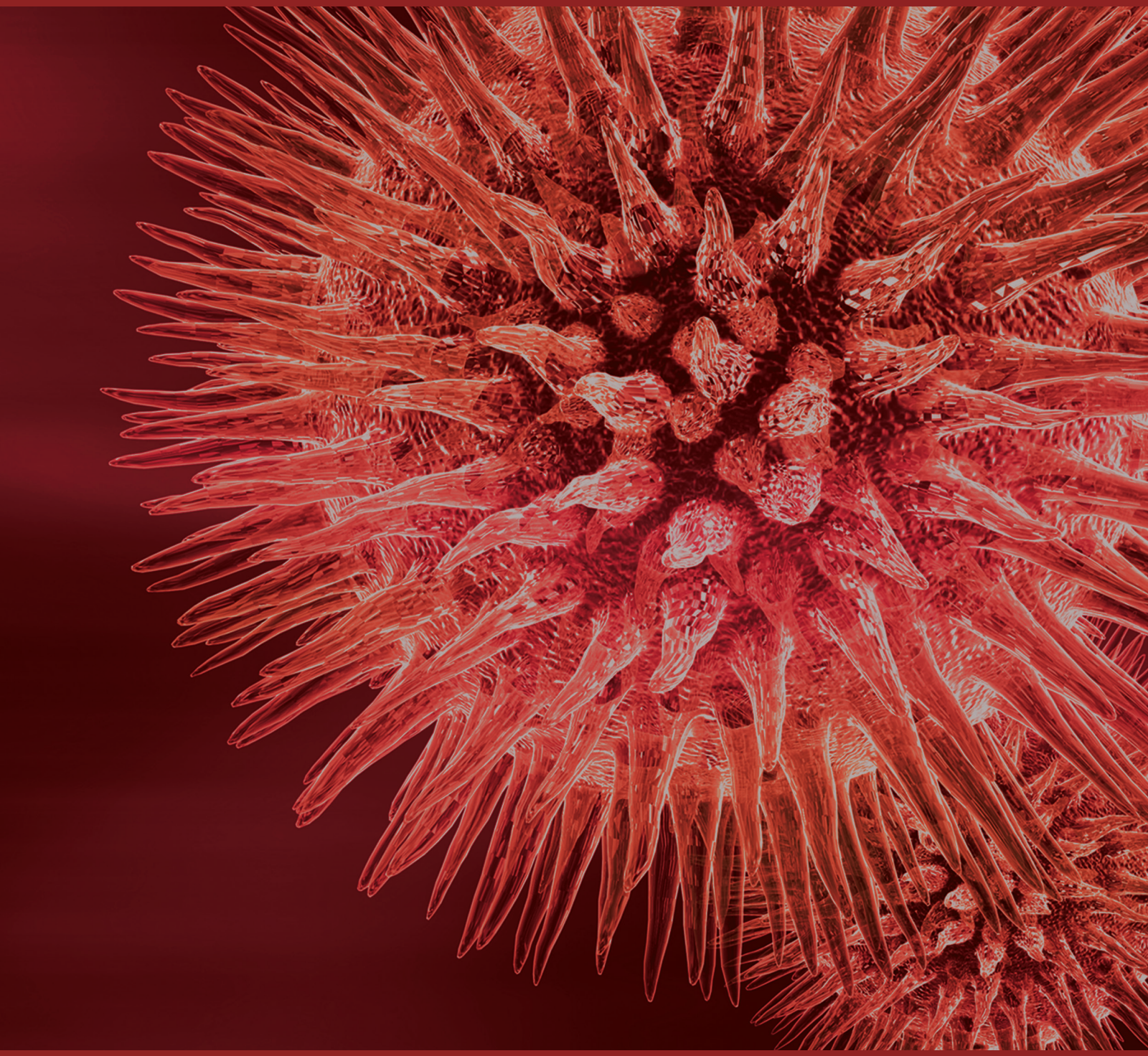
BioMed Research International

Ovarian Cancer

Guest Editors: Yong Sang Song, Hee Seung Kim, Daisuke Aoki, Danny N. Dhanasekaran, and Benjamin K. Tsang



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Editorial

Ovarian Cancer

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Ovarian cancer is the most lethal gynecologic malignancy and is the seventh leading cause of cancer deaths in women worldwide. Despite advances in surgery and chemotherapy, overall cure rate has remained very low. The poor clinical outcomes mainly come from the high percentage of cases being diagnosed at an advanced stage disease due to the lack of effective screening methods and frequent emergence of chemoresistance. Recent evidences have suggested that cancer stem cells may also contribute to the development of chemoresistance. However, there are still many questions on ovarian carcinogenesis and mechanisms of chemoresistance of ovarian cancer, which need to be resolved to improve the treatment outcomes of ovarian cancer.

The research topics include molecular genetics of ovarian carcinogenesis, autophagic reaction in ovarian cancer, chemoprevention using phytochemicals, tumor heterogeneity issue, and dual carcinogenesis of the ovary (type I versus II). The paper entitled "*BRCA-associated ovarian cancer: from molecular genetics to risk management*" by G. Girolimetti et al. demonstrated that ovarian cancer arising in BRCA 1 or 2 mutation carriers may have peculiar molecular, pathological, and clinical features. They also suggested that BRCA 1 or 2 mutational analyses would be helpful in tailoring ovarian cancer management based on BRCA status in the future.

The work by G. Valente et al. showed that the positive expression of BECLIN 1 with a crucial role in the regulation of both autophagy and cell death was correlated with the presence of LC 3 positive autophagic vacuoles and was inversely correlated with the expression of BCL-2 inhibiting the autophagy function of BECLIN 1. They also suggested that a low level of autophagy might favor cancer progression and that ovarian cancer with upregulated autophagy might have a less aggressive behavior and be more responsive to chemotherapy in the paper entitled "*Expression and clinical significance of the autophagy proteins BECLIN 1 and LC3 in ovarian cancer*."

The work by V. D. Martinez et al. assessed DNA copy-number loss (CNL), promoter hypermethylation, mRNA expression, and sequence mutation of KEAP1/CUL3/RBX1 complex as a regulator of the NFE2-related factor 2 (NRF2) pathway initiating response to oxidative stress in a cohort of 568 serous ovarian carcinomas form The Cancer Genome Atlas. They suggested that a remarkably high frequency of DNA and mRNA alterations may affect components of the KEAP1/CUL3/RBX1 complex, through a unique pattern of genetic mechanisms in the paper entitled "*Unique pattern of component gene disruption in the NRF2 inhibitor KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex in serous ovarian cancer*."

The paper entitled "*Phytochemicals: a multitargeted approach to gynecologic cancer therapy*" by L. Farrand et al. demonstrated molecular mechanisms of phytochemical action in cancer prevention and phytochemical-based approaches to overcome chemoresistance and phytochemical analogues and chemical modifications for greater efficacy. They suggested that high-throughput screening methods,

rational modification, and developments in regulatory policies would accelerate the development of novel therapeutics based on phytochemical compounds, which would likely improve overall survival and quality of life for patients with gynecologic cancers.

C. Yuan et al. performed a meta-analysis to examine whether the *XRCC3* polymorphisms are associated with ovarian cancer risk in the paper entitled "*Analyzing association of the XRCC3 gene polymorphism with ovarian cancer risk*." They found no association between XRCC3 rs861539 polymorphisms and ovarian cancer, whereas they observed a significant correlation with ovarian cancer risk using the homozygote comparison (T2T2 versus T1T1), heterozygote comparison (T1T2 versus T1T1), and the recessive genetic model (T2T2 versus T1T1 + T1T2). For XRCC3 rs1799796 polymorphisms, they also found a significant correlation with ovarian cancer risk using the heterozygote comparison (T1T2 versus T1T1).

G. Shuvayeva et al. demonstrated that single amino acid arginine deprivation triggered profound prosurvival autophagic response in cultured human ovarian cancer SKOV3 cells in the paper entitled "*Single amino acid arginine deprivation triggers prosurvival autophagic response in ovarian carcinoma SKOV3*." They also found that a significant drop in viability of arginine-starved SKOV3 cells was observed when autophagy was inhibited by either coadministration of chloroquine or transcriptional silencing of the essential autophagy protein BECLIN 1, suggesting that arginine deprivation-based combinational treatments that include autophagy inhibitors may produce a stronger anticancer effect as a second line therapy for a subset of chemoresistance ovarian cancers.

The work by R. Titone et al. demonstrated that the mRNAs of several autophagy-related genes contain the target sequence for miRNAs belonging to different families with either oncosuppressive or oncogenic activities in the paper entitled "*Epigenetic control of autophagy by microRNAs in ovarian cancer*." Furthermore, they emphasized that plasma and stroma-cell derived miRNAs in tumor-bearing patients could impact autophagy.

The work by M. Koshiyama et al. mentioned a recent theory of dual carcinogenesis of the ovary in the paper entitled "*Recent concepts of ovarian carcinogenesis: type I and type II*." In this review, they demonstrated that low grade serous carcinomas may be thought to evolve in a stepwise fashion from benign serous cystadenoma to a serous borderline tumor while the serous tubal intraepithelial carcinomas of the junction of the fallopian tube epithelium with the mesothelium of the tubal serous undergo malignant transformation to high grade serous carcinomas due to their location and metastasize to the nearby ovary and surrounding pelvic peritoneum.

The paper entitled "*Application of microRNA in diagnosis and treatment of ovarian cancer*" by K. Banno et al. suggested that many miRNAs have altered expression in ovarian cancer compared to normal ovarian tissues and these changes may be useful for diagnosis and treatment. Thus, they expect that chemotherapy targeting epigenetic mechanisms associated with miRNAs may also be effective to reverse gene silencing.

The paper entitled "*Expression profiles of epithelial-mesenchymal transition-associated proteins in epithelial ovarian carcinoma*" by M.-K. Kim et al. investigated the expression of Snail and Slug, the key regulators of epithelial-mesenchymal transition (EMT), in the primary ovarian cancer samples to assess the clinical significance of EMT-associated proteins. They found that Snail was differentially expressed according to the histologic subtype and was predominantly expressed in serous and endometrioid types. In the serous and endometrioid adenocarcinomas, the expression of Snail remained high across the stage and grade, suggesting its role in the early phase of carcinogenesis.

S. Mehrabi et al. assessed the levels of oxidative modified proteins in 100 primary serous epithelial ovarian carcinomas and normal/surrounding tissues using spectrophotometric, dinitrophenylhydrazone (DNPH) assay, two-dimensional gel electrophoresis, and Western blot analyses in the paper entitled "*Oxidatively modified proteins in the serous subtype of ovarian carcinoma*." They showed that the levels of reactive protein carbonyl groups increased as stages progressed to malignancy, and the levels of protein carbonyls in serous ovarian carcinoma among African Americans are 40% higher reactive to Caucasian at similar advanced stages.

In summary, molecular genetics and autophagic reaction in ovarian carcinogenesis, multitargeted approaches using autophagic reaction and phytochemicals, and dual approaches considering types I and II ovarian carcinogenesis are of paramount importance. This special issue presents new perspectives on carcinogenesis and chemoresistance of ovarian cancer, which will be helpful in overcoming the limitations of diagnosis and treatment of ovarian cancer in the future.

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Review Article

BRCA-Associated Ovarian Cancer: From Molecular Genetics to Risk Management

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Ovarian cancer (OC) mostly arises sporadically, but a fraction of cases are associated with mutations in BRCA1 and BRCA2 genes. The presence of a BRCA mutation in OC patients has been suggested as a prognostic and predictive factor. In addition, the identification of asymptomatic carriers of such mutations offers an unprecedented opportunity for OC prevention. This review is aimed at exploring the current knowledge on epidemiological and molecular aspects of BRCA-associated OC predisposition, on pathology and clinical behavior of OC occurring in BRCA mutation carriers, and on the available options for managing asymptomatic carriers.

1. The BRCA1 and BRCA2 Genes

1.1. Functions and Structure. BRCA1 and BRCA2 are separate genes mapping on two different chromosomes (17q21 and 13q12.3, resp.). They have distinctive primary sequences nevertheless disruption of either BRCA gene leads to similar pathophysiological effects [1], as well as to similar cancer spectra.

BRCA1 and BRCA2 are considered tumor suppressor genes, since they are deputed to the maintenance of genomic stability and hence to the control of cell growth [2]. The BRCA1 and BRCA2 proteins are mainly involved in the repair of DNA double-strand breaks (DSBs) via the homologous recombination (HR) pathway [3, 4]. DSBs are repaired by two major pathways: nonhomologous end-joining (NHEJ) and HR [4]. NHEJ usually results in changes in the DNA sequence at the break site [4]. In presence of a double-strand break, HR allows the exchange of the same genetic sequence from the healthy homologous sister chromatid to the damaged one [5] and therefore generally results in accurate repair of the break [6]. Both BRCA1 and BRCA2 proteins are critical for the recovery of DSBs by HR. Deficiency of BRCA1 or BRCA2 function leads to a high degree of chromosome instability, such as chromosome breaks, severe aneuploidy, and centrosome amplification [7–9] probably because it triggers the use of alternative pathways for the repair of DSBs such as NHEJ, resulting in accumulation of mutation events [4]. Genetic aberrations occur spontaneously favored by DNA-damaging agents that induce DSBs, in particular DNA cross-linking agent, mitomycin or platinum compounds [10], which explains why OC patients carrying BRCA1 or BRCA2 mutations display a better response to

platinum-based chemotherapy when compared to patients with sporadic OC [11, 12].

Several proteic interactors of BRCA1 and BRCA2 have been identified. RAD51 is responsible for repair mechanism of DSBs and is one of the most important players in HR; its functions are ultimately complemented by the proteins encoded by the two BRCA genes [13, 14]. A number of studies demonstrated the BRCA2 role in the regulation of intracellular transport, enzymatic activity, and function of RAD51 [15]. BRCA1 exhibits a physical association with RAD51 to create a complex responsible for resected single-stranded DNA at double-strand repair sites [16]. Other studies suggest a BRCA1 role in altering chromatin structure in the presence of a DNA damage to allow access for repair. It was shown that following damage, histone H2AX becomes extensively phosphorylated and forms foci at break sites [17]. BRCA1 is recruited to these foci before every other factor, such as RAD51, suggesting that H2AX and BRCA1 initiate repair by modifying local chromatin structure, thereby allowing DNA repair proteins to access the damage site [18]. Moreover, BRCA1 and BRCA2 exhibit a transcriptional coregulator and chromatin remodeling function [14, 19] and BRCA1 seems to have the ability to coactivate endogenous p53-dependent stimulation of p21 [20].

BRCA1 is a very large gene that generates several different transcripts. The full-length form is a 2843 amino acids (p220) protein and a shorter (1399 amino acids) form, named BRCA1-IRIS, may have an oncogenic activity. BRCA2 is even larger, counting 3418 amino acids, but has fewer recognized motifs [21]. BRCA1 and BRCA2 genomic regions harbor a very high density of repetitive DNA elements that contribute to genetic instability [22]. In particular the BRCA1 region consists of 42% Alu sequences and 5% non-Alu repeats [23]. The BRCA2 genomic region is 47% repetitive DNA: 20% Alu sequences and 27% LINE and MER repetitive DNA. Alu-dense regions of the genome are associated with a high density of genes and localize predominantly to R bands of metaphase chromosomes, which are involved in homologous and nonhomologous chromosomal exchange [24]. Based on the density of repeat elements in these genes, Alu-mediated genomic rearrangements within BRCA1 and genomic rearrangements in BRCA2 have been observed [25, 26].

1.2. Mutational Analysis. Disease-associated mutations are scattered across the entire length of the BRCA1 and BRCA2 genes and usually result in a truncated protein. Deleterious missense mutations occur frequently in exons-encoding domains that interact with BRCA1-binding proteins, such as BARD1, BRIP1, and PALB2, which (along with RAD51C, RAD51D, and possibly RAP80 and FAM175A) are also breast and/or ovarian cancer susceptibility genes [21].

The BRCA genes are routinely tested by Sanger sequencing of exons and exon-intron junctions. Whether a clearly pathogenic variant has not been identified, the multiplex ligation probe assay (MLPA) should be applied in order to exclude the presence of large BRCA1 deletions, involving one or more full-length exons. The MLPA assay is a rapid and robust method for copy number quantification and methylation status analysis of genomic sequence. It can be easily multiplexed and requires only a small amount of input DNA [26].

The silencing of BRCA1 gene trough promoter hypermethylation may occur in sporadic breast and ovarian cancers [27]. The analysis of DNA methylation patterns of BRCA1 may also be a useful predictive marker of the response to the PARP1 inhibitor therapy [28].

In a few years the next generation sequencing (NGS) technology, including different high-throughput sequencing systems, will likely replace Sanger sequencing as the technique of choice for genetic testing of BRCA genes because it undoubtedly offers advantages in terms of sensitivity, scale, and costs. However, the large number of false positive/negative insertions and deletions (indels) due to the high frequency of homopolymers in BRCA genes, which cause sequencing errors, has slowed down the usage of NGS for clinical genetic testing. The specificity of indels detection in NGS data is improving by the application of different filtering criteria for the variants calling [29].

Based on the diagnostic criteria used in the United States to select patients to be tested, clearly pathogenic mutations in either BRCA1 or BRCA2 are found in 10% to 15% of hereditary breast and ovarian cancer families. Rare patients with mutations in both BRCA1 and BRCA2 genes were described [30], which has led to recommending that the analysis of both genes should be completed even after the finding of one mutation.

The major challenge in the diagnostic testing of BRCA genes concerns the interpretation of unknown variants, the so-called "variants of uncertain significance" (VUS). The pathogenic variants can be nonsense mutations, small indels causing a frameshift, splicing site mutations that occur inside of the canonical splice sites, large deletions or known deleterious missense variants. VUS are alterations in the DNA sequence that have unknown effects on the protein function and disease risk; they usually are missense substitutions, splicing site mutations that occur outside of the canonical splice sites, small in-frame indels. Their frequency varies depending on the patient's ethnicity: in the United States, in individuals of European ancestry VUS account for approximately 5% of the alterations reported from BRCA genetic testing, but the estimate is as high as 20% among individuals of African ancestry [31]. To guide the clinical management, a statistically rigorous model that provides pathogenicity score for each variant has been proposed by the International Agency on Cancer Research (IARC) of the World Health Organization. It is a five-level system where classes 1 and 2 are managed as neutral variants and classes 4 and 5 are managed as pathogenic variants. Class 3 variants still have insufficient evidence to be considered either neutral or pathogenic and they require reclassification.

The method for VUS classification begins with a prior probability based on an *in silico* evaluation of the effect of each variant at the protein or mRNA level. Observational data, such as personal and family history, cosegregation of VUS with cancer phenotype in pedigrees, cooccurrence with other known pathogenic mutations, tumor immunohistochemistry, and histological grade, are summarized as

likelihood ratios in favor of pathogenicity and used to update the prior probability [32, 33]. The VUS database was built on a modified Leiden Open source Variation Database (LOVD) v.2.0 system, providing a flexible environment for the creation of locus-specific databases [34].

2. Clinicopathological Features of BRCA-Associated Ovarian Cancer

2.1. Prevalence of BRCA1/2 Mutations among Ovarian Cancer Patients. OC patients with a family history of breast or ovarian cancer present high probability of carrying a mutation in BRCA1 or BRCA2. The rate of mutations found in families with multiple cases of breast and ovarian cancer varies from 9% to 46%, depending on selection criteria and ethnicity [35–41]. Among families with at least two cases of epithelial OC, 43% were found to harbor BRCA germline mutations (36% BRCA1 and 7% BRCA2 mutations) [42].

In addition, many efforts have focused on assessing the contribution of BRCA mutation to the total burden of OC. Early population-based studies reported BRCA1 mutation rates in OC patients varying from 1.9 to 7.2% [43–46].

In a recent population-based cohort of 1001 Australian OC patients, 141 (14.1%) were found to carry a pathogenic mutation in BRCA1 (88 patients) or BRCA2 (53 patients), the proportion being 16.6% when only serous carcinomas where considered and increasing to 17.1% in patients diagnosed with high-grade serous cancers. 44% of mutation carriers failed to report any family history of breast or ovarian cancer [47]. Another large population-based study was conducted in Ontario, Canada: among 1342 unselected OC patients, 176 (13%) carried a BRCA1/2 mutation. Higher prevalence of mutations was associated with Italian, Jewish, or Indo-Pakistani origin, serous histology, younger age of onset, and, obviously, a family history of breast or ovarian cancer; among women without family history of such cancers, prevalence was 7.9% [48].

Several smaller studies have been carried out in different countries: in a Greek cohort of 592 patients with sporadic OC, a targeted screening of the commonest BRCA1 mutations detected 27 mutation carriers (4.6%) [49]. In Belgium, de Leeneer et al. tested 193 sporadic cases of breast and ovarian cancer for BRCA1/2, finding 3 carriers among 7 women with both breast and ovarian cancer (42.9%) but none among 6 patients with OC only [50]. In Poland, BRCA1/2 mutations were identified in 21 out of 151 consecutive OC patients (13.9%) [51], while in a sample of 74 Russian patients, the prevalence was as high as 19% [52]. In Korean OC patients, BRCA1/2 mutations were detected in 13 of 40 (33%) reporting a strong family history and in 23 of 283 (8%) without significant family history [53].

2.2. Pathology of BRCA-Associated Ovarian Cancer. The vast majority of OC associated with germline BRCA mutations reported in the literature are high-grade and advanced-stage serous carcinomas [54]. Most studies, however, do not include a complete pathology review of all material and rely only on pathology report. Nevertheless, the studies that incorporate a systematic histopathologic review confirm and emphasize the greater frequency of high-grade serous carcinomas in BRCA1-associated tumors, with a frequency ranging from 67% to 100%. Frequencies of endometrioid and clear cell carcinomas seem to be similar to the general population. Although other tumor types have been observed, they are extremely rare, accounting for <10% of all tumors [55–59]. However, these data may have been biased by the most recent updates about pathogenesis and clinicopathologic diagnosis of OC and by the significant interobserver variation that affects histopathological typing of all tumors, including OC, with categorization being particularly difficult for high grade lesions.

Also borderline BRCA1 carriers ovarian tumors are very rare [46], which reinforces the increasing evidence that BRCA1 mutations do not play a role in the development of this type of tumors. Fallopian tube cancer and peritoneal carcinomas are also part of the BRCA-associated disease spectrum.

A large dataset, aimed at expanding the knowledge of OC pathology in these patients, has been recently reported by the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) that represent the largest collaborative study of BRCA1 and BRCA2 mutation carriers, involving more than 37 groups from more than 20 countries [60]. Tumor pathology data has been collected through several mechanisms, including medical records and pathology reports. Laboratory methods for tissue preparation, immunohistochemistry and biochemical assays, scoring systems, and data interpretation vary widely; nevertheless data collated by CIMBA seem to be more representative of typical assessment of pathology conducted in routine practice. CIMBA results confirm that over 70% of OCs in BRCA1 and BRCA2 mutation carriers are grade 3 serous carcinoma (Table 1).

Moreover, according to the recent update on the different pathogenesis and clinicopathologic features of ovarian low-grade and high-grade serous carcinomas, as well as to the fundamental molecular differences between both categories of tumors, the vast majority of BRCA-related hereditary ovarian tumors are high-grade serous carcinoma. Low-grade serous carcinoma and noninvasive micropapillary serous carcinoma do not seem to be related to germline mutations of BRCA [61].

2.3. Molecular Pathology of BRCA-Associated Ovarian Cancer. A high frequency of loss of heterozygosity (LOH) in or near the BRCA1 region has been reported in BRCA1-mutation positive OC [62]. In addition, LOH of BRCA, as well as TP53 mutations, have been demonstrated as early events in high-grade serous carcinomas in patients with germline mutations; accordingly, mutations and/or loss of heterozygosity of TP53 and BRCA have been identified in early carcinomas and epithelial inclusions of the ovary [63, 64]. More recently, attention has been drawn to a lesion in the fallopian tube that has the cytologic appearance of high-grade serous carcinoma of the ovary and has been designated tubal intraepithelial carcinoma (TIC). These lesions are almost always detected in the fimbriae of the fallopian tube. The fimbriae are in close proximity to the ovarian surface, and it has been suggested that the tube is the origin of a subset of "ovarian"

Table 1: Distribution of morphology and grade of ovarian tumors arising in BRCA1 and BRCA2 mutation carriers from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) [60].

Factor		BRCA1 *n* (%)	BRCA2 *n* (%)	Total *n* (%)
Morphology	Serous	534 (66)	191 (70)	725 (67)
	Mucinous	11 (1)	4 (1)	15 (1)
	Endometrioid	94 (12)	33 (12)	127 (12)
	Clear cell	8 (1)	8 (3)	16 (1)
	Other	166 (20)	36 (13)	202 (19)
	Total	**813**	**272**	**1,085**
Grade	1	17 (3)	11 (6)	28 (4)
	2	104 (20)	37 (21)	141 (20)
	3	407 (77)	128 (73)	535 (76)
	Total	**528**	**176**	**704**

high-grade serous carcinomas. This is supported by some data: early serous carcinomas in prophylactic bilateral salpingooophorectomy specimens from women with BRCA mutations have been detected in the tube, especially the fimbriae, in the absence of an ovarian tumor; identical TP53 mutations have been reported in TIC and synchronous ovarian high-grade serous carcinomas, and identical TP53 mutations have been reported in TICs and in small foci of histologically normal tubal epithelium that diffusely expresses p53, which has been termed "p53 signature." It has been suggested that p53 signatures are precursors of TICs which in turn precede the development of high grade serous carcinoma. Moreover, it has been proposed that when there is a synchronous TIC and ovarian high-grade serous carcinoma, the fallopian tube is the primary site of origin for the "ovarian" tumor [61].

2.4. BRCAness. The term "BRCAness" has been used to describe the phenotypic characteristics that some sporadic OCs share with tumors found in the setting of BRCA germline mutations. The term also reflects that this common biologic behavior comes from molecular defects in the cellular machinery similar to those caused by BRCA mutation [12, 65]. The notion began to form in 1996 after studies of BRCA1/2 genes in sporadic OC showed multiple defects in the BRCA1/2 pathway that would explain a BRCA-like phenotype. BRCA1 and BRCA2 germline mutations are the fundamental defect in hereditary OC where the normal allele of the carrier is inactivated in cancer cells [66, 67]. In sporadic forms, BRCA1/2 somatic mutations are not very common [68] but still are a significant causative gene defect, as shown in extensive genomic analyses of ovarian carcinoma by the Cancer Genome Atlas Research Network [69]. Either genetic or somatic mutations of BRCA1 and BRCA2 are found in approximately 20% of all ovarian tumors [70]; when considering all kinds of BRCA1/2 alterations, including mutations, these have been reported in up to 82% of ovarian tumors [66]. In the absence of BRCA mutations, the BRCAness pattern of biological and clinical behavior seems to be the result of different epigenetic processes. Indeed, epigenetic mechanisms of transcriptional silencing are known to inactivate tumor suppressor genes. BRCA1 protein and mRNA levels in ovarian tumors are decreased or absent in as many as 90% of patient cases without evidence of germline BRCA1 mutations or family history of BRCA-associated diseases [71]. Baldwin et al. demonstrated that aberrant methylation in cytosine residues of CpG dinucleotides in the BRCA1 promoter leads to decreased BRCA1 expression in 5%–30% of ovarian tumors, resulting in BRCAness. Moreover, they showed that 12 of 81 (~15%) ovarian tumors in patients without a family history of OC had evidence of BRCA1 promoter methylation. None of the 12 normal ovaries had evidence of methylation. Tumor and genomic DNA from patients with BRCA1 promoter methylation were screened for the three key founder mutations in the Ashkenazi Jewish population: BRCA1 185delAG, 5382insC, and BRCA2 6174delT. None of the tumors that demonstrated BRCA1 promoter methylation had concurrent BRCA1 mutations. Baldwin et al. also performed immunohistochemistry on paired paraffin-embedded tumor tissues. BRCA1 expression was detected in the nuclei of adjacent stromal cells but in none of the 12 tumors, suggesting BRCA1 inactivation through promoter methylation. Only 5 of these 12 methylated samples exhibited LOH at the BRCA1 locus. While discordant findings between these studies may be a product of biases introduced by population sampling, they suggest the possibility of alternative sites of inactivating BRCA1 methylation not detected in either group's assay [72]. A subsequent study indicated that BRCA1 promoter methylation can be an unfavorable prognostic factor compared to either BRCA1 germline mutation or no loss [73]. A more recent report found epigenetic silencing of BRCA1 and BRCA1/2 mutations to be mutually exclusive; patients with epigenetic BRCA1 silencing showed a similar prognosis as noncarriers [69]. Although LOH for the BRCA locus has been noted in sporadic breast cancer [74], the importance of this mechanism has not been verified in OC. BRCAness could also emerge from defects in genes whose function either affects or is affected by normal BRCA gene function.

2.5. Treatment of BRCA-Associated Ovarian Cancer. Despite being more aggressive than sporadic ovarian carcinomas, those arising in BRCA mutation carriers show higher

susceptibility to platinum-salts and other DNA-damaging agents. Platinum-salts interfere with DNA cross-links creating double-strand breaks in the DNA helix, which cannot be repaired in BRCA due to HR deficiency. Studies demonstrated an improved long term-survival in women with OC treated with platinum-salts if compared to sporadic OC [75]. Intraperitoneal cisplatin chemotherapy has been shown to lead to favorable long term outcome in advanced OC women with BRCA mutation [76]. Similar outcomes were seen using pegylated liposomal doxorubicin [77–79].

In the last years the inhibition of the poly(ADP-ribose) polymerase enzyme (PARP) has emerged as a promising therapeutic approach in BRCA-associated OCs. PARP is a class of proteins which produces large branched chains of poly(ADP) ribose (PAR) from NAD+. They are involved in a number of cellular pathways including transcriptional regulation, DNA replication, and DNA damage repair [4, 80]. Of the numerous PARP protein detected, PARP-1 and PARP-2 were found to be mostly involved in DNA stability [81]. PARP-1 is a highly conserved nuclear enzyme whose main task is to assist the repair of single strand breaks (SSB) through the BER pathway therefore contributing to the maintenance of genomic integrity [82]. Inhibition of PARP generates DNA lesions caused by lack of an efficient repair of SSB lesions that may cause DSBs or collapsed replication forks. This damage requires functional BRCA1 and BRCA2 for DNA repair [3]. In the presence of a BRCA1 or BRCA2 defective background, HR is impaired and therefore PARP inhibition may results in the generation of replication associated DNA lesions that cannot be effectively repaired, leading to decreased chromosomal stability, cell cycle arrest, and/or cell death [81]. It was shown that cell lines lacking wild-type BRCA1 or BRCA2 and the tumors that they form are 1000-fold more sensitive to PARP inhibitors compared to heterozygous mutant or wild-type cells [81, 83]. Wild-type and BRCA1 or BRCA2 heterozygous cells are able to repair DSBs maintaining cell viability. Patients with BRCA-associated cancers usually lack wild-type BRCA1 or BRCA2 in tumor cells but normal cells retain a single wild-type copy of the gene. PARP inhibitors are, therefore, highly selectively lethal to cells that lack functional BRCA1 or BRCA2 and are associated with minimal toxicity to normal cells [4].

The oral PARP inhibitor olaparib has shown to be well tolerated in phase I studies. A phase II study recruiting patients with BRCA1/2 mutations with recurrent OC demonstrated the efficacy of single agent olaparib with a median duration of response of 9.5 months and with a 66% of clinical benefit rate with the dose of 400 mg twice a day [84]. At ASCO 2013 annual meeting Ledermann et al. [85] presented the results of a preplanned subgroup analysis of maintenance therapy with olaparib in platinum-sensitive relapsed serous OC and BRCA mutation from a previous randomized phase II study [86]. Olaparib maintenance treatment led to the greatest clinical benefit in patients with BRCA mutation. Besides olaparib other promising PARP inhibitor agents being investigated are niraparib, veliparib, and rucaparib. Preclinical data have shown a degree of synergy between PARP inhibitors and chemotherapy; however a recent randomized trial of olaparib in association with paclitaxel and carboplatin failed to show substantial benefit [87]. Future challenges in BRCA mutated OC will include the overcoming of PARP inhibitors resistance that ultimately develops in all patients and the identification of biomarker other than BRCA to select patients with homologous recombination defect that will benefit the most from targeted treatment.

2.6. Prognosis. Evidence exists that OC patients carrying germline BRCA mutations have an improved prognosis in comparison to sporadic cases. A pooled analysis of 26 observational studies was recently carried out to explore survival differences between BRCA mutation carriers (1213 overall) and noncarriers (2666); BRCA mutation carriers showed a more favorable prognosis than noncarriers [88].

A favourable prognosis for BRCA mutation carriers with OC was also supported by a recent retrospective study on 190 ethnically heterogeneous patients, 90 of whom were BRCA mutation carriers. A significantly longer overall survival was reported in mutation carriers compared with noncarriers (median overall survival: 93.6 months versus 66.6), although no difference was observed in progression-free survival [89]. Improved rates of progression-free and overall survival in BRCA1/2 carriers were also found in an Australian series of OC patients tested for BRCA regardless of family history [47].

By comparing OC outcome in BRCA1 versus BRCA2 mutation carriers, Liu et al. [90] failed to find any difference in event-free survival, while a nonsignificant increase in 5-year overall survival was observed in BRCA2 (75%) in comparison to BRCA1 carriers (61%).

An Italian retrospective study found a survival advantage in BRCA2-positive versus BRCA1-positive OC patients, the median progression free survival being 45.46 months in the former and 27.2 in the latter [91]. The better prognosis of BRCA2 mutation carriers is supported by another retrospective study on 190 patients (47 mutation carriers and 143 noncarriers), where multivariate analysis showed improved overall survival in BRCA2 carriers but not in BRCA1 carriers, in comparison with noncarriers [92].

A study on the outcome of primary surgical cytoreduction in 69 BRCA mutated in comparison to 298 wild-type patients with high-grade serous ovarian carcinoma (FIGO stage IIIC-IV) demonstrated that BRCA status was not associated with residual tumor volume on multivariate analysis, which led the authors to conclude that improved survival in BRCA carriers was not attributable to an increased rate of optimal tumor debulking [93].

Taken together, these studies consistently support a significantly improved survival in BRCA2 mutation carriers in comparison to sporadic OC patients, while for BRCA1 carriers the advantage, if any, seems smaller.

2.7. Correlations between BRCA Deficiency and Mitochondrial Function and Potential Implications for Therapy. A recent study demonstrates that BRCA1 mutations in breast cancer drive oxidative stress and glycolytic transformation of the tumor. Loss of BRCA1 function leads to hydrogen peroxide generation in both epithelial breast cancer cells and neighboring stromal fibroblasts and promotes the onset of a reactive glycolytic stroma. Importantly, these metabolic changes can

be reversed by antioxidants, which potently induce cancer cell death [94]. Furthermore our group has recently contributed to a study in which a correlation was shown between expression levels of BRCA2 and mitochondrial DNA (mtDNA) in prostate cancer [95]. MtDNA-depleted and HR-deficient cells appear to exhibit more than 50% reduction in BRCA2 protein expression through a posttranslational mechanism. For these reasons mtDNA-depleted cell lines die after PARP inhibition. Taken together these studies suggest a biologic relationship between mitochondria and BRCA genes. Mitochondrial dysfunction and loss of BRCA2 in sporadic cancer lead to nuclear genomic instability, cumulative mutations, and tumor progression but also enhance cancer cells sensitivity to apoptosis induced by PARP inhibitors [95]. Furthermore, by studying prostate tissue specimens from prostate cancer patients a direct correlation between presence of mtDNA large deletions and loss of BRCA2 protein *in vivo* was found, suggesting that mtDNA status might serve as a marker to predict therapeutic efficacy to PARP inhibitors. Further studies are warranted to confirm this correlation.

3. Prevention of BRCA-Associated Ovarian Cancer

3.1. Identification and Cancer Risk Assessment in BRCA Mutation Carriers. The detection of a mutation in a patient with breast or ovarian cancer allows for identification of asymptomatic mutation carriers in the family. Such predictive genetic testing should be performed in the framework of a comprehensive genetic counseling process, aimed at facilitating informed decisions about testing and risk-reducing options.

Carriers of BRCA mutations are at increased risk of both breast and ovarian cancer; such risks are consistently estimated to be higher in BRCA1 than in BRCA2 mutation carriers; moreover, the risk is higher for breast than for OC. Estimates of cancer risk are variable across different studies; nevertheless, two large meta-analyses, resulting from a total of 31 published studies, estimated an OC risk to age 70 of 49% for BRCA1 and 18% for BRCA2 [96] and of 39% for BRCA1 and 11% for BRCA2 [97]. The risk for breast cancer was 55% to 65% for BRCA1 and 45% to 47% for BRCA2 mutation carriers. However, the penetrance of BRCA mutations is likely to be overestimated in published studies, as carriers are mostly ascertained based on the clustering of cancer in the family, which may reflect the presence of additional risk factors modifying the penetrance.

In Europe and the United States, the onset of familial OC is generally reported to be 5 to 10 years earlier than that of sporadic OC, but the early onset is probably limited to BRCA1 mutation carriers. Indeed, Risch and colleagues reported a mean age at OC onset of 51.2 years for BRCA1 mutation carriers and of 57.5 years for BRCA2 positive women [98]. Similarly, in the series described by Liu et al, the median age at OC diagnosis was significantly younger in BRCA1 (51.1) in comparison to BRCA2 carriers (55.4) [90]. In Japan, age at onset of familial OC is similar to that in sporadic OC, although BRCA2-related OC tends to develop at a later age than sporadic OC [99].

3.2. Management of BRCA Mutation Carriers. Options to reduce the risk of OC or fallopian tube cancer in BRCA mutations carriers include surveillance, chemoprevention, and surgery [100]. Nevertheless, screening and prevention guidelines are generally based on nonrandomized trial and observational studies [101]. Although based on expert consensus (lowest level of evidence), the National Comprehensive Cancer Network (NCCN) guidelines provide the most comprehensive guidelines currently available [100].

3.2.1. Surveillance for Ovarian, Fallopian, and Primitive Peritoneal Cancer. Recommended surveillance should be performed with pelvic examination, transvaginal ultrasound, and CA-125 levels every 6 months beginning at age 30 or 5–10 years earlier than the youngest relative diagnosed with OC [100, 102]. Little is known about the mechanism or timing of progression from localized to disseminated ovarian cancer. Early detection of the ovarian cancer is less effective than breast cancer screening and these surveillance methods have not been demonstrated to reduce OC mortality [101]. Three large randomized controlled trials to determine whether screening for OC, compared with no screening, can achieve earlier diagnosis and decreased mortality have been or are being conducted in the United Kingdom, Japan, and the United States. Mortality data from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial in the UK have been reported, showing no change in the stage of cancer detected by screening and no decrease in cancer-specific or overall mortality for women who underwent annual screening (four years of transvaginal ultrasound and six years of CA-125 serum levels) [103–105]. The potential risks associated with screening for OC must also be considered. A positive screening result for OC most often is followed by surgery (either laparoscopy or laparotomy). Invasive procedures are associated with physical and psychological morbidity, a small risk for serious complications, and substantial financial costs. The PLCO trial reported that 15 percent of women who underwent surgery for false positive findings experienced a serious complication related to surgery [103].

In conclusion surveillance for OC in BRCA positive patients with pelvic exam, transvaginal ultrasound, and serum levels of CA-125 is not invasive, but its impact on early detection and mortality is low and may be associated with emotional distress [106].

3.2.2. Risk Reduction Surgical Options. Since surveillance for ovarian, peritoneal, and fallopian tube cancer has not been proven to be effective, prophylactic salpingooophorectomy is actually recommended to BRCA positive women by the age of 40 years and upon completion of child bearing [100].

Salpingooophorectomy in BRCA patients should be performed considering specific issues and adequate surgical details. Specific preoperative counselling and informed consents should be obtained [107]. This surgery reduces the risk of ovarian and fallopian tube cancer by 75–96% and of breast cancer by approximately 50% if performed after natural menopause [108]. The procedure, however, does not eliminate the risk of primary peritoneal cancer, that, after

prophylactic surgery, has been estimated to be between 2 and 4% [109]. The procedure begins with a thorough inspection of the peritoneal cavity, comprehensive of pelvic organs, gastrointestinal surfaces, liver, omentum, and pelvic, abdominal, and diaphragmatic peritoneal surfaces. Any suspected lesion should be biopsied and sent for frozen section. Abdominal cavity should be washed with saline and a sample obtained for cytology. This procedure is generally adequately performed by laparoscopy, even in presence of adhesions due to previous abdominal procedures.

Salpingooophorectomy requires complete removal of the ovary and the fallopian tube; the procedure should therefore be preceded by an accurate periadnexal adhesiolysis to obtain clear margins and simple handling of the structures. Peritoneum should be entered laterally to the ovarian vessels and visualization of ureter, ovarian artery, and veins obtained. Ovarian vessels should be coagulated and cut 1-2 cm away from the ovary to obtain clear margins and to verify the absence of vascular and lymphatic involvement. The peritoneum next to the ovary has to be cut with large margins to identify microscopic neoplastic foci. Finally, uteroovarian ligament and fallopian tubes should be coagulated and sectioned as close to the uterine wall as possible [110].

Fallopian tube and ovary are removed intact through an endobag to avoid spillage in the abdominal cavity and sent for frozen section. For logistic or organization issues frozen section could be missed, but the necessity of second surgery has to be discussed with the patient during preoperative counselling [111].

The removal of the uterus is not mandatory when preoperative imaging has excluded endometrial or myometrial disease; the risk of remnant of the intramural fallopian tube is negligible, but it should be discussed with the patient. Hysterectomy simplifies hormone replacement therapy and eliminates the possible increased risk of endometrial cancer and the possible increased risk of serous carcinoma of the uterus [112, 113].

Because of the tubal origin of serous ovarian carcinoma [114], some authors advocate the removal of the fallopian tubes, or fimbriectomy, as first step in prophylactic surgery for BRCA 1 and BRCA 2 patients as a bridge procedure for young patients willing to postpone oophorectomy after the fifth decade of life [115]. The procedure does not imply hormonal deficiency, but the safety and validity of this procedure should be confirmed by a multi-institutional study. Women who undergo radical fimbriectomy should continue to receive regular surveillance.

Finally, single port procedures are now available and this low aesthetic impact surgical access could be proposed to BRCA 1 and BRCA 2 patients [116].

The pathologic examination of specimens from risk-reducing salpingooophorectomy requires special attention. To this aim, the BRCA mutation status should be always shared with the pathologist, since in patients with benign gynecologic disease only one slide from the fallopian tube and ovary is normally reviewed, while in the setting of a known genetic predisposition to OC, most pathologists will submit the entirety of the fallopian tubes and ovaries for microscopic examination. The SEE-FIM (sectioning and extensively examining the fimbriated ends) protocol is widely used. Basically, this involves serially sectioning the tube meticulously, stopping before the fimbriae. The fimbria is amputated and sectioned longitudinally, thereby maximizing exposure. Deeper sections need to be obtained if foci of atypia are to be identified histologically. Foci of in situ or invasive occult carcinoma may be very subtle and are often less than 1 mm in maximum diameter. Microscopic occult carcinomas have been identified in these specimens in about 2% to 11% of BRCA mutation carriers, generally involving the tubal fimbriae [117–119].

4. Conclusions

Literature data demonstrates that OC arising in BRCA1/2 mutation carriers have peculiar molecular, pathological, and clinical features. Since a nonnegligible proportion of newly diagnosed OC patients are expected to carry such mutations, BRCA1/2 mutational analysis would help, in the future, to tailor OC management according to BRCA status. In addition, this would allow the subsequent identification of asymptomatic carriers who would benefit from targeted interventions for high-risk women.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Expression and Clinical Significance of the Autophagy Proteins BECLIN 1 and LC3 in Ovarian Cancer

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Autophagy is dysregulated in cancer and might be involved in ovarian carcinogenesis. BECLIN-1, a protein that interacts with either BCL-2 or PI3k class III, plays a critical role in the regulation of both autophagy and cell death. Induction of autophagy is associated with the presence of vacuoles characteristically labelled with the protein LC3. We have studied the biological and clinical significance of BECLIN 1 and LC3 in ovary tumours of different histological types. The positive expression of BECLIN 1 was well correlated with the presence of LC3-positive autophagic vacuoles and was inversely correlated with the expression of BCL-2. The latter inhibits the autophagy function of BECLIN 1. We found that type I tumours, which are less aggressive than type II, were more frequently expressing high level of BECLIN 1. Of note, tumours of histologic grade III expressed low level of BECLIN 1. Consistently, high level of expression of BECLIN 1 and LC3 in tumours is well correlated with the overall survival of the patients. The present data are compatible with the hypotheses that a low level of autophagy favours cancer progression and that ovary cancer with upregulated autophagy has a less aggressive behaviour and is more responsive to chemotherapy.

1. Introduction

Epithelial ovary cancers (EOCs) represent the vast majority (approximately 90%) of all ovary tumours. Based on morphological criteria, EOCs are classified as serous (of low and high grade), clear cell, endometrioid, mucinous transitional (Brenner type), mixed mesodermal, and undifferentiated histologic subtypes [1]. The histogenesis of EOC is still debated. Very recently, the traditional view that EOCs arise from the metaplastic transformation of the mesothelium overlying the ovaries has been challenged by a new paradigm suggesting that these carcinomas indeed arise in extraovarian sites and involve the ovaries secondarily [1]. Based on genetic and clinical features, ovarian carcinomas are classified as type I that comprise the low-grade serous, low-grade endometrioid, clear cell, mucinous, and transitional (Brenner) histologic types and as type II that comprise the high-grade serous, high-grade endometrioid, undifferentiated, and mixed mesodermal histologic types [1]. Type I ovarian carcinomas are genetically more stable and clinically indolent and less aggressive than type II ovarian carcinomas [1].

Ovarian cancer ranks as the sixth to eighth most frequent cancer in developed countries [2] and, in spite of the recent progresses made in understanding the genetic and biologic

bases [3, 4], it remains the most lethal among all the gynaecologic malignancies, with a 5-year survival of less than 30% [5]. Bad prognosis is essentially due to the fact that diagnosis of ovarian cancers often occurs at a late stage (because of the lack of precocious alarming symptoms) and also due to the recurrence of chemoresistant tumours. Therefore, new biomarkers for early detection and for monitoring the progression of ovarian cancers [6], as well as new therapeutic strategies that could specifically target the chemoresistant clones [3, 4], are needed.

Autophagy, a lysosomal-dependent pathway for the degradation of redundant or damaged cell components, has recently been suggested to play a role in ovarian carcinogenesis and to be a potential therapeutic target to combat this cancer [7]. Autophagy begins with the production of double-membrane vacuoles (named autophagosomes) that entrap the material to be degraded and eventually fuse with lysosomes (reviewed in [7]). The autophagosomes are characteristically marked by the presence of protein LC3 (deriving from posttranslational modifications of a microtubule-associated protein precursor) on their membranes [8]. Among the many proteins that directly or indirectly regulate the autophagy process, BECLIN 1 seems to be of particular relevance in ovarian carcinogenesis. BECLIN 1 was initially isolated as an interactor of the oncogenic antiapoptotic protein BCL-2, and it was reported to be deleted in up to 75% of human ovarian cancers [9, 10]. The monoallelic deletion of BECLIN 1 in mice caused the spontaneous development of tumours, including ovarian cancer, in association with reduced autophagy [11].

To trigger autophagy, BECLIN 1 must release BCL-2 and form dimers which interact with PI3-kinase class III (or Vps34), thus forming an oligomeric complex that can be evidenced by immunohistochemistry or immunofluorescence as definite spots in the cytoplasm [12, 13]. Autophagy-active BECLIN 1 has been proposed as a potential prognostic biomarker in several tumours [13–15]. However, the prognostic significance of BECLIN 1 expression in ovarian carcinomas appears controversial. Shen et al. [16] found that BECLIN 1 expression was significantly higher in benign and borderline ovarian tumours than in malignant EOC, which was consistent with the view that a decreased capacity of autophagy could favour tumorigenesis in the ovary. Recently, this same group confirmed this observation in a larger cohort of patients and also found that low expression of BECLIN 1 and high level of expression of BCL-2 were associated with advanced clinical stage at diagnosis and poor prognosis [17]. In contrast, another study found that BECLIN 1 expression was increased in malignant versus benign ovary tissues and that such high expression was associated with worse prognosis [18]. Increased expression of BECLIN 1 was found also to be associated with the most aggressive endometrioid adenocarcinomas and poor 5-year overall survival, probably because of concomitant tumour hypoxia [19]. In this same line, it was reported that the high expression of LC3A, the marker of autophagosomes, was associated with hypoxia and poor prognosis in clear cell, but not other examined subtypes, ovarian cancers [20].

In this work, we assessed by immunohistochemistry and immunofluorescence the expression of BECLIN 1 and of LC3 in various histologic subtypes of ovarian cancer. The ratio of BECLIN 1 and BCL-2 expression was also determined by western blotting in some selected cases. We noted that type I ovarian carcinomas that are clinically less aggressive than type II were more frequently expressing high level of BECLIN 1. Conversely, low level of BECLIN 1 expression correlated with histologic grade III tumours. No statistically significant association with patient survival was found in the cases judged negative for BECLIN 1 expression. On the other hand, granular-like positivity of BECLIN 1 and LC3, which is indicative of ongoing autophagy, was more frequently observed in tumours from patients with a better survival. These data suggest that ovarian cancer progression is facilitated by low level of intrinsic autophagy and that ovarian cancers with upregulated autophagy are more likely to respond to therapeutic treatments and to progress more slowly.

2. Materials and Methods

2.1. Patients, Therapeutic Treatments, and Tissue Collection. The present retrospective study includes 61 cases of ovarian carcinomas selected in the years 1999–2004 from the archived materials of the Department of Gynecology of Università di Torino (Italy). All cases were classified according to the current WHO Classification of Neoplasms. All patients underwent surgery. With the exception of those staged as pT1/G1 (for whom no further treatment was required), all patients were thereafter subjected to a standard chemotherapy regimen which included Carboplatin AUC 5/6 and Paclitaxel 175 mg/mq every 3 weeks for 6 cycles, outside of clinical trials. Follow-up ended in 2009. Biopsies were obtained at the time of the first surgery. Formalin-fixed paraffin-embedded tissue sections were prepared and used for diagnostic purposes and for the present investigation. No oral or written informed consent was obtained from the patients for the use of these retrospective samples, since it was not deemed necessary by the local ethics committee. All samples were treated anonymously.

2.2. Tissue Immunoreactivity for BECLIN 1, LC3, and BCL-2. Immunohistochemistry and immunofluorescence were performed in deparaffinized tissue sections following our published protocol [13]. Proteins of interest were revealed by subsequent incubation of the tissue with a primary (first step) and a secondary (second step) antibody. In the first step the following primary antibodies, either alone or in appropriate combination, were used: (a) anti-BECLIN 1 mouse monoclonal (BD Pharmingen, San Diego, CA; dilution 1 : 100) or anti-BECLIN 1 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1 : 100); (b) anti-LC3 rabbit polyclonal (Novus Biological, Littleton, Colorado; dilution 1 : 500); (c) anti-BCL-2 mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1 : 100). Appropriate secondary antibodies, goat-anti-mouse IgG or goat-anti-rabbit IgG (Sigma-Aldrich Inc., St. Louis, MO; dilution

1 : 200), labelled with horse-radish-peroxidase (for immunohistochemistry) or with FITC or Texas Red fluorescent dye (for immunofluorescence), were used in the second step. The section subjected to immunofluorescence were also stained with the fluorescent dye 4-6-diamidino-2-phenylindole-dihydrochloride (DAPI, 1 : 500 from a stock solution 20 mg/mL; 1 h) to evidence the nucleus. The sections were mounted with Slow-FAD (Light AntiFADE kit, Molecular Probes Invitrogen, Carlsband, CA, USA), observed under a fluorescence microscope (Leica DM1600, Leica Microsystem, Heidelberg, Germany) and representative areas were imaged with a digital camera.

2.3. Evaluation of Tissue Positivity for Autophagy Proteins. To assess the immunoreactivity for BECLIN 1 and for LC3 proteins, at least five fields randomly chosen (approximately 5000 cells) per section were evaluated independently by three investigators (GV and CI for IHC; GN and CI for IF). The sample was considered positive only when the immunoreaction presented with a granular-like pattern. For this purpose, high magnification images were used. Only neoplastic cells were counted. The proportion of positive cells over the total number of cells present in the imaged areas were expressed as percentage. A final hybrid score (H) was assigned to each sample, based on the product of a 0–3 scale of staining intensity and of the percentage of positive cells (0–100%), with a possible range of results from 0 to 300. Each biopsy was tested at least two times.

2.4. Tissue Western Blotting of BECLIN 1 and of BCL-2. For some samples a frozen biopsy was also available and used for western blotting detection of BECLIN 1 and of BCL-2, following our published protocol [13]. Essentially, a piece of frozen biopsy was homogenized by several cycles of freeze-thawing and sonication in a phosphate buffer containing detergents and protease inhibitors. A 30 μg of protein homogenate was resolved by SDS-polyacrylamide gel electrophoresis and thereafter electrotransferred into a nitrocellulose membrane. Standard procedure for western blotting was used [21] to detect BECLIN 1 and BCL-2, respectively, with a monoclonal antibody (BD Pharmingen; dilution 1 : 250) and a rabbit polyclonal antiserum (Santa Cruz Biotechnology; dilution 1 : 100). After stripping, the filter was reprobed to detect actin, used as a loading marker. Appropriate peroxidase-conjugated secondary antibodies (purchased from Sigma-Aldrich; dilution 1 : 20.000) were used to reveal the immunocomplexes through peroxidase-induced chemiluminescence reaction (Biorad, Hercules, CA, USA).

2.5. Statistical Analysis. BECLIN 1 and LC3 granular-like positivity as assessed by IHC and/or IF was correlated to the clinical outcome referred to as complete remission (CR) and overall survival (OS) at 5 years. The odds ratio, the relative risk, and the Chi-square were calculated using the Microsoft Excel XLStat 2010 software. The Fisher's exact test was also employed for pairwise comparison of distributions of categorized groups. A P value lower than 0.05 was taken to indicate data statistically significant.

3. Results

3.1. Histologic Type and Main Clinical Characteristics of Ovary Carcinomas Included in the Study. This retrospective study included 61 cases of ovary carcinomas of various histologic types selected from our archived materials. The tumours were grouped as type I and type II [1]. All patients were subjected to surgery and chemotherapy, following standard criteria based on clinical stage and patient performance status. The following information was available: clinical stage at first diagnosis, histologic type, objective response to chemotherapy, and clinical outcome. Response to therapy regimen was evaluated according to the international guidelines. Clinical outcomes were classified as complete remission (CR) or alternatively as not evidence of disease (NED), that is, disappearance of any evidence of disease during the follow-up or for at least four years), partial remission (PR, ≥ 50% decrease of tumour lesions for at least 24 months), and DOD (dead of disease). Overall survival (OS) was calculated from the time of first diagnosis to the end of the follow-up, which terminated in 2009. The database with the histologic, clinical, and patients' main information of the cases included in the present study is reported in Supplementary Table ST1 (see Supplementary Table ST1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/462658).

3.2. Immunodetection of BECLIN 1 in Ovary Cancer Tissues. The presence and the cytoplasmic distribution of BECLIN 1 were first analysed by immunohistochemistry (IHC) in paraffin-embedded tissue sections of ovary carcinomas. BECLIN 1 immunoreactivity in tumour cells presented as a faintly detectable staining diffused in the cytoplasm or as discrete stained puncta (referred to as granular-type) clearly evident in the vicinity of the nucleus. The former immunoreactivity pattern was considered as negative, whereas the latter was considered as positive in terms of BECLIN 1 macroaggregates and indicative of active autophagy. A parallel analysis of BECLIN 1 expression was conducted by immunofluorescence (IF) in the same sections. Results from both techniques overlapped, though some cases judged negative on IHC appeared faintly positive on IF, owing to the highest sensitivity of the latter technique. Representative images of BECLIN 1 expression and cellular distribution, as assessed by IHC and IF in selected cases, are shown in Figures 1 and 2, respectively.

3.3. Correlation of BECLIN 1 Expression with Histologic Type. Based on the proportion of BECLIN 1-positive cells within the tumour tissue, the samples were initially stratified into four ranges of positivity: <10%; 10–20%; 20–40%; >40%. Based on the intensity (on a 0 to 3 scale) and on the proportion of the cells positive for BECLIN 1 as assessed by IHC, hybrid score (H) was assigned to each section independently by two pathologists (GV and CI). To indicate positivity for BECLIN 1 expression the final threshold was set at ≥20% of cells showing a granular-like staining of intensity ≥2 (H ≥ 40). A high proportion of BECLIN 1-positive cells was reported in 41 out of 61 tumours. Of note, while type II tumours showed an approximately equal distribution of BECLIN 1 positivity (11

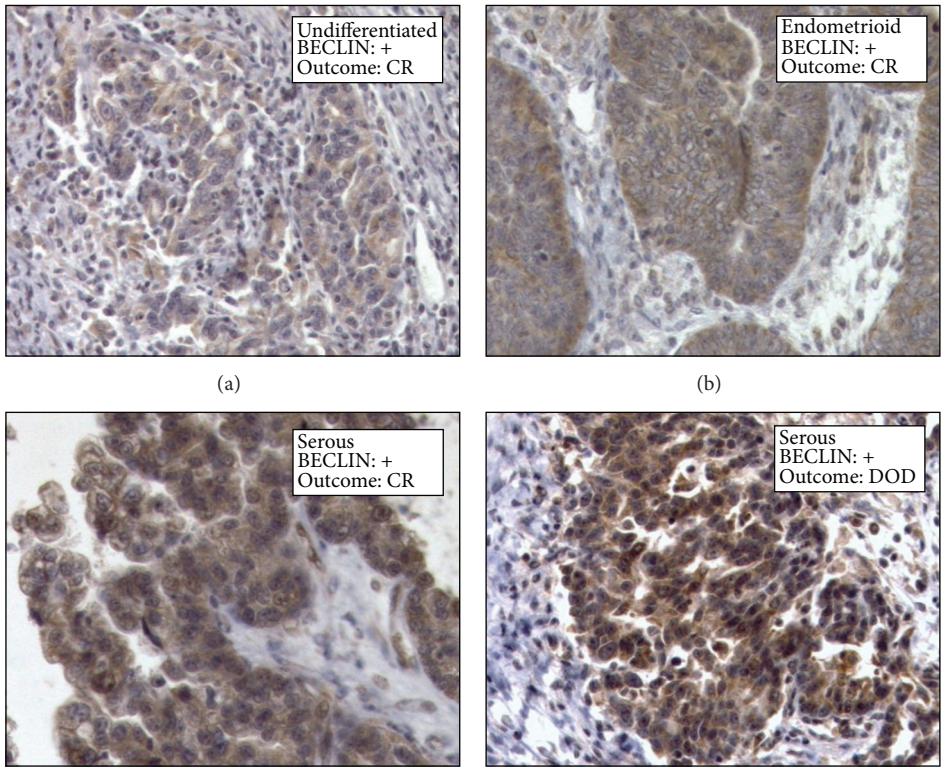


Figure 1: Immunohistochemical detection of BECLIN 1. Selection of representative cases. The histologic type and the clinical outcome (CR: complete remission; DOD: dead of disease) are indicated. Magnification 220x.

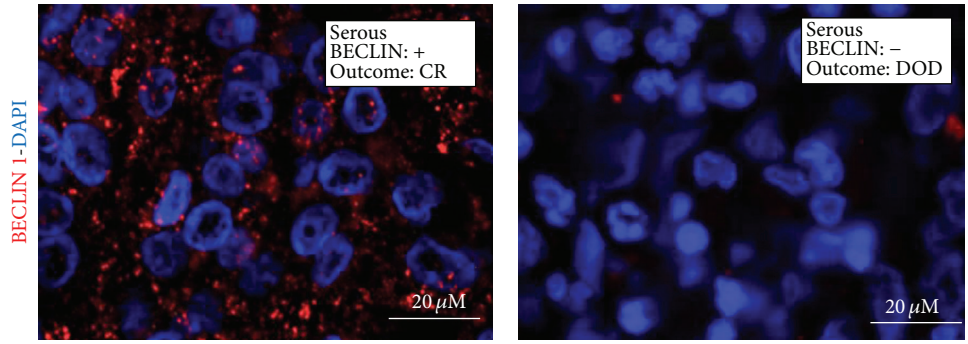


Figure 2: Immunofluorescence detection of BECLIN 1. Selection of representative cases. The histologic type and the clinical outcome (CR: complete remission; DOD: dead of disease) are indicated. The nuclei are evidenced by DAPI staining.

positive and 8 negative), as many as 20 out of 23 type I tumours were found highly expressing BECLIN 1. More in detail, >20% BECLIN 1-positive cells (>40 H) were found in the majority of endometrioid adenocarcinomas (11/13) and of serous cystadenocarcinomas (19/27). However, there was no statistically significant association between the extent of BECLIN 1-positive cells and a particular histologic type of ovarian cancer (Table 1).

3.4. BECLIN 1 Expression Correlates with Histologic Grading but Not with Pathological Staging at Diagnosis. Next, we looked for any correlation between the extent of BECLIN 1 expression and the aggressiveness of ovarian cancers as mirrored by the histologic grading and the pathological stage at diagnosis. It was found that while tumours with a high expression of BECLIN 1 were equally distributed in I-II and III grade, tumours negative or low expressing BECLIN 1 more frequently (18 out of 20) belonged to grade III (Table 2(a)). This correlation was statistically significant ($P = 0.004$). With regard to the pathological staging, it was found that of the 20 carcinomas with <20% of BECLIN 1-positive cells, 10 were classified as I-II stage and 10 as III-IV stage; of the 41

Table 1: Distribution of BECLIN 1 positivity (in terms of H ≥ 40) among ovarian carcinoma histologic types.

BECLIN 1 positive	Yes	No	Number of cases
Histologic type I			
Serous (low grade)	8	0	8
Endometrioid (low grade)	8	0	8
Clear cell	2	2	4
Mucinous	2	1	3
Transitional (Brenner)	0	1	1
Histologic type II			
Serous (high grade)	11	8	19
Endometrioid (high grade)	3	2	5
Undifferentiated	6	5	11
Mixed mesodermal	1	1	2
Total	**41**	**20**	**61**

Table 2: Correlation of BECLIN 1 positivity (in terms of H ≥ 40) with clinical-pathological characteristics. (a) Statistical correlation with histologic grade; (b) statistical correlation with pathologic stage at diagnosis.

(a)

Grade BECLIN 1	I-II	III	Number of cases
+	21	20	41
−	2	18	20
Total	**23**	**38**	**61**

Chi-square = 8.05
DF = 1
$P = 0.0046$
Fischer's test $P = 0.002$.

(b)

Stage BECLIN 1	I-II	III-IV	Number of cases
+	24	17	41
−	10	10	20
Total	**34**	**27**	**61**

Chi-square = 0.13
DF = 1
$P = 0.7$
Fischer's test $P = 0.59$.

carcinomas with ≥20% BECLIN 1-positive cells, 24 were of I-II stage and 17 of III-IV stage (Table 2(b)). No significant correlation was found between the positivity for BECLIN 1 and the pathological stage ($P = 0.7$). On the whole, these findings indicate that the absence of BECLIN 1 expression, which likely determines defective autophagy, favours a more malignant phenotype of the tumour, though other factors, independent of the intrinsic autophagy capacity, influence the evolution of the disease and the accompanying general symptoms that lead to the first diagnosis.

3.5. Ovarian Carcinomas Highly Expressing BECLIN 1 Associate with Better Patient's Clinical Outcome. We asked about the clinical significance of BECLIN 1 expression in terms of the impact on the posttherapy outcome. The patients were all subjected to surgical removal of the ovaries and annexes, followed by a standard chemotherapeutic treatment protocol. Chemotherapeutics included Carboplatin and Paclitaxel. For seven patients, staged as pT1 and bearing a G1 tumour, no adjuvant chemotherapy was administered. We first correlated the expression of BECLIN 1 with the patient's overall survival (OS) at 5 years. Patients bearing a tumour with a low expression of BECLIN 1 (H < 40) showed no differences in terms of OS, with 9 being dead and 11 still alive at the time of the end of the follow-up (Table 3(a)). By contrast, a statistically significant correlation was found between the high expression of BECLIN 1 (i.e., tumours with ≥20% of positive cells) and patient's OS. In particular, of the 41 patients bearing a tumour highly expressing BECLIN 1, 34 (~83%) were still alive at the end-point of the study and only 7 (17%) died during the observation period. These correlations were statistically significant ($P < 0.03$). We then considered the clinical outcome separately as CR (or NED), PR, and DOD to see any correlation with the expression of BECLIN 1. Amongst the 61 cases, 32 patients (52%) underwent CR. Of these, as many as 24 (75%) were bearing an ovary cancer with ≥20% BECLIN 1-positive cells. Conversely, only 8 out of 32 (25%) patients in CR were bearing a cancer with a ≤20% of BECLIN 1-positive cells (Table 3(b)). PR was more frequently observed in the group of patients bearing a cancer with a high proportion of BECLIN 1-positive tumour cells than in the group of patients bearing a BECLIN-negative cancer (25% versus 15%), and DOD was also less frequent in the former than in the latter group of patients (17% versus 45%). These correlations were, however, not statistically significant ($P < 0.06$). Altogether, these observations support the content that the high expression of BECLIN 1 in ovarian carcinomas associates with a better prognosis. However, no correlation with the clinical outcome was found in the group of patients bearing a tumour negative or low expressing BECLIN 1. We have also performed the analysis of the overall survival probability of the patients by the Kaplan-Meier method (Supplementary Figure 1, SF1). Log-rank test indicated that the association of high expression of BECLIN 1 in the tumour with a good prognosis was statistically significant. Yet, a larger number of patients should be studied in order to substantiate the above finding.

3.6. BECLIN 1 and LC3 Double Positivity Predicts a Favourable Prognosis in Ovarian Cancer. In autophagy active cells, the microtubule-associated LC3 protein is posttranslationally translocated into the membranes of autophagosomes [8]. Therefore, the detection of a granular-like staining of LC3 can be assumed *bona fide* as the proof of the presence of autophagic vacuoles (either autophagosomes or autophagolysosomes) in the cell. We analysed by immunofluorescence the expression of LC3 in selected BECLIN 1-positive ($n = 30$) and BECLIN 1-negative cases ($n = 12$). Cells were considered positive for ongoing autophagy when showing a granular-like staining for LC3 and the tumour was considered autophagy-active when ≥20% of the cells were LC3 positive.

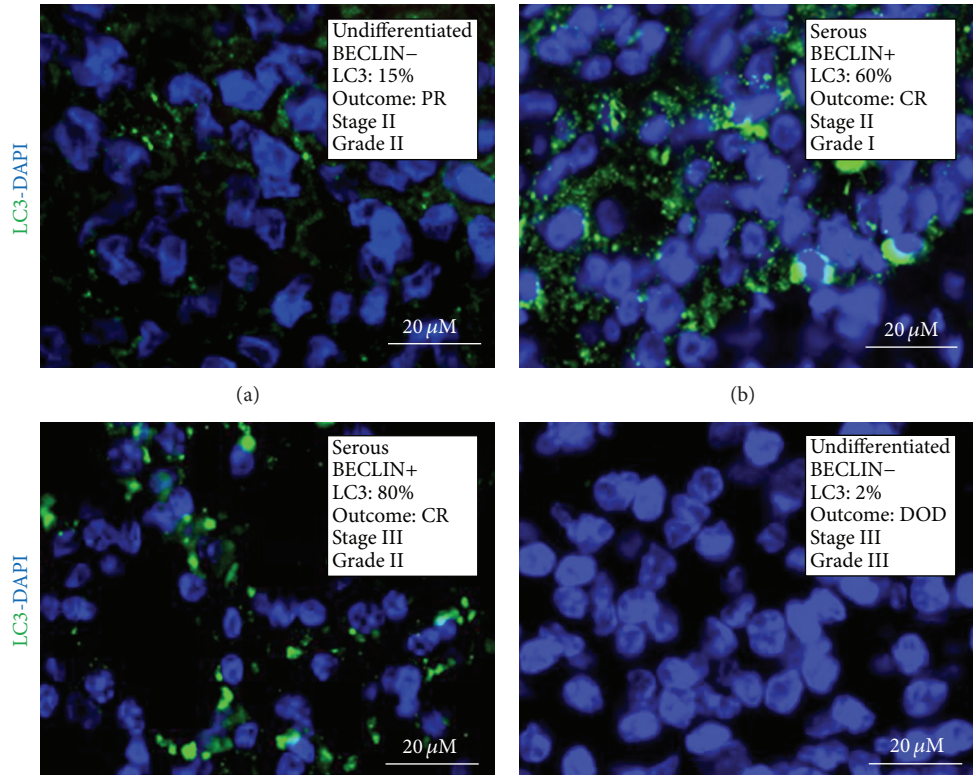


Figure 3: Immunofluorescence staining of LC3 in ovarian cancer tissue sections. Selection of representative cases. The histologic type, the positivity for BECLIN 1 aggregates, the percentage of cells positive for vacuolar LC3, the clinical outcome (CR: complete remission; PR: partial remission; DOD: dead of disease), the pathological stage, and the histologic grade are reported. The nuclei are evidenced by DAPI staining.

Table 3: Correlation of BECLIN 1 expression with clinical outcome in patients.

(a)

Clinical outcome BECLIN 1	Survivors	DOD	Number of cases
+	34	7	41
−	11	9	20
Total	**45**	**16**	**61**

Chi-square = 4.07
DF = 1
$P = 0.04$
Fischer's test
$P = 0.03$.

(b)

Clinical outcome BECLIN 1	CR	PR	DOD	Number of cases
+	24	10	7	41
−	8	3	9	20
Total	**32**	**13**	**16**	**61**

Chi-square = 5.4
DF = 2
$P = 0.066$.

Examples of LC3 staining in BECLIN 1-positive tumour cells are shown in Figure 3. With a few exceptions, cases judged positive for BECLIN 1 were highly positive also for LC3. On the whole, we found a concordance of 70% between the expression of both BECLIN 1 and LC3.

To further substantiate the involvement of autophagy in the progression and chemotherapeutic response of ovarian carcinomas, we correlated the expression of LC3 with the clinical outcome. When restricted to the group of BECLIN 1-positive tumours, it was found that 20 out of 21 patients bearing a tumour also positive for LC3 were still alive, while 6 out of 9 of those patients bearing a tumour negative for LC3 were DOD, at 5 years after diagnosis (Table 4(a)). These correlations were statistically significant ($P < 0.0002$). Statistics was then applied to the whole group of tumours analyzed for LC3 positivity, including both the BECLIN 1 positive and BECLIN 1 negative. On the whole, 23 out of 24 patients with an LC3-positive tumour were still alive, while 11 out 18 patients with an LC3-negative tumour were DOD, at 5 years after diagnosis (Table 4(b)). Of note, in this case the correlations were even more significant ($P < 0.0002$).

3.7. Coexpression of BECLIN 1 and BCL-2 in relation to Autophagy in Ovarian Cancers. The interaction of BECLIN 1 with BCL-2 abrogates the induction of autophagy [22]. On the other hand, high expression of BCL-2 inhibits not only

Table 4: Correlation of LC3 expression with patients overall survival. (a) Group of BECLIN 1 positive tumours; (b) group of BECLIN 1 positive and negative tumours.

(a)

% LC3 positive	Survivors 5 y	DOD	Number of cases
<20%	3	6	9
≥20%	20	1	21
Total	**23**	**7**	**30**

Chi-square = 13.5
DF = 1
$P = 0.0002$.

(b)

% LC3 positive	Survivors 5 y	DOD	Number of cases
<20%	7	11	18
≥20%	23	1	24
Total	**30**	**12**	**42**

Chi-square = 16.34
DF = 1
$P = 0.0001$.

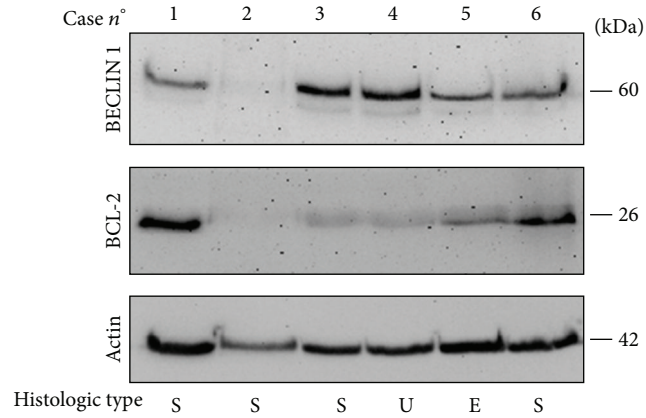


Figure 4: Western blotting analysis of the expression of BECLIN 1 and of BCL-2 proteins in ovarian carcinomas. Selection of representative cases. Tissue homogenates were subsequently probed for BECLIN 1, BCL-2, and actin (the latter was used as reference of homogenate protein loading). The molecular weight of proteins detected with the specific antibodies is indicated. Histologic type: S: serous; U: undifferentiated; E: endometrioid.

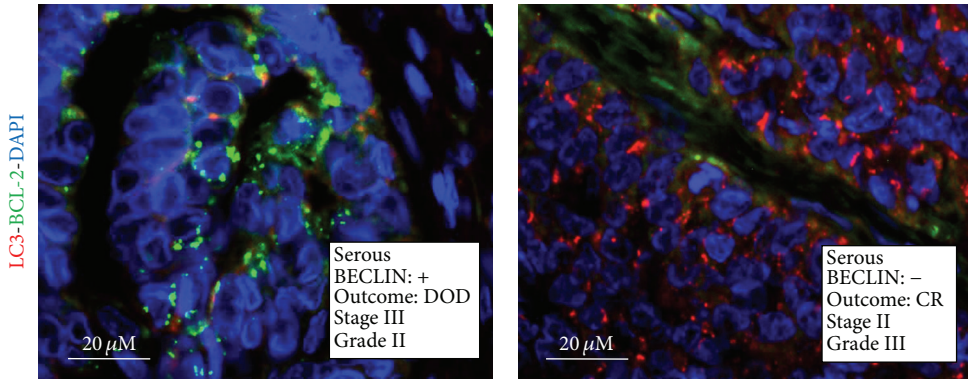
autophagy but also apoptosis, thus influencing the cytotoxic response of ovarian cancer cells to chemotherapeutics [17, 21]. Thus, evaluating the level of expression of BECLIN 1 may not be sufficient to draw conclusions about the capacity of the cell to activate autophagy. We have analysed by western blotting the expression of BECLIN 1 and of BCL-2 in a small subset of carcinomas for which the frozen biopsy was available (representative cases are shown in Figure 4). In general, the expression of these proteins was inversely related. To seek for a functional relationship between the two proteins, we performed the immunostaining of BECLIN 1, BCL-2, and LC3 in two paradigmatic situations among the cases analysed by western blotting. In case 1, the expression of BCL-2 was quite high, which could account for inhibition of BECLIN 1 proautophagic activity, and in fact this tumour was negative for LC3 staining (Figure 5(a)). On the opposite, BCL-2 and BECLIN 1 were not detectable (by western blotting) in the tumour case 2, and in spite of this the tumour was intensely LC3 positive (Figure 5(b)), which possibly was associated with BECLIN 1-independent autophagy.

4. Discussion

Autophagy, a cell homeostatic process for the lysosome-driven degradation of aged, damaged, and redundant self-constituents, may either suppress or facilitate carcinogenesis [7, 23]. The heterozygous deletion of the autophagy gene *BECLIN 1* in transgenic mice predisposes to the development of spontaneous tumours, including ovarian cancers [11, 24]. Accordingly, the expression of the BECLIN 1 protein and also of the autophagosome protein LC3 was found much lower in malignant ovarian cancers compared to benign ovary epithelial tissues [16, 17]. In our series, we also have found that 18 out of 20 ovarian cancers of histologic grade III were negative or low expressing BECLIN 1. This is consistent with the view that defective autophagy might favour cancer progression. In this same line, a decreased level of BECLIN 1 expression, especially in conjunction with increased expression of BCL-xL, was correlated with poor prognosis in ovarian cancer bearing patients [17]. Here we have analysed the tissue expression of BECLIN 1 in a series of 61 cases of ovarian carcinomas of various histologic types. BECLIN 1 staining presented with either a cytoplasmic diffused pattern (regarded as negative) or a granular-like pattern (regarded as positive). The latter likely reflected the engagement of BECLIN 1 in the oligomeric interactome with PI3-kinase class III [12], which preludes to the initiation of autophagy [22]. Fourteen (of the 61) cancers examined showed positive for BECLIN 1 in a percentage of cells ranging from 20% to 90%. The expression of BECLIN 1 was not correlated with patient's age at the time of diagnosis, nor was it correlated with a particular histologic type. It is to be noted, however, that in our series some histologic types were underrepresented so that no conclusion could be drawn with regard to the association between autophagy and histotypes. On the other hand, being autophagy, an evolutionary conserved and ubiquitous process, it is conceivable that it is not restricted to a particular subtype of cancer. Setting the cut-off at 20% of positive cells (in terms of BECLIN 1 macroaggregates), a positive correlation was found between negative expression and high histologic grade. In general, the clinically indolent type I tumours were more frequently expressing BECLIN 1 at high level.

However, no statistically significant correlation was found between the positive expression and the pathological stage at diagnosis. Thus, while defective autophagy likely favours the emergence of highly malignant clones, other factors influence the general evolution of the disease in the patient.

Tumours negative for BECLIN 1 showed no correlation with prognosis (11 survivors and 9 DOD), whereas of the 41



(a) (b)

Figure 5: Immunofluorescence staining of LC3 and BCL-2. Selection of representative cases. The histologic type, the positivity for BECLIN 1 aggregates, the clinical outcome (CR: complete remission; DOD: dead of disease), the pathological stage, and the histologic grade are reported. The nuclei are evidenced by DAPI staining.

patients bearing a BECLIN 1-positive tumours as many as 34 showed a favourable prognosis (24 CR and 10 PR). Seen from a different point, of the 32 patients that underwent CR as many as 24 were bearing a BECLIN 1-positive cancer and only 8 were bearing a BECLIN 1-negative cancer. These correlations were statistically significant. While our data seem to be consistent with the findings reported by Shen et al. [16] and Lin et al. [17], other authors have reported opposite findings. In one study [18], the expression of BECLIN 1 was inversely correlated with the histologic grade of differentiation of ovarian carcinomas and the high level of BECLIN 1 expression was associated with a lower relapse-free survival rate of the patients. High level of BECLIN 1 was also found associated with invasive endometrioid cancers and poor 5-year survival [19]. However, both in these studies BECLIN 1 was not an independent prognostic factor. In our series, we have indeed observed that seven patients bearing a cancer with >20% of BECLIN1-positive cells deceased within the follow-up period. Assuming that BECLIN 1 main function was to drive autophagy and that autophagy was playing a positive role in the response to chemotherapy treatments, we considered the possibility that failure in the chemotherapy response in those patients could arise from impaired (or insufficient) induction of autophagy in the tumour cells expressing BECLIN 1. To better detect autophagy active cells in the tumour, we stained the cells for LC3, an autophagosomal protein considered to be hallmark of ongoing autophagy [8]. In general, a high concordance between BECLIN 1 and LC3 positivity was observed in the large majority of the cases. In some cases, LC3 was negative in spite of the positivity for BECLIN 1. This fact was likely due to the concomitant high expression of BCL-2, which is known to nullify the autophagy function of BECLIN 1 [22], as was proven in at least some of the cases. We found that the BECLIN 1-positive cancers associated with the patients deceased during the study were indeed negative for vacuolar LC3 staining and highly expressing BCL-2. Though not statistically relevant because of the small number of cases, indirectly our finding agrees with that reported by Lin et al. [18], who showed that low expression of BECLIN 1 in combination with high expression of BCL-xL predicts a poor survival in ovarian cancer patients. Of note, also LC3 positivity significantly correlated with patient's overall survival at 5 years after diagnosis, thus supporting the contention that the patients bearing a tumour with a high proportion of autophagy-active cells had a better prognosis. In this regard, it is to be mentioned that, in clear cell ovarian cancer histotypes, but not in other examined histotypes, the high expression of LC3A was found to significantly correlate with hypoxia and poor prognosis [20]. We could not compare with this study, as in our series we had only 4 cases of clear cell carcinomas, 2 each either BECLIN 1 positive or BECLIN 1 negative.

It remains to be explained through which molecular pathway the ongoing autophagy in cancer cells could turn of benefit in the chemotherapeutic response so that the patient experiences a better prognosis. The two-hit model predicts a synergistic death effect of two proautophagic stimuli [25]. In fact, although autophagy is in principle a prosurvival pathway, it might also lead to cell death if dysregulated [12, 23, 26]. In particular, cells in which autophagy is basally upregulated may undergo apoptosis if subjected to an additional metabolic or genotoxic stress that hyperinduces autophagy [25]. We hypothesize that autophagy-active cancer cells may succumb in response to drugs that hyperstimulate autophagy. This is the rationale for the use of mTOR inhibitors in ongoing clinical trials for the treatment of ovarian cancers [7]. With relevance to our chemotherapy protocol, it has been reported that the transgenic overexpression of BECLIN 1 sensitizes cervical cancer cells to carboplatin and to paclitaxel by promoting apoptosis and autophagic cell death [27, 28]. BECLIN 1 and BCL-2 occupy a central role in the complex cross-talk between autophagy and apoptosis [29], and chemotherapeutic drugs could be more effective in those cells with an altered ratio between these two proteins. Consistent with our hypothesis, it was recently shown that the Src/Abl kinases inhibitor Dasatinib arrested the growth of ovarian cancer xenograft by inducing BECLIN 1-dependent autophagic cell death, and hyperstimulation of autophagy was associated with downregulation of BCL-2 expression [30].

This could also explain the poor survival reported in women bearing an ovarian cancer expressing low level of BECLIN 1 and high level of BCL-xL [17]. Besides, the high expression of BECLIN 1 could enhance the cytotoxic response to a chemotherapeutic drug in ovarian cancer cells also via an autophagy-independent mechanism [31]. Additionally, the hypothesis that tumour with intrinsic high level of basal autophagy may have a better prognosis even without chemotherapy cannot be excluded. Though we could not test directly this hypothesis, we note that of the 7 patients for whom chemotherapy was not deemed (because they were staged as pT1 and the tumour was of grade 1) 6 were bearing a BECLIN 1-positive tumour and underwent CR, whereas 1 was bearing a BECLIN 1-negative tumour and was DOD.

In conclusion, while on one hand the upregulation of basal autophagy associated with a higher ratio of BECLIN 1 versus BCL-2 proteins expression enables the cancer cells to overcome the metabolic stresses caused by the lack of oxygen and nutrients, it on the other hand also renders these cells more susceptible to chemotherapeutic drugs that overstimulate autophagy. Given the role of mitochondria in the apoptotic response to chemotherapeutics [32], we suspect that in the latter case apoptosis ensues because of the exaggerated mitophagy. Thus, to improve the chance to cure ovarian carcinomas, one should carefully consider whether to employ autophagy inhibitors or autophagy-enhancer drugs in the chemotherapy cocktail depending on the ratio of BECLIN 1 and BCL-2 expression and the actual level of autophagy in the cancer cells.

Conflict of Interests

The authors declare that no conflict of interests exists.

Acknowledgment

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Research Article

Unique Pattern of Component Gene Disruption in the NRF2 Inhibitor KEAP1/CUL3/RBX1 E3-Ubiquitin Ligase Complex in Serous Ovarian Cancer

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The NFE2-related factor 2 (NRF2) pathway is critical to initiate responses to oxidative stress; however, constitutive activation occurs in different cancer types, including serous ovarian carcinomas (OVCA). The KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex is a regulator of NRF2 levels. Hence, we investigated the DNA-level mechanisms affecting these genes in OVCA. DNA copy-number loss (CNL), promoter hypermethylation, mRNA expression, and sequence mutation for *KEAP1*, *CUL3*, and *RBX1* were assessed in a cohort of 568 OVCA from The Cancer Genome Atlas. Almost 90% of cases exhibited loss-of-function alterations in any components of the NRF2 inhibitory complex. CNL is the most prominent mechanism of component disruption, with RBX1 being the most frequently disrupted component. These alterations were associated with reduced mRNA expression of complex components, and NRF2 target gene expression was positively enriched in 90% of samples harboring altered complex components. Disruption occurs through a unique DNA-level alteration pattern in OVCA. We conclude that a remarkably high frequency of DNA and mRNA alterations affects components of the KEAP1/CUL3/RBX1 complex, through a unique pattern of genetic mechanisms. Together, these results suggest a key role for the KEAP1/CUL3/RBX1 complex and NRF2 pathway deregulation in OVCA.

1. Introduction

Reactive oxygen species (ROS) participate in normal hormonogenesis and physiological functions of the ovaries, such as steroid hormone production, ovulation, and essential preovulatory responses [1–3]. Hence, tight regulation of ROS levels in the ovaries is required.

The NFE2-related factor 2 (NRF2) pathway is the primary regulator of cellular ROS levels (reviewed in [4–7]). Under basal conditions, NRF2 protein—encoded by the *NFE2L2* gene—is rapidly targeted for proteasomal degradation through interaction with an E3-ubiquitin ligase protein complex, whose protein components include Kelch-like ECH-associated protein 1 (KEAP1), Cullin 3 (CUL3), and ring-box 1, E3-ubiquitin protein ligase (RBX1) (Figure 1). KEAP1 acts as a substrate adaptor, interacting with NRF2 through ETGE and extended DLG motifs [8, 9]. Subsequently, NRF2 interacts with the CUL3 N terminal region, while RBX1 recruits the catalytic function of ubiquitin-conjugating enzyme (E3) [10]. An abnormal increase in ROS levels induces the formation of disulfide bonds between cysteine residues of KEAP1, which liberates NRF2, although some studies have suggested that electrophilic modification of Keap1 does not lead to complex disruption [11, 12]. Moreover, a cyclic degradation model involving sequential binding of NRF2 first to the ETGE motif and then through the DLG motif has been proposed [13]. This allows its translocation to the nucleus and subsequent induction of cytoprotective genes [6, 14, 15].

Besides its protective role, an emerging concept is that constitutive activation of NRF2 and its target genes can result in promotion of tumor growth and resistance to oxidants and anticancer drugs in a number of tumor types [6, 16, 17]. Constitutive activation of NRF2 is associated with acquisition of malignant features and has been demonstrated in various tumor types, including serous ovarian carcinoma

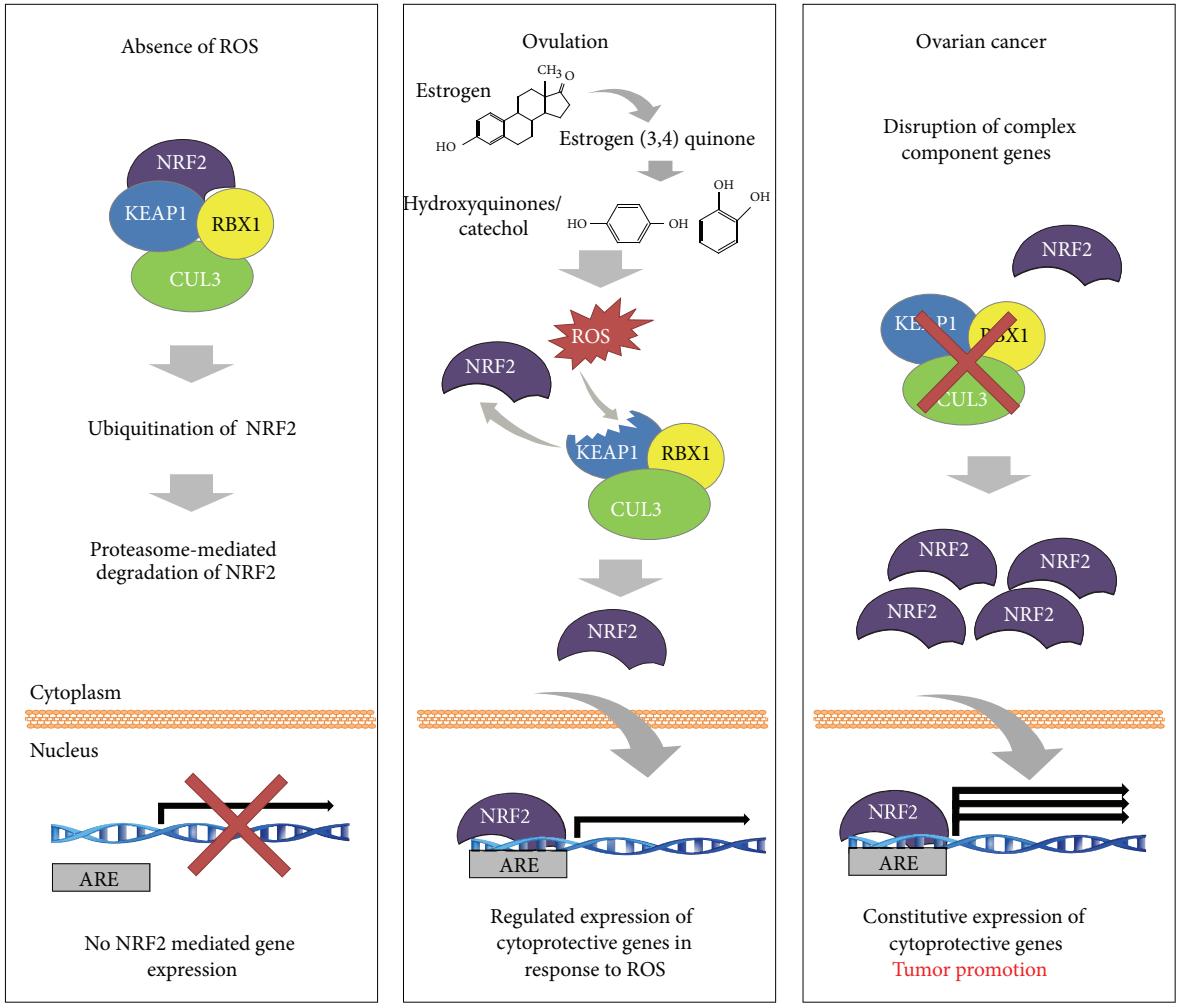

Figure 1: *KEAP1/CUL3/RBX1 E3-ligase protein complex*. In the absence of ROS (a), NRF2 is regulated by the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex which targets NRF2 for proteasomal degradation and inhibits expression of NRF2-controlled genes. The oxidative metabolism of estrogen through the catechol pathway induces the generation of reactive oxygen species (ROS, b). These oxidative species induce conformational changes in KEAP1, which disrupt the activity of the inhibitory complex. As a consequence, NRF2 is stabilized and translocates to the nucleus, where it induces expression of cytoprotective genes containing NRF2-regulatory sequence motifs (e.g., antioxidant response elements, AREs). When the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex is compromised by genetic alteration in any of its component genes (c), NRF2 is stabilized and accumulated and transported to the nucleus. Under these conditions, the activation of cytoprotective genes becomes constitutive, which has been associated with tumor promotion.

(OVCA) [4, 18]. Gain-of-function mutations in *NFE2L2* and inactivating *KEAP1* mutations are the most frequent NRF2 activation mechanisms observed in breast, gallbladder, and lung tumors, among other cancer types [19–23]. Notably, multiple inactivating genetic mechanisms affecting components of the KEAP1/CUL3/RBX1 inhibitory complex are also known to occur, and the disruption of even a single complex component has been shown to compromise its function and stimulate substrate accumulation in lung tumors [24].

Traditional approaches for identifying driver alterations usually focus on high frequency, single-gene disruption. However, this approach may overlook biologically significant events, for example, when multiple gene products are required for proper multiprotein complex function [24–26]. For instance, a single component of a multiprotein complex or pathway may be disrupted at low frequency, but a high cumulative frequency of functional disruption may occur when alterations to individual complex components are simultaneously considered.

Genetic and epigenetic mechanisms underlying NRF2 activation in OVCA remain to be elucidated. A previous study identified heterozygous missense *KEAP1* mutations in 5 of 27 (19%) ovarian carcinomas, although frequencies differ across subtypes (29% and 8% in clear cell and serous tumors, resp. [27]). Interestingly, the same study noted 50% of tumors without *KEAP1* mutations exhibited nuclear localization of NRF2

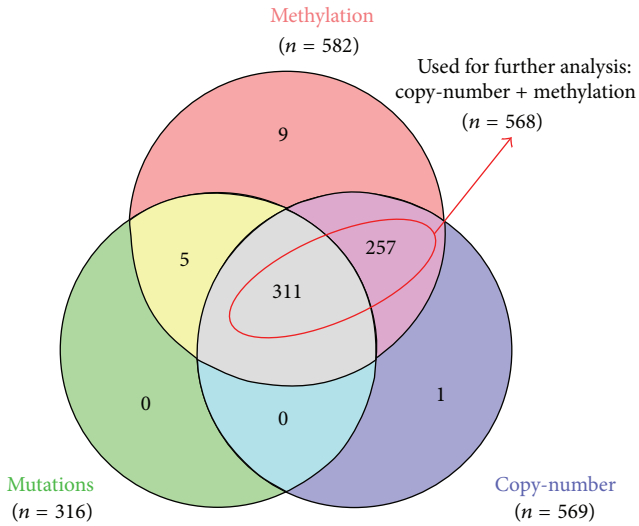

FIGURE 2: *Number of samples with the various types of data.* Information for DNA sequence mutation (green, $n = 316$), copy-number (purple, $n = 569$), and methylation (orange, $n = 582$) were retrieved from the cBio portal for Cancer Genomics. For subsequent frequency calculations comparing genetic and epigenetic mechanisms, we focused on the cases with both copy-number and methylation data ($n = 568$, i.e., cases circled in red).

protein (denoting pathway activation), suggesting that other mechanisms are likely driving NRF2 pathway activation in ovarian tumors. We hypothesized that DNA-level disruptions affecting the master NRF2 inhibitory complex may account for this discrepancy. Therefore, we assessed different types of DNA-level inactivating alterations (DNA sequence mutation, copy-number loss, and DNA hypermethylation) affecting the component genes of the CUL3/KEAP1/RBX1 E3-ubiquitin ligase complex in 568 OVCA cases from The Cancer Genome Atlas (TCGA) project.

2. Materials and Methods

2.1. Tumor Samples and Data Analysis. Genomic and epigenomic information for OVCA were obtained from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) [28, 29] and the cBio portal for Cancer Genomics [30]. Level 3 data for DNA sequence mutation (somatic mutation calls for each participant), copy-number (putative copy-number calls, per sample), methylation (calculated beta values mapped to the genome, per sample), and mRNA (expression calls for genes, per sample) were used for analysis of different 'omics dimensions (Figure 2).

2.2. DNA Sequence Mutations. Mutation data (derived from exome sequencing) were obtained for 316 cases (Figure 2). Mutation status and predicted functional impact was assessed through the cBioPortal for Cancer Genomics [31]. Non-synonymous DNA sequence mutations with medium/high predicted functional impact scores were considered.

2.3. DNA Copy-Number Alterations. A total of 569 DNA copy-number profiles (Affymetrix GenomeWide SNP 6.0 platform) were obtained (Figure 2). In addition, copy-number data generated by the GISTIC algorithm [32] were also obtained through the cBio portal [31]. Both heterozygous (-1) and homozygous (-2) copy-number losses were considered when assessing inactivating DNA-level alterations affecting OVCA cases.

2.4. DNA Methylation Status. Methylation profiles (Illumina BeadArray 27K platform) for 582 samples were obtained from TCGA (Figure 2). Additionally, 8 profiles derived from organ-specific controls for ovarian tissue were retrieved for comparisons. Beta values (from probes located at promoter regions for each gene) were compared with beta values derived from organ specific controls. Differences (tumor-normal) ≥0.15 were considered hypermethylated in tumors.

2.5. mRNA Expression Profiling. Affymetrix U133 microarray and RNA sequencing data for *KEAP1*, *CUL3*, and *RBX1* mRNA expression were obtained from the cBio portal and TCGA data portal, respectively. Data from the Affymetrix U133 microarray ($n = 370$) were used for comparisons, since the number of samples with data available was higher than those available with RNA sequencing data for genes of interest.

2.6. Normalization of Expression Levels. In order to perform comparisons across the sample set, expression values were rank-normalized, in order to preserve the ordering of genes in a sample while removing any other factor affecting the set. For this, we used the "RankNormalize" package available through GenePattern [33].

2.7. Gene Set Enrichment Analysis. These analyses were performed using a single-sample gene set enrichment analysis (ssGSEA) [34]. Using rank-normalized expression levels, ssGSEA calculates separate enrichment scores (ES) that represent the degree to which each gene in a gene set is coordinately up- or downregulated within a sample. For enrichment analysis, we used 3 published gene sets (SINGH_NFE2L2_TARGETS, BIOCARTA_ARENRF2_PATHWAY, and V$NRF2_Q4) that contain genes either altered upon inactivation of NRF2 or genes that contain the NRF2 recognition motif (NTGCTGAGTCAKN) in the vicinity of its transcription start site [±2 kb].

2.8. Statistical Analysis. A comparison of the distributions of mRNA expression levels between samples with and without DNA-inactivating alterations in genes encoding complex components was performed in GraphPad Prism 6 (La Jolla, CA) using a Mann-Whitney U test. This analysis compared the differences in the median expression values between groups (no alterations versus any alterations) with 95% confidence.

2.9. Comparison of E3-Ubiquitin Ligase Complex Component Disruption across Different Tumor Types. To investigate whether patterns of disruption to the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex were specific to ovarian cancer, we assessed the frequency of genomic alterations in additional TCGA tumor types with (1) the largest number of

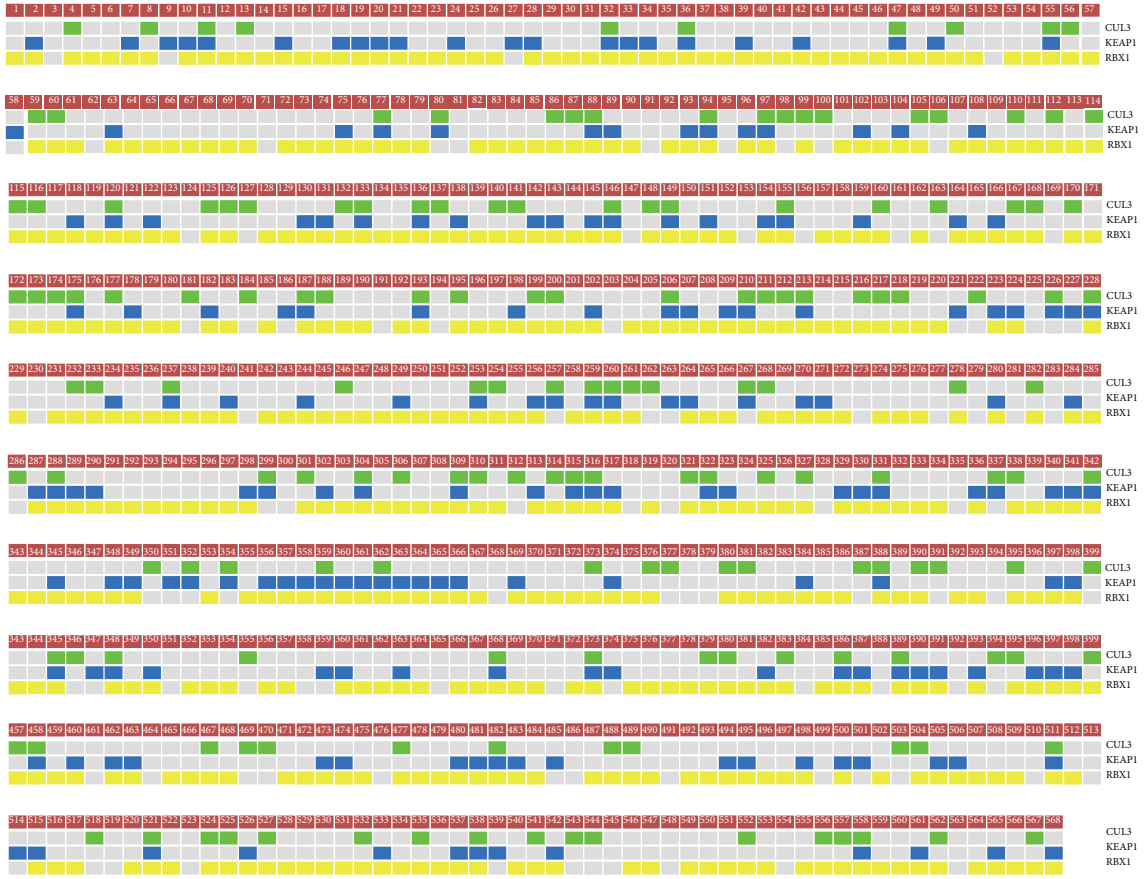

Figure 3: *DNA-level alteration affecting components of the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex in OVCA tumors*. Alteration status of individual complex component genes (*KEAP1* in blue, *CUL3* in green, and *RBX1* in yellow) across a panel of 588 ovarian tumors is indicated by colored boxes.

samples with available multidimensional data through cBio portal and (2) data status indicating "No restrictions; all data available without limitations." These included breast invasive carcinoma (BRCA), kidney renal clear cell carcinoma (KIRC), glioblastoma multiforme (GBM), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), head and neck squamous cell carcinoma (HNSC), thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC).

3. Results

3.1. CUL3/KEAP1/RBX1 E3-Ubiquitin Ligase Complex Is Frequently Disrupted by Multiple DNA Mechanisms in OVCA. First we investigated the frequency of DNA-level alterations (i.e., sequence mutations, copy-number loss, and hypermethylation) affecting each component gene of the CUL3/KEAP1/RBX1 E3-ubiquitin ligase complex. The results are summarized in Table 1. The disruption status of individual samples considering the various data types is shown in Figure 2.

Copy-number loss (CNL) was by far the most prominent inactivating mechanism affecting all complex components. Deletion of *CUL3*, *KEAP1*, and *RBX1* was detected in 26.0%, 32.7%, and 81.5% of samples, respectively (Table 1). Aberrant DNA methylation also affected component genes but at a much lower frequency than CNL. Somatic DNA mutations with significant predicted effects on protein function (according to Mutation Assessor) were found in only 2 samples (Table 1). Due to the low number of cases harboring mutations, we decided to focus our analysis on 568 samples with both copy-number and methylation data (Figure 3). Remarkably, when CNL and hypermethylation were considered concurrently, 90.5% of the OVCA cases examined sustained one or more alterations affecting any of the three components of the CUL3/KEAP1/RBX1 E3-ubiquitin ligase complex (Figure 3). The frequencies of individual alteration mechanisms were different among complex component genes (Figure 4).

3.2. DNA Alterations Affect Complex Component Gene Expression. We next evaluated the impact of DNA-level alterations on mRNA expression of *CUL3*, *KEAP1*, and *RBX1*, by comparing mRNA levels in samples with and without inactivating DNA-level alterations affecting any of the complex component genes for samples with available expression data for these genes ($n = 37$) (Figure 5). For the *CUL3*, *KEAP1*, and *RBX1* genes, mRNA levels were lower among samples harboring inactivating DNA-level alterations compared to those lacking these alterations (P value <0.01, Mann Whitney

Table 1: Frequency of OVCA cases affected by individual genetic mechanisms.

Gene complex component	Sequence mutation (n = 316)	Copy-number loss (n = 569)	Hypermethylation (n = 582)
CUL3	1 (0.3%)	148 (26.01%)	30 (5.15%)
KEAP1	1 (0.3%)	186 (32.69%)	5 (0.86%)
RBX1	0 (0%)	464 (81.54%)	41 (7.04%)

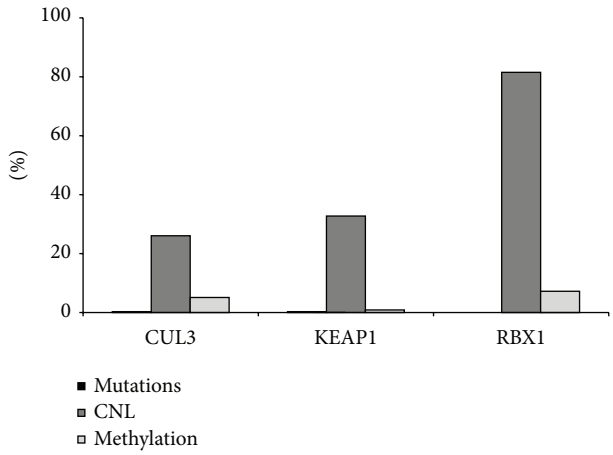

Figure 4: *Loss of function alterations affecting each complex component gene*. The frequency of DNA sequence mutations (black), DNA copy-number loss (CNL, dark grey), and promoter methylation (light grey) affecting each complex gene is shown.

test), the vast majority of which were copy-number loss (Table 1).

3.3. Activation of NRF2 Target Genes Is Apparent in Samples Harboring DNA-Level Disruption of Complex Components. We assessed activation of NRF2 target genes in each sample harboring DNA-level disruption affecting any component of the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex (N = 502) using single-sample gene set enrichment analysis (ssGSEA). ssGSEA assessed whether NRF2 gene sets were enriched in genes expressed in individual tumor samples (based on ranked gene expression levels within a tumor) (Figure 6(a)). Results for three different datasets from the Molecular Signatures Database revealed that 90.2% of samples harboring DNA-level alterations in complex components exhibited a positive enrichment for NRF2 target genes (Figure 6(b)).

3.4. OVCA Displays a Unique Pattern of NRF2 Inhibitory Complex Gene Disruption. Given the importance of NRF2 activation in other malignancies, we next sought to determine how the spectrum of alterations in OVCA compares to other tumor types. We evaluated the frequency of *CUL3*, *RBX1*, and *KEAP1* disruption across multiple tumor types from the TCGA, selected based on data availability from TCGA (Section 2). Intriguingly, the frequency of complex disruption differed considerably across tumor types (Figure 7(a)), with an extremely high frequency of disruption in lung, thyroid, uterine, and ovarian tumors. Our analysis on individual complex component genes revealed that, in addition to different frequencies of disruption, each tumor type displays a distinctive pattern of CUL3/KEAP1/RBX1 E3-ubiquitin ligase complex alterations (Figure 7(b)). Overall, *KEAP1* (range: 2%–97.3%) and *CUL3* (range: 8.6%–98.4%) were typically the most frequently disrupted complex components. However, the frequency of alterations affecting *RBX1* in OVCA was the highest of any complex component gene in any of the tumor types analyzed, with CNL of *RBX1* observed in 81.5% of 568 cases (Figure 7(c)).

4. Discussion

Given the role of the NRF2 pathway in regulating cellular response to ROS, this pathway is likely critical to normal physiological ovarian function. However, the low reported frequency of inactivating *KEAP1* mutations does not account for the reportedly high frequency of NRF2 protein activation in ovarian cancer [18, 27, 35]. In this study, we provide evidence that inactivating genetic alterations affect multiple components of the CUL3/KEAP1/RBX1 E3-ubiquitin ligase NRF2 inhibitory complex in a remarkably high number of OVCA cases. These events are associated with a concordant reduction in component mRNA expression levels and positive enrichment of NRF2 target gene expression. Moreover, we note that OVCA sustains a unique pattern of complex component gene disruption compared to other cancer types, including those for which NRF2 activation through complex disruption are well known.

DNA-level inactivating alterations affecting gene components of the CUL3/KEAP1/RBX1 E3-ubiquitin ligase NRF2 inhibitory complex resulting in reduction of mRNA expression levels has been previously shown in thyroid, head and neck, and non-small cell lung tumors [24, 36, 37]. Moreover, these alterations were associated with a consequential increase in activated forms of complex ligands [24]. Concordant with our findings, we also observed a positive enrichment of NRF2 target genes in ~90% of OVCA samples harboring alteration in any of the individual complex component genes. This provides evidence of the potential effect of complex disruption on this pathway in disrupted tumors.

Interestingly, we did not observe DNA sequence mutations in the *NFE2L2 or KEAP1* genes in OVCA, even though this mechanism of NRF2 activation is well established in many tumor types [19–23]. This is consistent with the low frequency of *KEAP1* mutations observed in serous ovarian tumors in a previous study with a much smaller cohort [27]. Likewise, only 48 of the 568 samples (8.45%) exhibited segmental amplification and concurrent overexpression of

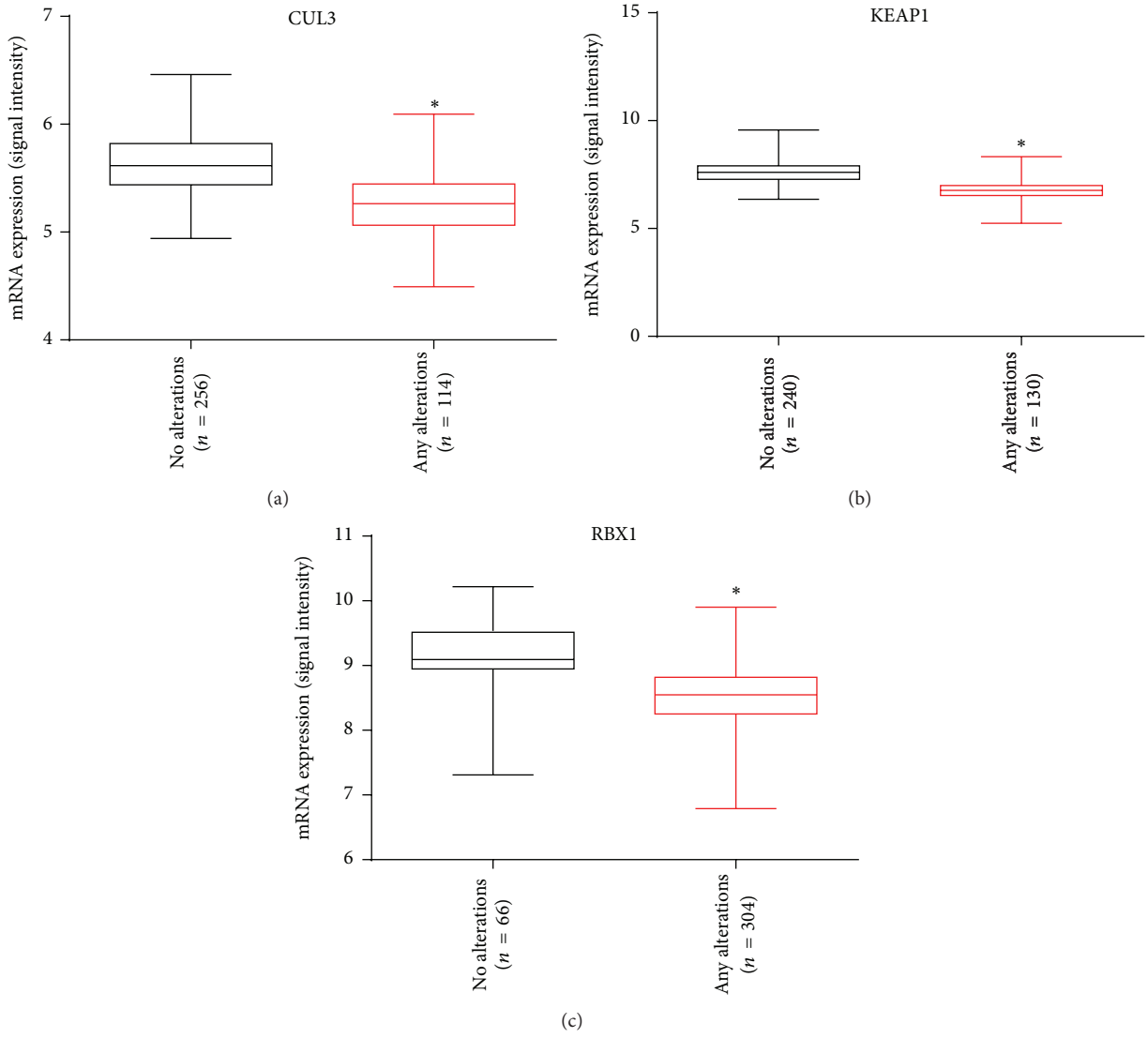

FIGURE 5: *Impact of DNA-level alteration on mRNA expression levels*. mRNA levels (measured as normalized array signal intensity) between OVCA groups with (black) and without (red) DNA-level alteration(s) were compared. * indicates statistically significant differences ($P < 0.01$), assessed through the Mann-Whitney test.

NFE2L2 suggesting other genetic mechanisms contribute to NRF2 protein and pathway activation in OVCA.

Analysis of the frequency of KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex component gene disruption in a broad spectrum of cancer types revealed that component gene alteration is a common phenomenon in cancer, albeit at varying frequencies, suggesting this NRF2 inhibitory complex is important to many cancer types (Figure 7(a)). The frequency of disruption for ovarian tumors was comparable to the high frequencies observed in uterine carcinoma and lung squamous cell carcinoma (LUSC), where disruption of the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex is well established. Given that NRF2 inhibitory complex alterations are known to drive NRF2 pathway activation, we speculate that the alterations we have identified may contribute to the high frequency of aberrant NRF2 activation reported in ovarian cancer [18].

We found that *RBX1* sustained an extremely high frequency of copy-number loss, representing a characteristic of NRF2 inhibitory complex component disruption unique to OVCA. *RBX1* was altered in 81.5% of the OVCA tumors analyzed, compared to 26.05% and 32.74% for *CUL3* and *KEAP1*, respectively (Figure 7(b)). After OVCA, the highest frequency of CNL affecting *RBX1* was observed in breast cancer (BRCA), at 45.5%, while other gynecological tumors, such as uterine corpus endometrial carcinoma (UCEC), showed *RBX1* CNL in only 17.05% of cases (Figure 7(c)). Of note, the frequency of complex disruption in OVCA was similar to that seen in thyroid carcinoma (THCA) (Figure 7(a)), another organ that requires ROS for normal physiological function, hormonogenesis, and proliferation [36, 38, 39]. Taken together, these results demonstrate that the frequencies and patterns of alteration affecting KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex components are tumor-type and

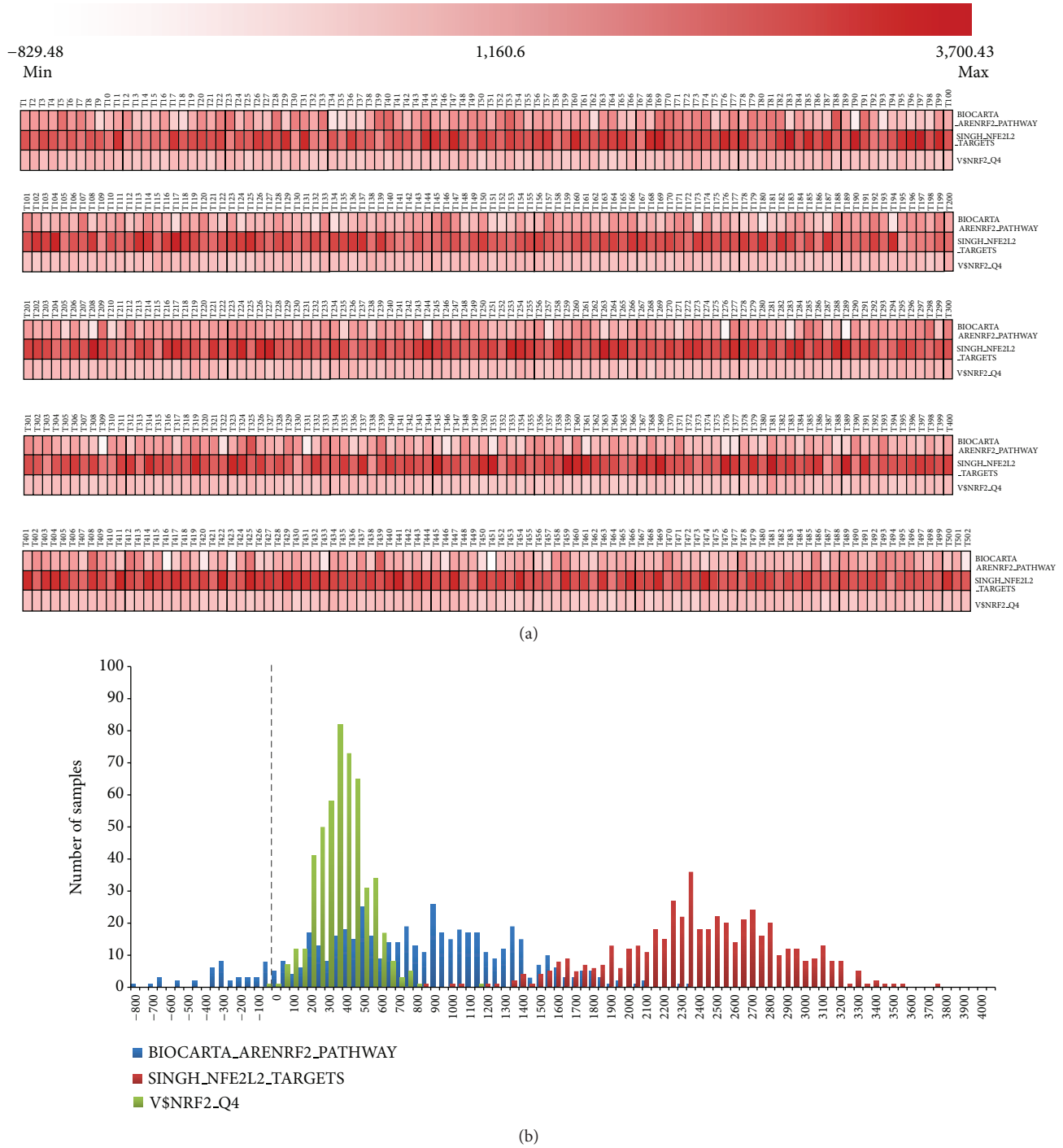

Figure 6: *Enrichment of NRF2 target genes in samples harboring complex component gene alteration*. (a) Enrichment of the different NRF2 target gene sets (Section 2). Enrichment scores (ES) are depicted for each sample. Increasing shades of red denote a larger ES. White boxes denote negative enrichment of NRF2 target genes (ES < 0). (b) Histogram for ES values across 502 samples with alterations affecting complex components genes.

tissue specific and that, in OVCA, copy-number loss affecting *RBX1* is the most prominent mechanism likely contributing to the increased NRF2 activation observed in ovarian cancer.

Given the extensive role of CUL3/KEAP1/RBX1 complex component proteins in other cellular pathways and functions, biological consequences of disruption to these genes certainly extend beyond the NRF2 pathway. For example, somatic disruption of KEAP1/CUL3 E3-ubiquitin ligase complex components also promote activation of NF-κB in lung cancer, by compromising degradation of the NF-κB activator, IKBKB [24]; given the extensive functions of NF-κB, this may have broad implications to a multitude of biological systems of

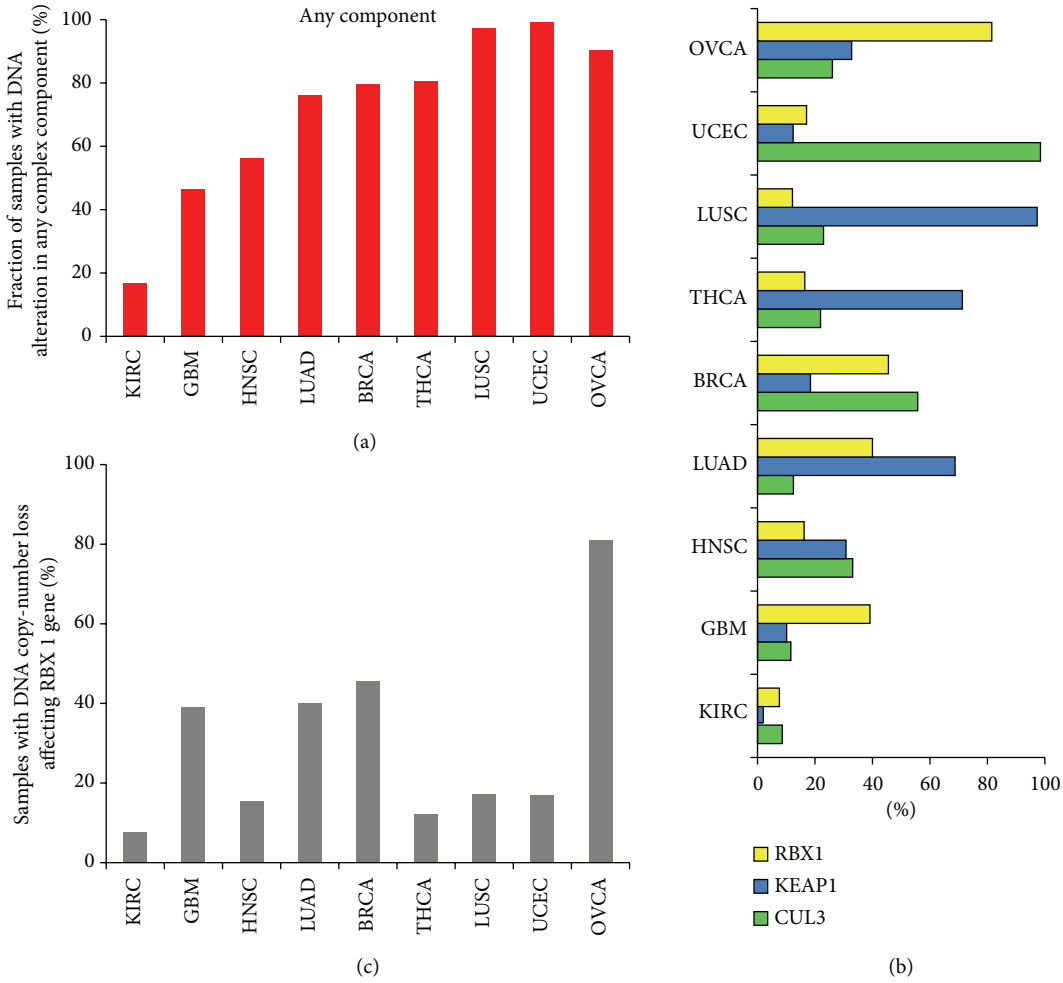

Figure 7: *Pan-cancer comparison of DNA alteration frequency affecting components of the KEAP1/CUL3/RBX1 E3-Ubiquitin ligase complex.* Frequency of DNA-level disruption (inactivating mutation, CNL, or hypermethylation) in ovarian carcinomas (OVCA) was compared to breast invasive carcinoma (BRCA), kidney renal clear cell carcinoma (KIRC), glioblastoma multiforme (GBM), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), head and neck squamous cell carcinoma (HNSC), thyroid carcinoma (THCA), and uterine corpus endometrial carcinoma (UCEC). (a) Proportion of tumors with 1 or more complex component genes disrupted by inactivating DNA-level mechanisms. (b) Frequency of disruption of individual complex component genes. (c) Frequencies of DNA copy-number loss (CNL) affecting the *RBX1* gene across multiple tumor types.

particular relevance to cancer. Moreover, KEAP1 interacts with other "ETGE" containing proteins that may be affected by KEAP1 disruption. Dipeptidyl peptidase 3 (DPP3), an ETGE containing protein, competes with endogenous NRF2 for binding to KEAP1 and is able to activate NRF2-mediated transcription [40]. Thus, it is plausible that DNA-level inactivation of KEAP1 may result in activation of DPP3 and subsequently pose an alternative pathway for NRF2 target gene activation. CUL3 can assemble with numerous substrate receptors with N-terminal BTB domains to form ubiquitin ligases complexes [41], whereby a shared catalytic core is able to recruit a variety of substrates (reviewed in [42, 43]). RBX1 is also a component of the von Hippel-Lindau (VHL) tumor suppressor complex, which interacts with Elongin B, Elongin C, and CUL2 [44]. Taken together, the potential implications of DNA-level alterations affecting components of the CUL3/KEAP1/RBX1 protein complex are broad and, cumulatively, may have profound implications in tumor biology.

While the frequency of DNA and mRNA level disruption we observed for KEAP1-CUL3-RBX1 complex components and correlation of these events with association NRF2 target gene transcriptional activation in ovarian cancer is compelling, we also acknowledge that other mechanisms might also impact NRF2 levels. For example, NRF2 activity can be repressed through another ubiquitin protein ligase complex, composed of beta-transducin repeat containing E3-ubiquitin protein ligase (BTRC), Cullin 1 (CUL1), and S-phase kinase-associated protein 1 (SKP1) [45, 46]. NRF2 is phosphorylated by GSK3, creating a phosphodegron to which BTRC is recruited [45]. To assess the possibility that alteration to these components may be contributing to NRF2 activation in ovarian tumors, we evaluated DNA-level

alterations affecting the genes involved in this BTRC/SKP1/CUL1 complex. Interestingly, a high proportion of cases (84%) exhibited DNA-level alteration affecting at least one of the complex components. BTRC, GSK3A, and SKP1 exhibited a high frequency of DNA-level disruption, with 42%, 43%, and 45% of samples showing DNA copy-number losses, respectively. A significant effect on gene expression was also observed when samples with any alterations affecting BTRC, GSK3A, or SKP1 were compared against those cases without alterations affecting these complex components (see supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/159459). While the magnitude and frequency of KEAP1/CUL3/RBX1 complex component disruption were more prominent in the cohort we assessed, these results reveal the potential importance of alternative mechanisms of NRF2 activation in ovarian cancer and warrant consideration in future studies.

One of the main limitations of this study is the lack of protein level and/or localization analysis of complex components and NRF2 in OVCA tissues to confirm the biological effects of the DNA and mRNA level alterations we have described. We have addressed this by assessing enrichment of NFR2 target genes on each sample (ssGSEA), which has been previously used in OVCA cases from the TCGA project as well as in other studies [34, 47]. Use of this approach is especially relevant for cases where no clinical tissue specimens are available for immunohistochemical or similar analyses.

In conclusion, we have identified an extremely high frequency of genetic disruption affecting the KEAP1/CUL3/RBX1 E3-ubiquitin ligase complex in serous ovarian tumors, occurring predominantly through copy-number loss of *RBX1*. Disruption was associated with NRF2 pathway activation in the same individual tumors harboring complex alterations. Our observations highlight a potential mechanism underlying activation of NRF2 protein in OVCA. The high frequency of DNA-level complex disruption provides evidence that such disruption is selected in OVCA and further emphasizes the importance of NRF2 activation in this tumor type. Therapeutic targeting of NRF2 may represent a promising intervention point for serous ovarian tumor therapy; however, an improved understanding of the biological role of NRF2 in the context of ovarian tumor and nonmalignant (i.e., normal) cells must first be achieved, especially considering the importance of this pathway to normal ovarian function.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Phytochemicals: A Multitargeted Approach to Gynecologic Cancer Therapy

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Gynecologic cancers constitute the fourth most common cancer type in women. Treatment outcomes are dictated by a multitude of factors, including stage at diagnosis, tissue type, and overall health of the patient. Current therapeutic options include surgery, radiotherapy, and chemotherapy, although significant unmet medical needs remain in regard to side effects and long-term survival. The efficacy of chemotherapy is influenced by cellular events such as the overexpression of oncogenes and downregulation of tumor suppressors, which together determine apoptotic responses. Phytochemicals are a broad class of natural compounds derived from plants, a number of which exhibit useful bioactive effects toward these pathways. High-throughput screening methods, rational modification, and developments in regulatory policies will accelerate the development of novel therapeutics based on these compounds, which will likely improve overall survival and quality of life for patients.

1. Introduction

Gynecologic cancers are malignant neoplasms of the female reproductive system, the most common of which are endometrial, ovarian, and cervical cancers. Together, they constitute the fourth most common cancer type in women, with approximately 82,000 diagnosed in the USA annually [1]. Treatment outcomes for endometrial and cervical cancers are relatively more effective, due to the availability of more definitive screening methods and a faster onset of symptoms that generally prompt earlier intervention. In contrast, ovarian cancer is the most deadly, with more women dying of the disease than all other types of gynecologic cancer combined [2]. This can be attributable to a lack of symptoms and detectable biomarkers, frequently resulting in late-stage diagnoses.

2. Therapy and Chemoresistance

First-line treatment strategies for gynecologic cancers are administered depending on the stage and malignant cell type involved, but surgical intervention and chemotherapeutic agents such as paclitaxel and cisplatin-based derivatives are frequently included. Endometrial cancers are most effectively treated with surgery, via hysterectomy [3]. Clinical studies have demonstrated the efficacy of less aggressive surgical approaches, when decisions take into account lower grades outlined by the International Federation of Gynecology and Obstetrics [4]. In contrast, more advanced endometrial cancers can only be optimally debulked in 44–72% of cases [5, 6]. Neoadjuvant chemotherapy in such cases has yielded some positive outcomes [7], but the only large randomized trial involving chemotherapy for endometrial cancer found

no difference in survival between the groups that received doxorubicin or no further therapy following regular surgical intervention [8]. Similarly, the efficacy of radiotherapy remains controversial, with ambiguous and conflicting lines of evidence [9, 10].

Most cervical cancers are squamous cell carcinomas arising from the epithelial cells lining the cervix. Treatment strategies include radical surgery or radiotherapy; however, extensive clinical studies have shown that no treatment of choice exists for early stages of the disease [11]. A combination of surgery and radiotherapy results in higher morbidity, and the optimal therapy for each individual patient is reliant on clinical factors such as age and histological type. For advanced stages of the disease, pelvic radiation has become the currently accepted gold standard [12]. In addition, a combination of histone deacetylase inhibitor (vorinostat) and proteasome inhibitor (bortezomib) has been shown to significantly retard cervical tumor growth in a xenograft model, although such an approach in a clinical setting has yet to be attempted [13].

In contrast, cisplatin (CDDP: *cis*-diamminedichloroplatinum) and its derivatives are considered first-line treatments for ovarian cancer, following surgical debulking [14]. In most cases, however, recurrent disease emerges that fails to respond to further chemotherapy. This phenomenon is referred to as chemoresistance and often signals the end of the road in terms of viable treatment options. Chemoresistance arises from the dysregulation of signaling factors responsible for inducing cell death [15]. Current standards of treatment using chemotherapy primarily focus on ovarian cancer; however, all of the gynecologic cancer types may be susceptible to novel chemotherapeutic approaches. One concern that remains clear is that the current therapeutic options of radiotherapy, surgery, and chemotherapy for gynecologic cancers are insufficient for current patient needs. The severity of side effects and frequent development of infertility posttherapy necessitate the development of more sensitive and personalized strategies for higher standards of treatment.

3. Phytochemicals

Phytochemicals are a broad class of molecules with bioactive properties that are derived from botanical sources. In recent years, a growing number of studies have uncovered a plethora of potential applications for phytochemicals in signaling pathways related to cancer [16]. Bioactive compounds that can inhibit or antagonize factors that are dysregulated in malignant cells have the potential to enhance the effects of conventional therapy or be developed into a stand-alone therapeutic in their own right. One major advantage for the use of phytochemicals over synthetic compounds, in many cases, is their historical presence in the human diet. Due to this evolutionary exposure, severe adverse events are conceivably less likely to arise in therapeutic settings when compared to synthetic compounds that are entering the human body for the first time. Modern high-throughput screening techniques can also facilitate the screening of fractionated separations of plant extracts containing thousands of phytochemicals, while synthetic libraries require each candidate to be engineered separately. Some phytochemicals also exert influences on multiple targets within a common oncogenic signaling pathway [17]. Many oncogenic signaling pathways are shared by malignant cells across different tissue types, due to common functional requirements for sustained survival and proliferation. Therefore, phytochemicals that exhibit anticancer activity in one cell type may have potential for application in treating a wider range of cancers.

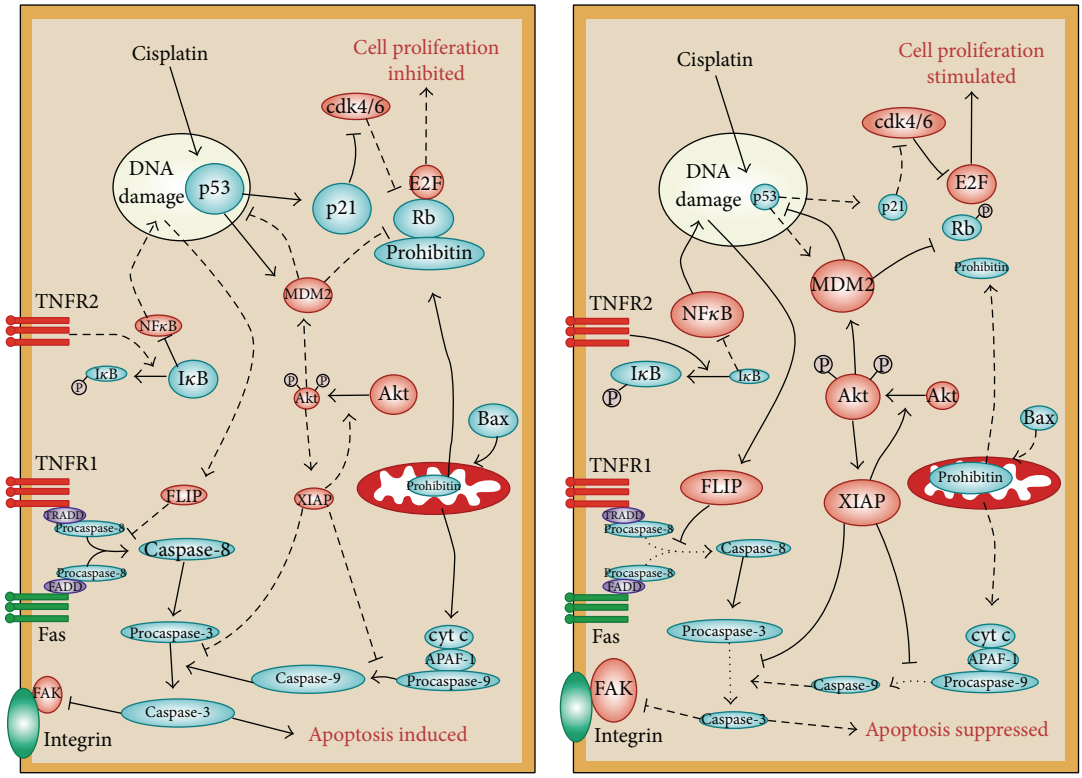
4. Molecular Mechanisms of Phytochemical Action in Cancer Prevention

The science of cancer prevention receives relatively little attention when compared to the field of cancer therapy. Whether a result of market forces or a lack of experimental precedent in developing preventative approaches is unclear. However, environmental factors including tobacco smoking and a sedentary lifestyle are known to contribute to a higher risk of many cancers. Epidemiological evidence also suggests that dietary behavior significantly influences the prevalence of specific cancer types in any given population [18]. A diet high in fruits and vegetables appears to broadly reduce cancer risk, and this can be at least partially attributable to the bioactivity of phytochemicals [19]. Perhaps the most recognized example is resveratrol, a phytoalexin found in the skins of red grapes. Resveratrol exhibits a number of striking bioactive properties beneficial for human health, including antitumor activity [20].

Luteolin is a flavonoid present in cruciferous vegetables that exhibits cancer chemopreventive activity. It inhibits protein kinase Cε and Src kinase activities, both of which have been implicated in oncogenic signaling [21]. Other phytochemicals may exert chemopreventive activities by targeting alternative hallmarks of cancer such as angiogenesis and inflammation. Myricetin is one of the major phytochemicals present in onions and berries and has been found to inhibit angiogenesis via the inhibition of PI3K and the suppression of matrix metalloproteinases responsible for vascular growth [22]. These findings have been supported by a mouse model of angiogenesis, in which myricetin topical treatment was sufficient to suppress UV-B induced blood vessel formation. Meanwhile, apigenin (another abundant flavonoid found in onions and berries) has been shown to counteract inflammatory processes via direct binding to cyclooxygenase 2, thereby suppressing downstream events [23]. Apigenin, as well as chalcone (a pigment of petunia flowers), can regulate MAPK pathways in endometrial cancer cells via selective action on activator protein-1 [24]. Similarly, sulforaphane (found in cruciferous vegetables) has been demonstrated to trigger cell cycle arrest in cervical cancer cells when treated at low concentrations [25].

5. Phytochemical-Based Approaches to Overcoming Chemoresistance

Chemoresistance arises in cancer cells via the downregulation of tumor suppressors and the stabilization or activation of



(a) Chemosensitive cell (b) Chemoresistant cell

Figure 1: A hypothetical model of chemoresistance in human ovarian cancer cells. In a chemosensitive ovarian cancer cell (a), cisplatin activates p53, leading to upregulation of proteins promoting cell cycle arrest, such as p21, and of proapoptotic proteins such as Bax and Fas. This activates both the intrinsic (mitochondrial) and the extrinsic (death-receptor) apoptotic pathways, the overall result of which is the activation of the execution caspase-3 (and caspase-7, not shown). In these cells, cell survival mediators such as Xiap, Akt, and Flip (shown in red) are downregulated or are in their inactive state. In chemoresistant cells (b), increased p53 ubiquitination by MDM2 results in the maintenance of low levels of p53, despite the presence of cisplatin. Moreover, cisplatin fails to downregulate Xiap, thereby maintaining an active state of the PI3K/Akt pathway. In addition, binding of TNFR2 by TNFα leads to upregulation of FLIP through the NF-κB pathway, thus inhibiting the proapoptotic actions of the cytokine through TNFR1. Overall, as a consequence of a failure to activate the caspase cascade in response to the chemotherapeutic agent, these cells have lost their capacity to undergo apoptosis and thus became chemoresistant. Taken from [15].

cell survival factors [26]. Mutation, overexpression, or gene deletions are responsible for dysregulating apoptosis signal pathways. The identification and targeting of such dysregulation with bioactive compounds may therefore represent a viable strategy for improving chemosensitivity.

The PI3K/Akt pathway is frequently overexpressed or activated in a number of cancers (Figure 1). The downregulation of Akt sensitizes chemoresistant ovarian cancer (OVCA) to CDDP-induced apoptosis, at least in part, by modulating cisplatin-induced, p53-dependent ubiquitination of Fas-associated death domain-like interleukin-1 beta-converting enzyme- (FLICE-) like inhibitory protein (FLIP) [27, 28]. Akt inhibition has been shown to sensitize chemoresistant ovarian cancer cells to paclitaxel [29], while other studies have shown that its downregulation stabilizes the p53-inducible protein phosphatase PPM1D, increasing its content in response to cisplatin challenge. In chemoresistant cells with high Akt expression, PPM1D stability is enhanced in response to protein synthesis inhibition, which is significantly decreased in the chemosensitive response [30]. The caspase-independent apoptosis pathway, regulated by the activity of apoptosis-inducing factor (AIF), is also influenced by Akt action through its attenuation of AIF nuclear translocation [31]. AIF is negatively regulated by the X-linked inhibitor of apoptosis protein (XIAP), another determinant of chemoresistance that is also stabilized by Akt [32, 33]. XIAP, in turn, can regulate Akt activity and caspase-3-dependent cleavage during CDDP-induced apoptosis [34]. The tumor suppressor p53 also plays a central role in apoptosis and its phosphorylation at serine residues 15 and 20 stabilizes it by preventing association with murine double minute 2 (MDM2) [28, 35]. Many lines of evidence show that a functional p53 significantly affects the capacity of cancer cells to undergo apoptosis [27, 36, 37]. It has been demonstrated that p53 promotes the ubiquitination and subsequent proteasomal degradation of FLIP by promoting its interaction with the E3 ligase Itch [38].

A crucial step in the induction of apoptosis is mitochondrial outer membrane permeabilization (MOMP). Proapoptotic Bcl-2 family members (including Bax and Bak), as well as the BH3-only proteins (Bid, Bim, and PUMA) permeabilize the membrane after activation [39]. When challenged

with cisplatin, p53 has been shown to translocate to the mitochondria and facilitate MOMP by interacting with membrane proteins that mediate pore formation [15, 37]. This results in the release of cytochrome c and second mitochondria-derived activator of caspases, leading to cell death. Dynamin-related protein 1 (Drp1) is a cytosolic GTPase responsible for the process of mitochondrial fission and is activated by cytosolic changes in calcium levels via its regulator, calcineurin [40]. Fission precedes cellular apoptosis in the majority of cases, and the speed at which it occurs can directly influence its induction [41]. Studies have shown that the phytochemicals piceatannol and piperlongumine (found in red grapes and the long pepper, resp.) can enhance cisplatin-induced apoptosis through enhanced levels of Drp1-dependent mitochondrial fission [42, 43]. Piceatannol, a natural stilbene and a metabolite of resveratrol, enhances cisplatin sensitivity in ovarian cancer, by increasing the p53-mediated expression of the proapoptotic protein NOXA, XIAP degradation via the ubiquitin-proteasome pathway, and promoting caspase-3 activation. These effects are also associated with increases in Drp1-dependent mitochondrial fission, a step that appears to improve the induction of apoptosis. A xenograft mouse model has shown that these events translate into additive reductions in tumor size when treatment includes both cisplatin and piceatannol in combination (Figure 2).

Curcumin has been shown to sensitize cervical cancer cells to paclitaxel treatment *in vivo* [44]. Curcumin exerts its effect via downregulation of the NF-κB, MAPK, and Akt pathways and, in combination with paclitaxel, induces a synergistic reduction in tumor incidence as well as tumor volume in a xenograft model using NOD-SCID mice. Moreover, preexposure of cervical cancer cells to curcumin was found to potentiate paclitaxel sensitivity in 3-methylcholanthrene-induced cervical carcinoma models. Similarly, both apigenin and emodin (a purgative resin found in Himalayan rhubarb) have been shown to control Fas and TRAIL sensitivity in endometrial cancer cells via the inhibition of casein kinase [45].

Of the gynecologic cancers, ovarian and cervical cancers have received the most attention in terms of phytochemical approaches to overcome chemoresistance. Hirsutenone, a diarylheptanoid from the bark of *Alnus hirsuta,* has been shown to sensitize chemoresistant ovarian and cervical cancer cells to cisplatin [46]. Hirsutenone activates p53 via phosphorylation at Ser 15 in cells with wild type-p53 and also has significant effects in p53-null and p53-mutant cell lines. CDDP-dependent apoptosis in chemoresistant cells was associated with ubiquitin/proteasome-mediated degradation of XIAP and enhancement of AIF translocation from the mitochondria to the nucleus. These effects appeared to in part be regulated by Akt, linking hirsutenone-dependent PI3K inhibition with its downstream apoptotic effectors AIF and XIAP (Figure 3). Other phytochemicals with an ability to overcome chemoresistance in ovarian cancer include the citrus flavonoid tangeritin [47], the turmeric compound curcumin [48], and the resveratrol [49].

To overcome the problem of chemoresistance, researchers have begun to focus on the identification of novel targets for inhibition by small molecules. Smac mimetics are synthetic compounds that mimic the role of second mitochondrial activator of caspases protein by binding to and inhibiting the activity of IAP family members like XIAP [50]. Studies have shown that such compounds can induce apoptosis in chemoresistant ovarian cancer cells by potentiating ligand-mediated death pathways [51]. New promising targets that have yet to be validated in clinical settings include the ubiquitin specific protease 8 (USP8) and pyruvate kinase M2 (PKM2) [52]. USP8 regulates the expression of receptor tyrosine kinases responsible for downstream activation of oncogenic signaling pathways including the PI3K/Akt and MAPK s pathways, and its inhibition with a small molecule has been shown to selectively kill cancer cells. PKM2 regulates aerobic glycolysis in tumor cells, providing the metabolic advantage required for rapid proliferation. Its knockdown with short hairpin RNA leads to a reversal of the Warburg effect and inhibits tumor growth in a xenograft model [53].

High-throughput screening of phytochemical libraries may identify potent compounds to inhibit such novel targets and overcome chemoresistance.

Recently there have also been reports that the US FDA is reconsidering its regulatory framework for the approval of novel cancer therapeutics [54]. Instead of the conventional approach of approving cancer drugs for a specific indication, there may be progress toward approval based solely on the molecular pathway that a drug is targeting. If this framework is implemented, considerable flexibility will be conferred to the pharmaceutical industry in the development and clinical testing of new drug candidates. The heterogeneity present in tumor cell populations also justifies more versatility in the choice of therapeutic regimen available to physicians.

6. Phytochemical Analogues and Chemical Modifications for Greater Efficacy

Some phytochemicals have multiple molecular targets, and such properties are not limited to application in gynecologic cancers alone. Geraniol is an effective plant-based mosquito repellant present in a number of essential oils including citronella. This acyclic monoterpene has been shown to independently induce apoptosis and autophagy via the inhibition of Akt and the activation of AMPK. It has also been demonstrated that the combined effect of Akt inhibition and AMPK signaling is more potent at suppressing prostate cancer cell growth than either action alone [55]. Moreover, when treated in combination with docetaxel, geraniol markedly improved chemosensitivity in a xenograft model [56].

A close structural analogue of geraniol is the more widely known compound menthol. Of particular note, menthol has been widely used in foods, cosmetic products, and topical therapeutic creams for centuries. Studies have shown that menthol binds and activates the TRPM8 Ca(2+)-permeable channel that exhibits abnormal expression patterns in a number of cancer types [57]. Menthol has also been found to markedly downregulate activity of the polo-like kinase 1 (PLK1), thereby inhibiting progression of the G2/M phase in malignant cells [58].

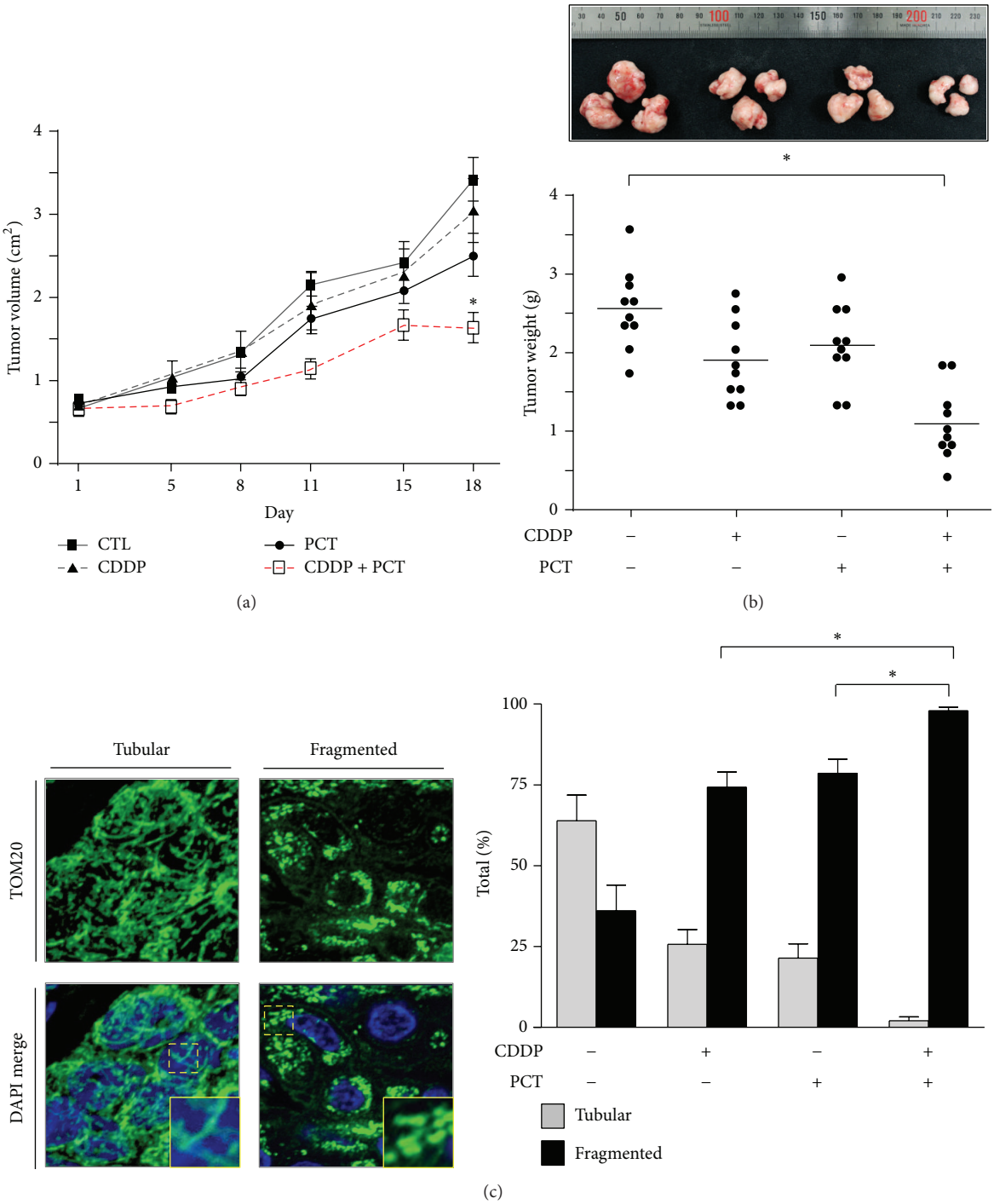


FIGURE 2: Effects of piceatannol and CDDP treatment on tumor growth in a mouse model of OVCA. (a) Effect of CDDP (1.8 mg/kg, once per week) and piceatannol (20 mg/kg, 5 times per week) on tumor volume. Tumors were formed by subcutaneous insertion of 1×10^6 OV2008 cells embedded in matrigel into the hind flanks of athymic nude mice. Tumors were measured over 18 days for the intervals indicated and volume was calculated using the equation $V = \pi/6(l \times h \times w)$. (b) Measurements of tumor weight on the day of sacrifice ($^*P < 0.05$). (c) Effect of CDDP and piceatannol treatment on mitochondrial morphology in recovered tumors. Taken from Farrand et al. [42].

In some cases, phytochemicals can be used to demonstrate proof of concept and to contribute to the development of rationally designed therapeutics. Although not structurally related to menthol, icilin is a synthetic superagonist that was rationally designed to target the same TRPM8 channel as menthol and also produces an extreme sensation of cold. Interestingly, icilin induces G1 arrest in the absence of cell death, via activation of JNK and p38 kinase pathways [59]. Imidazole is another organic compound present in many important biological molecules.

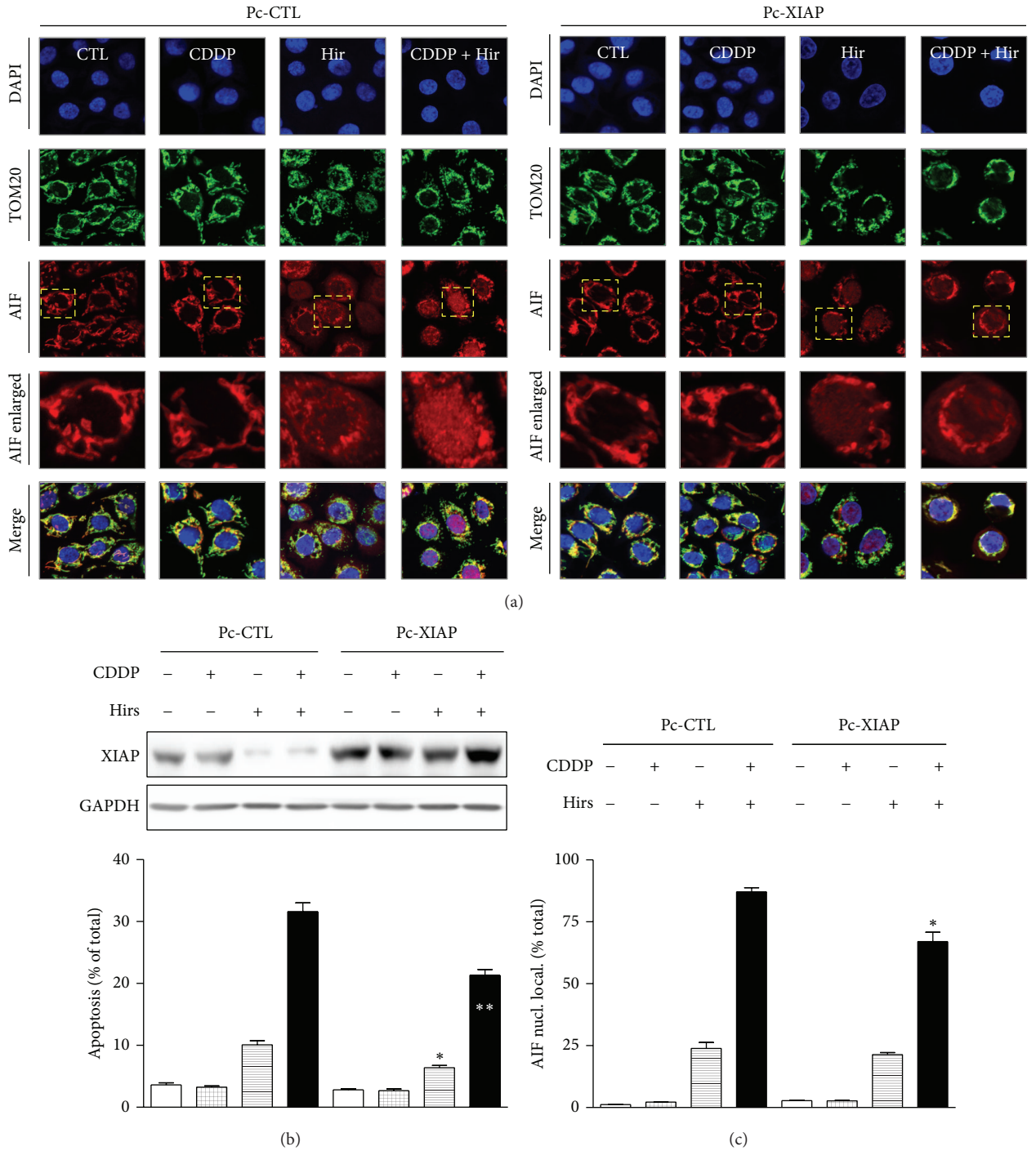


Figure 3: Hirsutenone-facilitated CDDP-induced apoptosis in chemoresistant OVCA cells is mediated by AIF and by suppressed XIAP-AIF interaction. (a) Effects of hirsutenone (10 μM) and CDDP (10 μM) treatment (12 h) on AIF nuclear translocation, in the presence and absence of XIAP overexpression. Chemoresistant cells were transfected with XIAP overexpression constructs (Pc-XIAP, 0.1 μg, 48 h) or GFP control constructs (Pc-GFP, 0.1 μg, 48 h), prior to treatment. Blue: DAPI, red: AIF, and green: TOM20 (mitochondrial membrane marker). (b) Effects of XIAP overexpression on apoptosis induced by hirsutenone (10 μM) and CDDP treatment (10 μM, 24 h). Chemoresistant cells were transfected with XIAP overexpression constructs (Pc-XIAP, 1 μg, 48 h) or GFP control constructs (Pc-GFP, 1 μg, 48 h), prior to treatment. (c) Quantification of AIF nuclear localization data shown in (a). Nuclear signal was quantified using Image J software ($^{*}P < 0.05$; $^{**}P < 0.01$ versus respective DMSO control (CTL)). Taken from [46].

SK&F 96365 is a synthetic imidazole derivative that also targets TRP calcium channels. Unexpectedly, its off-target effects promote necrosis rather than apoptosis, underlining the potential implications of relatively simple structural changes to phytochemical scaffolds [60]. Curcumin's clinical potential has been hampered by observations of poor bioavailability *in vivo*, sparking interest in chemical modifications to the scaffold that may improve such properties. One such derivative, EF24 (diphenyl difluoroketone), potently inhibits tumorigenesis in a mouse model of prostate cancer by downregulating NF-κB and miRNA-21 expression [61]. High-throughput screening approaches have identified another curcumin analog, B82 ((1E,4E)-1,5-bis(5-bromo-2-ethoxyphenyl)penta-1,4-dien-3-one), which exhibits strong antitumor activity against non-small-cell lung cancer cells *in vivo* by inducing ER stress [62]. Similar approaches using resveratrol derivatives have shown that a number of parameters including VEGF inhibition, cytotoxicity, and inhibition of angiogenesis can be improved with side chain modifications to the parent structure [63]. Further research into the relationships between the structure and function of key molecular scaffolds and active side chains is ongoing and will inevitably lead to the discovery of novel drugs with enhanced target specificity.

7. Future Directions

Progress toward better therapeutic strategies for the treatment of gynecologic cancers will be reliant on steady innovation in the areas of prevention, detection, and treatment. The rapid advance of the computer age is providing more powerful software tools for bioinformatics approaches and meta-analyses. These are already yielding benefits, evident in the rapid emergence of publications in the field of systems biology in recent years. However, further integration is necessary for more comprehensive therapeutic solutions. For gynecologic cancers in particular, a severe lack of accurate biomarkers is hampering the effort to improve screening procedures. The identification of novel biomarkers requires a deeper understanding of the molecular mechanisms responsible and will drive the development of better diagnostic medical devices. Coupled with genotyping approaches, these advances will create the strong foundations necessary for a fully fledged era of personalized medicine.

Like all malignant neoplasms, gynecologic cancers arise not as a result of the disruption of a single cellular target, but only after a critical combination of mutations occurs that result in self-sufficient proliferation and survival signaling. The development of optimal treatment strategies will therefore need to shift away from the historical shotgun approaches of cytotoxic chemotherapy and focus on the identification of specific elements at play in each individual case. The beginning of the era of personalized medicine is being accelerated with the development of medical devices capable of genotyping patients quickly and affordably. However, these advances have yet to be matched with similar progress in the area of targeted cancer therapies for known mutations. A major factor for this discrepancy is undoubtedly the complexity of cell signaling pathways, emphasizing the need for continuing research into how these pathways culminate in the evasion of apoptotic signaling.

The exact details of how future personalized therapeutic approaches will operate remain to be determined by the wider medical community. It appears likely to involve individual genotyping for verified oncogenic mutations followed by targeted therapies tailored to an *in silico* evaluation of an optimal strategy. In order to curb the side effects of medication, it may also be advantageous to make the distinction between full, partial, and even subtle inhibition of certain molecular signaling components. In such cases, then, a larger molecular toolbox will provide a more versatile arsenal with which to untangle the molecular mechanisms responsible for each malignancy and eliminate the threat of proliferation. Phytochemicals represent a large and relatively undiscovered resource that can be exploited to supplement such a toolbox. The vast combinations of molecular structures that exist in the evolutionary inventory in some cases have been further enhanced by the fact that some structures have evolved specifically to disrupt molecular signaling pathways in animal cells for defense reasons. A number of lines of evidence support the hypothesis that dietary intake of specific phytochemicals imparts cancer chemopreventive effects, and this may be one factor in explaining regional variations in cancer incidence across the globe.

With further progress in the area of phytochemical-based approaches to gynecologic cancer therapy, it may soon become convention to treat such patients with capsules or infusions containing a cocktail of phytochemicals and rationally designed therapeutics tailored to the specifics of every individual patient. This will require further investments in high-throughput screening and other platform technologies to accelerate the hit-to-lead process and subsequent clinical trials. There are also tantalizing hints that the US FDA may be moving toward a system of regulatory approval for novel cancer therapeutics based solely on their molecular targets rather than the traditional indication-based approach. Such advances will no doubt enhance the versatility afforded to the pharmaceutical sector during strategic drug development decisions. This will contribute to improved overall patient survival and better quality of life through significant improvements in efficacy and the minimization of harmful side effects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Analyzing Association of the *XRCC3* Gene Polymorphism with Ovarian Cancer Risk

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This meta-analysis aims to examine whether the *XRCC3* polymorphisms are associated with ovarian cancer risk. Eligible case-control studies were identified through search in PubMed. Pooled odds ratios (ORs) were appropriately derived from fixed effects models. We therefore performed a meta-analysis of 5,302 ovarian cancer cases and 8,075 controls from 4 published articles and 8 case-control studies for 3 SNPs of *XRCC3*. No statistically significant associations between *XRCC3* rs861539 polymorphisms and ovarian cancer risk were observed in any genetic models. For *XRCC3* rs1799794 polymorphisms, we observed a statistically significant correlation with ovarian cancer risk using the homozygote comparison (T2T2 versus T1T1: OR = 0.70, 95% CI = 0.54–0.90, $P = 0.005$), heterozygote comparison (T1T2 versus T1T1: OR = 1.10, 95% CI = 1.00–1.21, $P = 0.04$), and the recessive genetic model (T2T2 versus T1T1+T1T2: OR = 0.67, 95% CI = 0.52–0.87, $P = 0.002$). For *XRCC3* rs1799796 polymorphisms, we also observed a statistically significant correlation with ovarian cancer risk using the heterozygote comparison (T1T2 versus T1T1: OR = 0.91, 95% CI = 0.83–0.99, $P = 0.04$). In conclusion, this meta-analysis shows that the *XRCC3* were associated with ovarian cancer risk overall for Caucasians. Asian and African populations should be further studied.

1. Introduction

Ovarian cancer is the leading cause of the female reproductive system, with over 220,000 new cases and over 140,000 deaths worldwide in 2008 [1]. As most of the carcinomas, ovarian cancer is a multifactorial disease. Genetic factors are considered to influence the susceptibility of glioma genetic factors which all play significant roles in its susceptibility [2]. The genetic basis of ovarian carcinogenesis has been investigated in many studies. *BRCA1*, *BRCA2*, *MLH1*, *MSH2*, *SMAD6*, *RAD51C*, *RAD51D*, *RB1*, *LIN28B*, *CASP8*, and *MTDH* have all been implicated [3–11]. Recently, several common susceptibility alleles in four loci to be strongly associated with ovarian cancer risk have been found in three genome-wide association studies (GWAS) [12–14]. Examination of gene polymorphisms may explain individual differences in cancer risk [15].

XRCC3 (X-ray repair cross-complementing group 3) belongs to a family of genes responsible for repairing DNA double strand breaks caused by normal metabolic processes or exposure to ionizing radiation [16]. *XRCC3* interacts and stabilizes Rad51 and involves in HRR (homologous recombinational repair) for DBSs (double strand breaks of DNA) and cross-link repair in mammalian cells [17, 18]. The SNP rs861539 lead to Thr241Met amino acid substitution, that may affect the function and/or its interaction with other proteins involved in DNA damage and repair [17, 19]. The SNP rs1799794 (4541 $A > G$) is located in 5′UTR and the SNP rs1799796 (17893 $A > G$) is located in intron 5 [20]. So the two SNPs do not change the proteins of *XRCC3*. *XRCC3* polymorphism was associated with the risks of many cancers, such as lung cancer, breast cancer, and head and neck cancer [21–24]. The association between *XRCC3* polymorphism and ovarian cancer has been studied [20, 25–29]; however, those experimental results remain confusing. To summarize the effect of the *XRCC3* polymorphism on the risk for ovarian cancer, we performed a meta-analysis.

2. Methods

2.1. Search and Selection Process. The search of the PubMed database was performed using the following keywords: "X-ray repair cross-complementing group 3," "*XRCC3*," "rs861539," "T241M," "rs1799794," "a4541g," "rs1799796," "a17893g," "polymorphism," "ovarian cancer," and their combination. Two authors (Yuan and Wang) independently checked all the references retrieved to assess their appropriateness for the inclusion in this meta-analysis. In addition, we checked all the references cited in the articles and relevant reviews. For overlapping and republished studies, only the study with the largest samples was included. If an article reported results including different studies, each study was treated as a separate comparison in our meta-analysis.

Included studies met 3 criteria:

(1) evaluating the association between *XRCC3* polymorphisms and ovarian cancer risk;

(2) using sufficient published data to enable estimation of an odds ratio (OR) with its 95% confidence interval (CI);

(3) using respective or prospective cohort case-control studies.

2.2. Data Extraction. Two authors (Yuan and Wang) independently extracted data from selected articles according to the inclusion criteria and reached a consensus on all items.

The following information was extracted from each study if available: the first author, year of publication, countries, area of the cases, the ethnicity of the population, the cases source, the sample type of cases, the numbers of cases and controls, and the genotype distributions of *XRCC3* in both cases and controls.

2.3. Quality Score Assessment. Two authors independently evaluated the quality of the 8 studies according to the scale for quality assessment (Table 1), which has been described previously [30, 31]. Quality score assessment was performed according to "source of cases," "source of controls," "specimens of cases for determining genotypes," "Hardy-Weinberg equilibrium in controls," and "total sample size." Total scores ranged from 0 (worst) to 15 (best). Studies scoring ≥10 were defined as "high quality," and those <10 were defined as "low quality."

2.4. Statistical Analysis. Pooled ORs with 95% CIs were calculated to access the strength of association between *XRCC3* polymorphism and ovarian cancer susceptibility, according to the genotype frequencies of cases and controls groups [32]. $P < 0.05$ was considered statistically significant; all tests and CIs were two sided. If the heterogeneity was significant, the pooled ORs were initially measured by the random effects model. Else, the fixed-effects model was chosen [33].

The *XRCC3* polymorphism and ovarian cancer risk were performed for a homozygote comparison (T2T2 versus T1T1), heterozygote comparison (T1T2 versus T1T1), dominant genetic model (T1T2+T2T2 versus T1T1), and the recessive genetic model (T2T2 versus T1T1+T1T2). In addition, sensitivity analysis was performed by omitting each study. Publication bias was estimated using a funnel plot. The degree of asymmetry was examined by t Egger's test ($P < 0.05$ was considered significant publication bias) [34]. The analysis was carried out using Review Manager statistical software (RevMan version 5.0.17.0; The Nordic Cochrane Center, Rigshospitalet, Copenhagen, Denmark) and STATA software (version 11.2, Stata Corporation, College Station, TX, USA). Hardy-Weinberg equilibrium (HWE) was calculated using a web-based statistical tool (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Table 1: Scale for quality assessment.

Criteria	Score
Source of cases	
Population or cancer registry	3
Mixed (hospital and cancer registry)	2
Hospital	1
Other	0
Source of controls	
Population based	3
Volunteers or Blood bank	2
Hospital based (cancer-free patients)	1
Not described	0
Specimens of cases for determining genotypes	
Blood or normal tissues	3
Mixed (blood and archival paraffin blocks)	1
Tumor tissues or exfoliated cells of tissue	0
Hardy-Weinberg equilibrium in controls	
Hardy-Weinberg equilibrium	3
Hardy-Weinberg disequilibrium	0
Total sample size	
≥1000	3
≥500 and <1000	2
≥200 and <500	1
<200	0

3. Results

3.1. Study Characteristics. Through the literature search, 13 articles were found. Eight articles [35–42] were excluded as irrelevant study. One study [26] was excluded because it was carried out on overlapping populations with another, more samples eligible study [27]. Total 4 articles including 8 studies were selected on 5,302 ovarian cancer cases and 8,075 controls for 3 SNPs [20, 25–27] (Figure 1). These studies were all published in English. The main characteristics of the 4 studies are shown in Table 2. All subjects in these studies were Caucasians. The sample sizes (cases and controls) ranged from 1,478 to 5,906. Quality scores for all studies were high quality (≥10). Distribution of rs861539 polymorphisms genotype frequencies among ovarian cancer cases and controls of the 2 studies is shown in Table 3. Distribution of

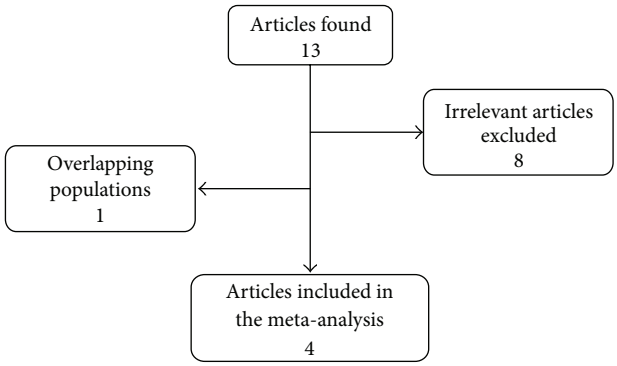

Figure 1: Study flow chart explaining the selection of the four articles included in the meta-analysis.

rs1799794 polymorphisms genotype frequencies is shown in Table 4 and distribution of rs1799796 polymorphisms genotype frequencies is shown in Table 5.

Hardy-Weinberg disequilibrium of genotype frequencies among the controls was calculated in three studies.

3.2. Association of Individual Polymorphisms with Ovarian Cancer. The heterogeneity analysis has been carried out. As it was shown in Tables 3, 4, and 5, the heterogeneities of 3 SNPs are all not significant. So the fixed-effects model was chosen for 3 SNPs.

The meta-analysis results of *XRCC3* rs861539 polymorphisms are shown in Table 3. No statistically significant associations between *XRCC3* rs861539 polymorphisms and ovarian cancer risk were observed in any genetic models (T2T2 versus T1T1: OR = 0.95, 95% CI = 0.85–1.06, $P = 0.37$; T1T2 versus T1T1: OR = 0.95, 95% CI = 0.88–1.03, $P = 0.22$; T1T2+T2T2 versus T1T1: OR = 0.95, 95% CI = 0.88–1.02, $P = 0.19$; T2T2 versus T1T1+T1T2: OR = 0.97, 95% CI = 0.88–1.08, $P = 0.63$).

For *XRCC3* rs1799794 polymorphisms, two studies [16, 18, 20, 21, 23, 24] (3,119 cases and 6,207 controls) were eligible. The meta-analysis results of rs1799794 polymorphisms are shown in Table 4. We observed a statistically significant correlation with ovarian cancer risk using the homozygote comparison (T2T2 versus T1T1: OR = 0.70, 95% CI = 0.54–0.90, $P = 0.005$), heterozygote comparison (T1T2 versus T1T1: OR = 1.10, 95% CI = 1.00–1.21, $P = 0.04$), and the recessive genetic model (T2T2 versus T1T1+T1T2 : OR = 0.67, 95% CI = 0.52–0.87, $P = 0.002$). However, no statistically significant associations were observed in dominant genetic model (T1T2+T2T2 versus T1T1: OR = 1.06, 95% CI = 0.96–1.15, $P = 0.24$).

For *XRCC3* rs1799796 polymorphisms, the meta-analysis results were shown in Table 4. We observed a statistically significant correlation with ovarian cancer risk using the heterozygote comparison (T1T2 versus T1T1: OR = 0.91, 95% CI = 0.83–0.99, $P = 0.04$). However no statistically significant associations were observed in homozygote comparison (T2T2 versus T1T1: OR = 1.07, 95% CI = 0.93–1.24, $P = 0.33$), dominant genetic model (T1T2+T2T2 versus T1T1: OR = 0.94, 95% CI = 0.86–1.03, $P = 0.16$), and the recessive genetic model (T2T2 versus T1T1+T1T2: OR = 1.13, 95% CI = 0.98–1.29, $P = 0.08$).

3.3. Publication Bias and Sensitivity Analysis. The publication bias was tested by Begg's funnel plot and Egger's test for three SNPs. Egger's test results did not show any evidence of publication bias for any of the genetic models of the three SNPs (data not shown). The shape of the four Begg's funnel plots showed no evidence of obvious asymmetry of the three SNPs (data not shown).

In the sensitivity analysis, the corresponding pooled ORs were not altered, when the fixed-effects model was changed to random-effects model. So it revealed that the results of this meta-analysis were stable.

4. Discussion

The *XRCC3* gene is required for genomic stability [36]. It was reported that the *XRCC3* polymorphism increased the risk of many cancers, including ovarian cancer [36]. However, the results have been inconsistent. We preformed the meta-analysis including 5,302 ovarian cancer cases and 8,075 controls for 3 SNPs of *XRCC3*.

For rs861539 polymorphisms, no correlation with ovarian cancer risk was observed in any genetic models. However, For *XRCC3* rs1799794 and rs1799796 polymorphisms, we observed a statistically significant correlation with ovarian cancer risk. It was shown that the difference between different SNP sites was considerable for *XRCC3*.

All of the literature was of high quality. All study subjects were Caucasian. The global multicenter studies can provide more valuable conclusions. So further studies should be done to explore the possible relationships between *XRCC3* polymorphisms and ovarian cancer risk in other ethnicities.

In conclusion, this meta-analysis shows that the *XRCC3* were associated with ovarian cancer risk overall for Caucasians. Asian and African populations should be further studied.

Abbreviations

CIs: Confidence intervals
HWE: Hardy-Weinberg equilibrium
ORs: Odds ratios
XRCC3: X-ray repair cross-complementing group 3.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 2: Main characteristics of the studies included in the meta-analysis.

First author	Year	Country	Area of the cases	Ethnicity	Cases source	Controls source	Sample type of cases	Total cases/controls	Quality score
Auranen [20]	2005	Mixed (UK-USA)	Royal Marsden Hospital in London and 6 counties in Northern California	Caucasian	Mixed (hospital and cancer registry)	Population	Blood	1665/4241	14
Beesley [26]	2007	Australia	New South Wales and Victorian Cancer Registries	Caucasian	Cancer registry	Population	Blood	731/747	15
Quaye [25]	2009	Mixed (DK-UK-USA)	MALOVA from Denmark-SEARCH from the UK-and GEOCS from the USA.	Caucasian	Mixed (hospital and cancer registry)	Population	Blood	1461/2299	14
Webb [27]	2005	Australia	New South Wales-Victoria and Queensland	Caucasian	Mixed (hospital and cancer registry)	Volunteers	Mixed (blood and archival paraffin blocks)	1445/788	12

Table 3: Distribution of *XRCC3* rs861539 genotype among ovarian cancer cases and controls included in the meta-analysis.

First author	Year	Genotypes distribution (Case source)			Genotypes distribution (Controls source)			P-HWE (Controls)	T2T2 versus T1T1		T1T2 versus T1T1		T1T2+T2T2 versus T1T1		T2T2 versus T1T1+T1T2	
		T1T1	T1T2	T2T2	T1T1	T1T2	T2T2		OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Auranen [20]	2005	676	762	227	1712	1946	583	Yes	0.99 [0.83, 1.18]	0.88	0.99 [0.88, 1.12]	0.89	0.99 [0.88, 1.11]	0.87	0.99 [0.84, 1.17]	0.91
Beesley [26]	2007	291	339	101	288	351	108	Yes	0.93 [0.67, 1.27]	0.63	0.96 [0.77, 1.19]	0.69	0.95 [0.77, 1.17]	0.62	0.95 [0.71, 1.27]	0.72
Quaye [25]	2009	545	612	175	784	958	282	Yes	0.89 [0.72, 1.11]	0.31	0.92 [0.79, 1.07]	0.27	0.91 [0.79, 1.05]	0.21	0.93 [0.76, 1.14]	0.51
Webb [27]	2005	591	656	198	307	375	106	Yes	0.97 [0.74, 1.28]	0.83	0.91 [0.75, 1.10]	0.32	0.92 [0.77, 1.10]	0.37	1.02 [0.79, 1.32]	0.87
Total		2103	2369	701	3091	3630	1079		0.95 [0.85, 1.06]	0.37	0.95 [0.88, 1.03]	0.22	0.95 [0.88, 1.02]	0.19	0.97 [0.88, 1.08]	0.63
									Test for heterogeneity	$P = 0.91$	**Test for heterogeneity**	$P = 0.88$	**Test for heterogeneity**	$P = 0.82$	**Test for heterogeneity**	$P = 0.77$

Table 4: Distribution of *XRCC3* rs1799794 genotype among ovarian cancer cases and controls included in the meta-analysis.

First author	Year	Genotypes distribution (Case source)			Genotypes distribution (Controls source)			P-HWE (Controls)	T2T2 versus T1T1		T1T2 versus T1T1		T1T2+T2T2 versus T1T1		T2T2 versus T1T1+T1T2	
		T1T1	T1T2	T2T2	T1T1	T1T2	T2T2		OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Auranen [20]	2005	1060	550	48	2551	1188	161	Yes	0.72 [0.52, 1.00]	0.048	1.11 [0.98, 1.26]	0.087	1.07 [0.95, 1.20]	0.29	0.69 [0.50, 0.96]	0.027
Quaye [25]	2009	940	484	37	1505	713	89	Yes	0.67 [0.45, 0.99]	**0.04**	1.09 [0.94, 1.25]	0.25	1.04 [0.91, 1.19]	0.57	0.65 [0.44, 0.96]	0.027
Total		2000	1034	85	4056	1901	250		0.70 [0.54, 0.90]	0.005	1.10 [1.00, 1.21]	0.04	1.06 [0.96, 1.15]	0.24	0.67 [0.52, 0.87]	0.002
									Test for heterogeneity	$P = 0.77$	**Test for heterogeneity**	$P = 0.83$	**Test for heterogeneity**	$P = 0.78$	**Test for heterogeneity**	$P = 0.80$

Table 5: Distribution of *XRCC3* rs1799796 genotype among ovarian cancer cases and controls included in the meta-analysis.

First author	Year	Genotypes distribution (Case source)			Genotypes distribution (Controls source)			P-HWE (Controls)	T2T2 versus T1T1		T1T2 versus T1T1		T1T2+T2T2 versus T1T1		T2T2 versus T1T1+T1T2	
		T1T1	T1T2	T2T2	T1T1	T1T2	T2T2		OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Auranen [20]	2005	769	692	203	1757	1776	433	Yes	1.07 [0.89, 1.29]	0.47	0.89 [0.79, 1.01]	0.062	0.93 [0.83, 1.04]	0.188	1.13 [0.95, 1.35]	0.17
Quaye [25]	2009	676	608	177	1040	1006	253	Yes	1.08 [0.87, 1.33]	0.5	0.93 [0.81, 1.07]	0.31	0.96 [0.84, 1.09]	0.536	1.11 [0.91, 1.37]	0.30
Total		1445	1300	380	2797	2782	686		1.07 [0.93, 1.24]	0.33	0.91 [0.83, 0.99]	0.04	0.94 [0.86, 1.03]	0.16	1.13 [0.98, 1.29]	0.08
									Test for heterogeneity	$P = 0.97$	**Test for heterogeneity**	$P = 0.65$	**Test for heterogeneity**	$P = 0.69$	**Test for heterogeneity**	$P = 0.90$

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Research Article

Single Amino Acid Arginine Deprivation Triggers Prosurvival Autophagic Response in Ovarian Carcinoma SKOV3

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Autophagy is a process of cytosol-to-lysosome vesicle trafficking of cellular constituents for degradation and recycling of their building blocks. Autophagy becomes very important for cell viability under different stress conditions, in particular under amino acid limitation. In this report we demonstrate that single amino acid arginine deprivation triggers profound prosurvival autophagic response in cultured human ovarian cancer SKOV3 cells. In fact, a significant drop in viability of arginine-starved SKOV3 cells was observed when autophagy was inhibited by either coadministration of chloroquine or transcriptional silencing of the essential autophagy protein BECLIN 1. Enzymatic arginine deprivation is a novel anticancer therapy undergoing clinical trials. This therapy is considered nontoxic and selective, as it allows controlling the growth of malignant tumours deficient in arginine biosynthesis. We propose that arginine deprivation-based combinational treatments that include autophagy inhibitors (e.g., chloroquine) may produce a stronger anticancer effect as a second line therapy for a subset of chemoresistant ovarian cancers.

1. Introduction

It is established that some types of tumours are deficient in the biosynthesis of certain amino acids and often exhibit elevated sensitivity to deprivation of a corresponding single amino acid (such as arginine, methionine, and asparagine), both *in vitro* and, importantly, *in vivo* (for recent reviews: [1–5]). This provided a rational basis for the development of metabolic anticancer therapies based on the application of recombinant amino acid degrading enzymes, such as asparaginase for the treatment of leukemias and other tumours [2, 5, 6]. First clinical trials with recombinant enzymes hydrolyzing amino acid arginine, human arginase I, and *Mycoplasma hominis* arginine deiminase have demonstrated therapeutic efficacy in controlling the growth of hepatocarcinomas and melanomas [7–9]. Recent *in vitro* studies also suggested that other types of cancers may be potentially sensitive to this therapy (pancreatic, prostate, renal carcinomas, and mesotheliomas) due to the transcriptional silencing of arginine anabolic enzyme of urea cycle, argininosuccinate synthetase (ASS) [10–13] (see Figure 2). It was also observed that the development of chemoresistance to platinum compounds in ovarian carcinomas leads to collateral appearance of arginine auxotrophy due to the downregulation of ASS [14], adding these tumours to the list of potential targets of arginine deprivation-based enzymotherapy.

Although metabolic enzymotherapy based on arginine deprivation is considered as nontoxic and selective, it is not free of certain limitations. One such limitation arises from the upregulation of ASS expression in many tumours in response to arginine starvation, leading to the appearance of the ASS-positive tumour relapse insensitive to the therapy [2]. Also, we recently observed that tumour cells become profoundly more resistant to arginine withdrawal in *in vitro* 3D spheroid models relative to respective monolayer cultures [15, 16]. This phenomenon is consistent with the results of animal studies and ongoing clinical trials which showed that arginine deprivation is effective in inhibiting tumour growth but not

in inducing tumour regression. The latter observation stimulates further search for more efficient rational combinational therapeutic approaches based on arginine deprivation.

Arginine, besides being required for protein biosynthesis, has other versatile functions in the cell as a precursor of nitric oxide, agmatine, and polyamines [17]. It was also demonstrated that arginine is an essential amino acid for cultured tumour cells due to their deficiency in arginine biosynthesis *de novo* [18]. Thus, arginine withdrawal profoundly affects tumour cell physiology. In this work we show that arginine deprivation strongly induces the autophagic process in ovarian carcinoma cells in monolayer culture. Autophagy, the selective process of lysosomal recycling of cell constituents, is known to have a prosurvival role under different stresses in tumour cells [19]. Therefore, we addressed the question whether inhibition of autophagy affects tumour cell survival upon arginine starvation. Such a strategy could be applied to enhance the therapeutic effect of enzymotherapy based on arginine deprivation.

2. Materials and Methods

2.1. Reagents. The following antibodies were used: polyclonal antibodies against MAP-LC3 (Novus Biologicals, CO, USA) and BECLIN 1 (BD Biosciences, CA, USA), mouse monoclonal anti-LAMP1 (BD) and anti-Golgin97 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), ph-4E-BP1 and ph-p70-S6k (Cell Signaling Technology, Beverly, MA, USA), FITC-conjugated polyclonal goat anti-rabbit (Santa Cruz) and Cy3-conjugated polyclonal goat anti-mouse (Santa Cruz), and horseradish peroxidise- (HRP-) conjugated polyclonal goat anti-mouse and anti-rabbit (both from Millipore Corporation, Bedford, MA, USA).

Monodansylcadaverine (MDC), 3-methyladenine (3MA), chloroquine (CQ), asparagine (Asn), and other bench chemicals were purchased from Sigma-Aldrich.

2.2. Cell Line and Culture Conditions. SKOV3 cells originating from human ovarian carcinoma tissues were obtained from ATCC (USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, Utah, USA) with 10% foetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria), 2 mmol/L glutamine, and 50 mg/L gentamycin (Sigma-Aldrich, Steinheim, Germany) and maintained in the incubator at 37°C with 5% CO_2. Where indicated, arginine-containing (0.4 mM; HyClone Laboratories, Logan, UT, USA) and arginine-free media were supplemented with 5% dialysed FBS (HyClone). To study the growth dynamics of ovarian carcinoma cells under standard and arginine-deprived conditions, the cells were seeded at a density of 20000 cells per well in regular medium in 96-well plates and allowed to adhere for 24 h; then the medium was aspirated and the cell monolayer was washed two times with PBS, and finally the cells were incubated with fresh complete medium or arginine-free medium (AFM). The cells were cultured for up to 96 h, and cell growth was assessed by counting the cells every 24 h in triple. Cell viability was assessed using the trypan blue (final concentration 0.05%) dye exclusion. Viable (unstained) and nonviable (blue-stained) cells were counted on a haemocytometer by light microscopy.

2.3. RT-PCR. Total RNA was isolated from cells by the method of Chomczynski and Sacchi [20]. First-strand cDNA synthesis was performed using First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and an oligo-dT primer according to the manufacturer's instructions. PCR was performed using a High Fidelity PCR Enzyme Mix (Fermentas) with the following primer pairs:

ASS-S, 5′-GGGGTCCCTGTGAAGGTGACC-3′;

ASS-AS, 5′-CGTTCATGCTCACCAGCTC-3′;

ASL-S, 5′-GAAGCGGATCAATGTCCTGC-3′;

ASL-AS, 5′-CTCTTGGTGAATCTGCAGCG-3′;

OTC-S, 5′-AATCTGAGGATCCTGTTAAACAATG-3′;

OTC-AS, 5′-CTTTTCCCCATAAACCAACTCA-3′;

GAPDH-S, 5′-CAAGGTCATCCATGACAACTTTG-3′;

GAPDH-AS, 5′-GTCCACCACCCTGTTGCTGTAG-3′.

PCR fragments were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The relative mRNA expression levels were estimated after normalization with GAPDH. The number of cycles was chosen at which PCR product amount was optimal and within the linear portion of the curve, well before saturation point.

ASS: number of cycles—26, size of the amplicon—448 bp,

ASL: number of cycles—27, size of the amplicon—502 bp,

OTC: number of cycles—40, size of the amplicon—1125 bp,

GAPGH: number of cycles—25, size of the amplicon—496 bp.

2.4. Visualization of Monodansylcadaverine-Labelled Vacuoles. MDC is an autofluorescent weak base that accumulates in acidic lysosomal vacuoles and autophagolysosomes [21]. Cells attached to glass coverslips were incubated with 0.05 mM MDC (Sigma-Aldrich) in PBS at 37°C for 10 min. After incubation, cells were washed three times with PBS and immediately analyzed with a fluorescence microscope (ZEISS, Axio Imager A1) equipped with Axio Vision Software (v. 4.6.3). Images were captured with a CCD camera and imported into Photoshop. Quantification of cell fluorescence was conducted using ImageJ 1.48v Software.

2.5. Immunofluorescence and Microscopy Analysis. Immunofluorescence staining was performed as previously described [22]. Essentially, cells cultured on glass coverslips were washed with PBS, fixed with cold methanol, and permeabilized with 0.2% Triton X-100 in PBS. The coverslips were then incubated with the indicated primary antibodies in PBS contained 0.1% Triton X-100 and 4% FBS overnight at 4°C and thereafter incubated with the appropriate secondary antibodies for 1 hr at room temperature. Nuclei were stained with DAPI. Coverslips were mounted on microscope slides and monitored under a ZEISS fluorescence microscope (Axio Imager A1) equipped with Axio Vision Software (v. 4.6.3). Images were captured with a CCD camera and imported into Photoshop. Pearson correlation coefficients were calculated using ImageJ 1.48v Software to assess the degree of colocalization of protein markers of autophagy.

2.6. Immunoblotting. The cell monolayer was washed with ice-cold PBS and lysed in Extraction Buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 5 mmol/L benzamidine, 1 mmol/L PMSF, 10 mg/mL aprotinin, 10 mg/mL Leupeptin, and 1 mg/mL Pepstatin A) at 4°C for 20 min. Cell extracts were obtained after centrifugation at 12,000 g at 4°C for 30 min and cellular proteins were quantified using Peterson's method [23]. Equal amounts of protein homogenates were loaded, separated by SDS-PAGE (concentration of acrylamide varied depending on the size of the protein to be detected), and transferred onto PVDF membrane (Millipore Corp., Billerica, MA, USA). The membranes were blocked with 5% nonfat dried milk in PBS containing 0.05% Tween-20 and probed with the indicated primary and secondary (horseradish peroxidase-conjugated) antibodies. β-Actin was used for protein loading control. The bands were visualized using the enhanced chemiluminescence reagent (Millipore Corp.). Band densitometry quantification was performed using the Gel-Pro analyzer (Version 32).

2.7. Small Interfering RNA Transfection. BECLIN 1 silencing was achieved using RNA interference as previously described [24]. Cells were transfected using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. Protein knockdown was determined by immunoblotting 48 hr after transfection. As control (sham transfection), a nonspecific scramble sequence siRNA was used.

2.8. Statistics. In each individual experiment triplicate wells were used for each treatment and control. All experiments were repeated at least three times. Statistical analyses were performed using Student's t-test. Results were expressed as means ± SD. Significance was established when the P value was less than 0.05.

3. Results

3.1. SKOV3 Cells Retain Viability and Proliferative Potential under Long-Term Arginine Deprivation In Vitro. Tumour cell lines in monolayer cell culture substantially differ in their sensitivity to a single amino acid withdrawal [18]. Therefore, we first analyzed whether and to what extent arginine deprivation affects the viability and the proliferative potential of SKOV3 cells. Upon shifting to a defined arginine-deficient medium (AFM), growth arrest and reduction in the proportion of viable cells were observed. It is to be noted that, even after 4 days of arginine starvation, SKOV3 cells were still able to resume cell proliferation in response to arginine resupplementation, though regrowth potential progressively declined in the course of incubation in AFM (Figure 1). No signs of PARP fragmentation as a reporter of apoptosis in arginine-starved SKOV3 cells were observed (data not shown). This observation suggested that a substantial fraction of SKOV3 cells remained viable even after the prolonged arginine withdrawal indicating that these cells are rather resistant to this metabolic stress. For comparison, hepatocellular carcinoma HepG2 cells lose their proliferative potential already after 2 days of arginine starvation and exhibit concomitant apoptosis [18].

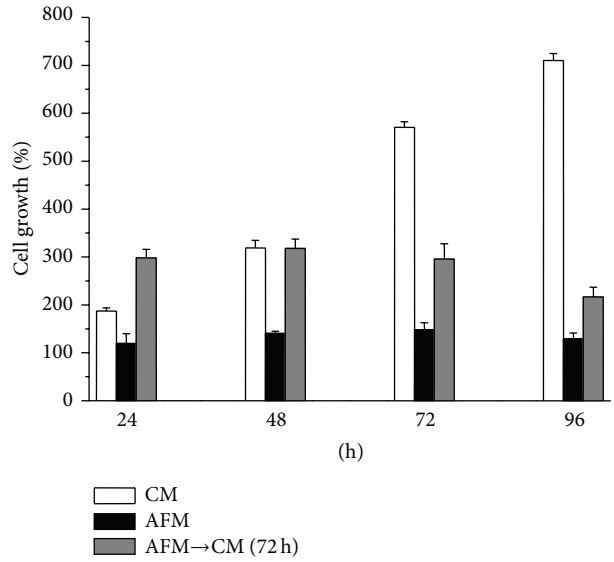

Figure 1: Effect of arginine deprivation on SKOV3 cell proliferation. The growth of SKOV3 cells in arginine-sufficient complete medium (CM), in arginine-free medium (AFM), and the ability of SKOV3 cells to rescue cell proliferation after different periods of arginine starvation was assessed by counting only the viable cells. After the indicated periods of incubation in AFM, cells were shifted to a fresh CM and allowed to proliferate for additional 72 h (AFM → CM (72 h)). Viable cells were determined by the trypan blue exclusion test. The initial number of cells (time-point zero) was considered as 100%. Data from three independent experiments in triplicate.

3.2. Arginine Is an Essential Amino Acid for Cultured SKOV3 Cells. The effect on growth arrest in AFM (Figure 1) suggested that arginine is an essential amino acid for SKOV3 cells. The RT-PCR analysis of arginine key anabolic enzymes of the urea cycle revealed that SKOV3 cells incubated in complete medium do not express mitochondrial arginine biosynthetic enzyme ornithine transcarbamylase (OTC; it converts ornithine to citrulline) but do express cytosolic

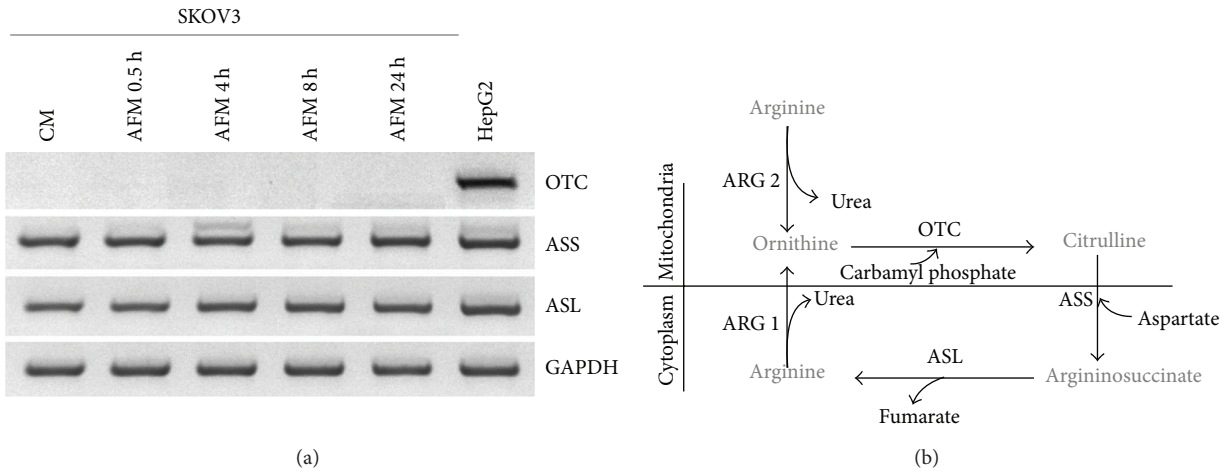

Figure 2: Expression of the key genes of arginine anabolism in SKOV3 cells. (a) Specific mRNA levels determined by RT-PCR analysis as described in Section 2. Cells were cultured in arginine-free medium (AFM) for 24 h or in arginine-sufficient complete medium (CM). Human hepatocarcinoma HepG2 cells (which express urea cycle enzymes) were used as a positive control. GAPDH expression was used as an internal loading control. (b) Scheme of arginine biosynthesis in the urea cycle. ARG1: arginase I, ARG2: arginase II, OTC: ornithine transcarbamylase, ASS: argininosuccinate synthetase, and ASL: argininosuccinate lyase.

argininosuccinate synthetase (ASS; it converts citrulline to argininosuccinate) and argininosuccinate lyase (ASL; it converts argininosuccinate to arginine) (Figures 2(a) and 2(b)). Arginine deprivation did not trigger an upregulation of ASS or ASL, often observed in other tumour cell lines, or induction of OTC transcription (Figure 2(a)). Human hepatocellular carcinoma HepG2 cells were used as a positive control [18]. OTC deficiency in cultured SKOV3 cells implies that they are deficient in endogenous arginine anabolism, and arginine can only be synthesized via ASS-mediated conversion of exogenously supplied citrulline (which is absent in standard DMEM medium). Accordingly, exogenous ornithine did not support proliferation of SKOV3 cells in AFM (data not shown). Therefore, we can assume that incubation in AFM surely induces arginine starvation in SKOV3 cells.

3.3. Arginine Deprivation in SKOV3 Cells Triggers Profound Autophagic Response. One of the prosurvival responses of tumour cells triggered upon amino acids limitation is the elevated intracellular protein recycling via autophagy [19]. In particular, arginine deprivation has been shown to induce autophagy in melanomas [25]. However, it is known that different tumour cells exhibit varying basal and stimulus-dependent inducible autophagic proficiency [26]. We addressed the question whether low sensitivity (in terms of cell survival and proliferation) of SKOV3 cells to arginine withdrawal as an essential amino acid (Figure 1) was associated with, or causally linked to, induction of autophagy. To monitor autophagy, we first employed a classical staining of acidic vacuoles, which includes lysosomes and autophagolysosomes, with the vital fluorescent dye MDC [21]. Arginine deprivation rapidly and profoundly induced the expansion of the autophagolysosomal compartment in SKOV3 cells (Figure 3). We observed MDC-labelled intracellular vacuoles in SKOV3 cells already after 30 min of arginine starvation. Treatment of the starved cells with 3-methyladenine (3MA, 10 mM), a classic inhibitor of autophagy that inhibits the formation of autophagosomes [22], strongly diminished the fluorescence signal (Figure 3). Upon treatment with chloroquine (CQ, 25 μM), another known inhibitor of autophagy [21], MDC staining of the cells further increased (Figure 3). This effect was expected, as CQ impairs the late stages of autophagy and leads to the accumulation of autophagosomes and of autophagolysosomes [21]. By contrast, a partial decrease in MDC staining in arginine-deprived SKOV3 cells was observed upon concomitant treatment with an excess of asparagine (Asn, 50 mM), which is known to downregulate autophagy through the stimulation of mTOR, a negative regulator of autophagy [21]. Quantification of cell fluorescence under arginine starvation and upon cotreatment with inhibitors using ImageJ 1.48v Software revealed an approximately four-time decrease in fluorescence between control arginine-starved and CQ-treated cells versus those treated with 3MA and Asn at 4 hours of incubation (data not shown). Taken together, the above data indicate that MDC staining is indeed mirroring the induction of autophagolysosomes in arginine-deprived SKOV3 cells. To definitively demonstrate the induction of autophagy, in a parallel experiment the cells cultured on coverslips were immunostained for LC3 (a hallmark of autophagosomes) and LAMP1 (a marker of lysosomes), as well as for BECLIN 1 (a component of the autophagy interactome) and Golgin97 (which labels the Golgi complex). Colocalization of LC3 and LAMP1 is a reliable indicator for the formation of autophagolysosomes, while formation of BECLIN 1 aggregates in the Golgi area is indicative of activation of the autophagy process [24].

We observed the colocalization of such signals (yellow fluorescence) already at 30 min of arginine deprivation (Figures 4(a) and 4(b)), indicating a fast upregulation of the autophagy process. Colocalization of autophagosome-

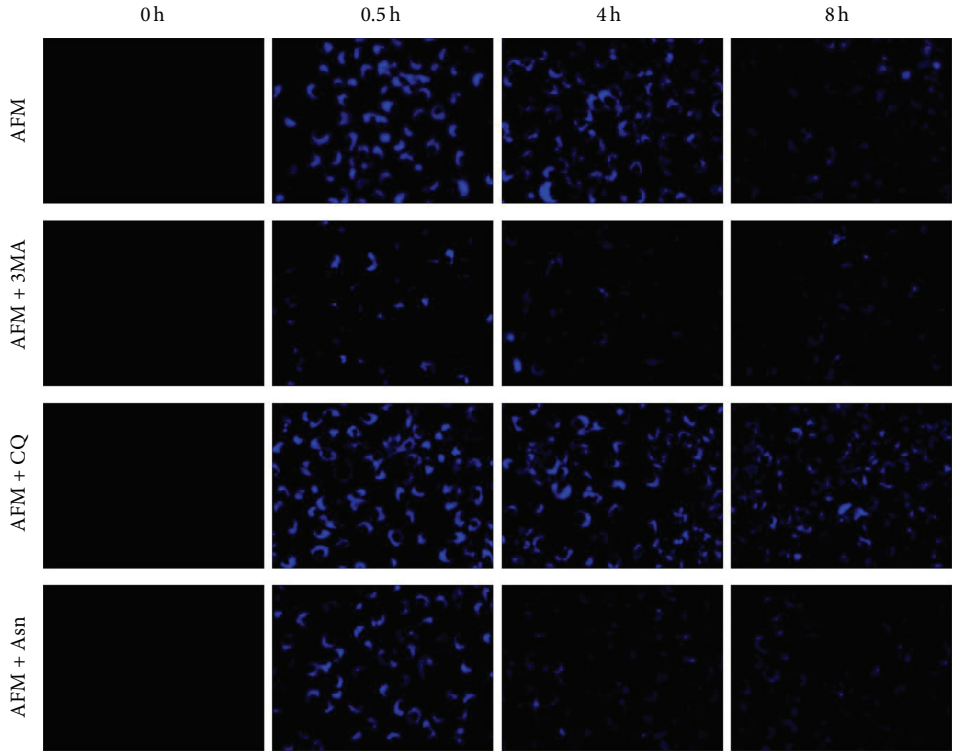

Figure 3: MDC staining of acidic vacuoles in ovarian carcinoma SKOV3 cells subjected to arginine deprivation (AFM). The cells were labelled with MDC as described in Section 2 at zero point and after 0.5, 4, and 8 hours of arginine withdrawal and immediately monitored under a fluorescence microscope. Magnification 400x.

and lysosome-associated proteins was evident during the whole time course of our analysis (Figures 4(a) and 4(b)). Importantly, in agreement with the MDC data (Figure 3), 3MA and Asn reduced while CQ increased the number and the size of LC3-positive vacuoles (i.e., autophagosomes) and also the aggregation of BECLIN 1 in the Golgi area (Figures 4(a) and 4(b)). The calculated values of Pearson correlation coefficient for the pairs LC3/LAMP1 and BECLIN 1/Golgin97 were ≥ 0.5 for arginine-starved cells, whereas for cells treated with inhibitors (3MA, CQ, and Asn) the coefficients values were 0 to ≤ 0.2. From these data we conclude that arginine deprivation strongly induces an autophagic response in ovarian carcinoma SKOV3 cells.

3.4. Effects of Arginine Deprivation and Autophagy Impairment on mTOR-Dependent Biosynthesis Pathway. Amino acid starvation is known to inhibit the biosynthetic pathway and, in parallel, to induce the autophagy degradation of redundant protein as an attempt to rescue the amino acids needed for the synthesis of vital proteins. The mTOR kinase is placed at the cross-point and is a master regulator of both these pathways [27, 28]. To get an insight into the relationship between the induction of autophagy and the biosynthetic pathway under arginine-deprivation conditions, we assayed the activation status of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and of p70-S6K (ribosomal p70 S6 kinase), two downstream substrates of mTOR that direct protein synthesis [29], in the presence of CQ, that hampers the last step of autophagy. In fact, in the presence of CQ the consumption of autophagosomes is interrupted as witnessed by the induced accumulation of LC3II both in CM and in AFM conditions, and this accumulation increases with time of incubation (Figure 5). However, in the cells subjected to arginine deprivation the level of LC3I decreases with time of incubation despite the fact that no further increase in LC3II is observed, indicating a rapid autophagy flux and consumption of autophagosomes. When these cells were concomitantly exposed to CQ the autophagosomes in fact accumulated with time (as indicated by increased LC3II at 8 versus 2 h). Next we looked at the translational activity in SKOV3 cells cultivated under these conditions. While phosphorylation of 4E-BP1 elicits its inhibition and therefore reliefs the inhibitory action of 4E-BP1 on protein synthesis, the phosphorylation of p70-S6K elicits the activation of the translational process [29]. Thus, the phosphorylation of both 4E-BP1 and p70S6k converges on triggering the initiation of protein synthesis. Both 4EBP and p70-S6K were highly phosphorylated in the cells cultivated in CM in the presence of CQ, while their activation status was greatly reduced under AFM culture condition regardless of whether CQ was or was not present (Figure 5).

3.5. Effect of Autophagy Inhibition by Transcriptional Silencing of BECLIN 1 or with CQ on the Sensitivity of SKOV3 Cells to Arginine Starvation. To elucidate the role of autophagy in maintaining SKOV3 cell viability under arginine deprivation, we manipulated the autophagy pathway by genetic and pharmacologic approaches. Transient transfection with a specific

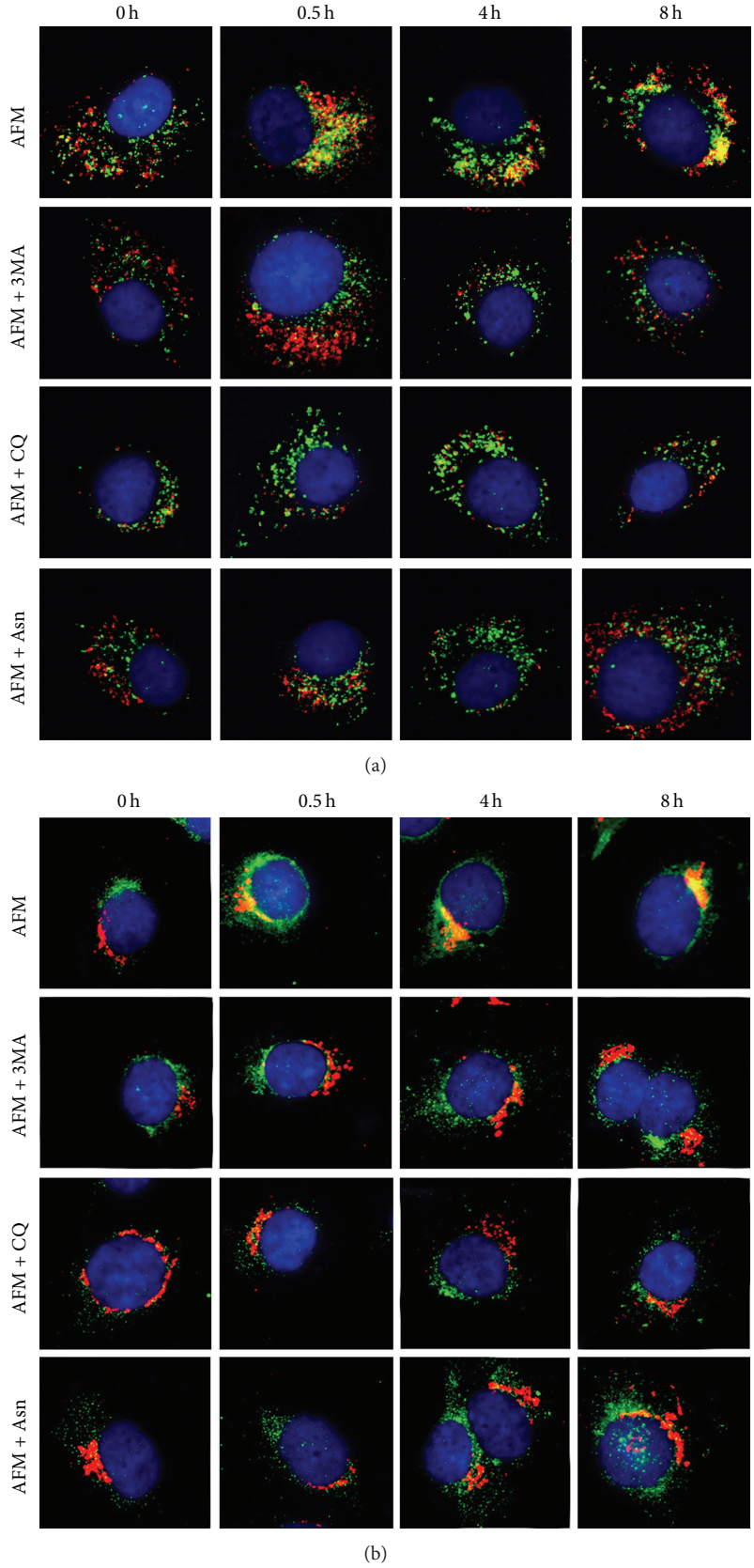

Figure 4: (a) Immunofluorescence staining of the autophagosomal protein LC3 (green fluorescence) and of the lysosomal protein LAMP1 (red fluorescence) and (b) of BECLIN 1 (green fluorescence) and Golgi-associated Golgin97 (red fluorescence) in SKOV3 cells subjected to arginine starvation. Nuclei were labelled with DAPI. Images were captured with ZEISS fluorescence microscope (Axio Imager A1) equipped with Axio Vision Software v. 4.6.3. Magnification 1000x.

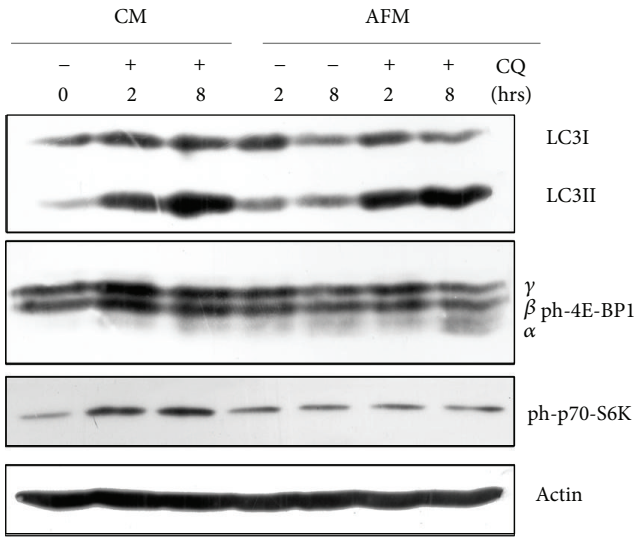

Figure 5: Effect of arginine deprivation and CQ treatment on accumulation of autophagosomal protein LC3II and phosphorylation of mTOR substrates. After indicated periods of treatment, the cells were washed and harvested for WB analysis. 50 μg of total sample protein was loaded per lane. β-Actin served as the loading standard.

siRNA elicited the transcriptional silencing (approximately 54%) of the autophagy protein BECLIN 1 (Figure 6(a)). After three days of incubation in AFM, the number of viable cells in siRNA-BECLIN 1 transfected culture decreased by approximately 40% as compared to the sham-transfected culture (Figure 6(b)). Arginine-deprived BECLIN 1-silenced cells also exhibited a decrease in their proliferative potential upon shift to the fresh CM relative to control cells (Figure 6(b)), thus suggesting a significant prosurvival role of autophagy in the cell response to arginine starvation.

Next, we addressed the question whether cotreatment with CQ, which impairs the late steps of the autophagy pathway, affects the survival of SKOV3 cells under arginine deprivation in a similar manner as observed with the silencing of BECLIN 1 expression. To this end, we monitored cell viability under the combined treatment and cell proliferation upon arginine resupplementation. The treatment with CQ (25 μM) dramatically decreased cell viability and the proliferative potential under arginine starvation (Figure 7). Importantly, CQ treatment rendered arginine-starved SKOV3 cells unable to resume proliferation in fresh CM already after two days of the combined treatment (Figure 7; cf. Figure 1). It is to be noted that a prolonged incubation with this same concentration of CQ was cytotoxic for SKOV3 cells also in CM medium (data not shown). No signs of apoptosis (as tested by western blotting of PARP and Annexin V staining) and no signs of senescence (as tested by positivity for beta-galactosidase staining) were detected in AFM plus CQ cotreated cells up to 144 h. However, SKOV3 cells double staining with ethidium bromide and Hoechst 33342 revealed that under this culture condition the majority of cells died via necrosis (not shown).

4. Discussion

Autophagy, a cytosol-to-lysosome membrane-trafficking process of degradation of cellular constituents, is a housekeeping homeostatic pathway and also plays a fundamental role to preserve cell viability upon different stress conditions [19]. One of such stresses is nutrient limitations, in particular amino acid restriction [30]. Autophagy is known to have dual function in cancerogenesis, playing both negative and positive roles in cancer progression and being implicated in chemoresistance of certain tumour types (for review: [26, 31–33]). It was also established that even single amino acid starvation triggers autophagic response in tumour cells [11, 34]. Previously reported [35] and our unpublished data suggest that the most profound autophagic response in tumour cells is triggered by starvation for arginine, methionine, lysine, and leucine relative to other amino acids. It remains to be elucidated whether specific regulation by this set of amino acids involves the mTORC1 complex or other mechanisms. Our data indicate that single arginine deprivation early affects the mTOR-dependent biosynthetic pathway.

Arginine, besides being required for protein biosynthesis, has other versatile functions in the cell as a precursor of nitric oxide, agmatine, and polyamines and as a regulatory molecule (for review: [17]). For cultured tumour cells arginine is an essential amino acid due to their deficiency in arginine biosynthesis *de novo* [18]. Ovarian carcinomas were recently added to the growing list of tumour types potentially sensitive to the treatment with recombinant arginine-degrading enzymes. In fact, it was demonstrated that relapses of ovarian carcinomas resistant to cisplatinum treatment concomitantly become deficient in argininosuccinate synthetase, a rate limiting enzyme of arginine biosynthesis, and thus potentially sensitive to arginine-degrading enzymes [14]. In this work we investigated how modulation of autophagy affects ovarian cancer cells viability under arginine deprivation. Human ovarian carcinoma SKOV3 cells were used as an experimental model. We found that SKOV3 cells exhibit high resistance to the stress exerted by arginine deprivation (Figure 1). This fact potentially allows studying the physiological role of autophagy under arginine withdrawal without interfering with processes of programmed cell death (apoptosis) that are often triggered to a different extent in cancer cells under such conditions [18]. Although SKOV3 cells exhibit high expression of argininosuccinate synthetase (as we show in Figure 2), they still are a suitable informative model for *in vitro* studies since SKOV3 cells are fully dependent on exogenous arginine supply due to the deficiency in ornithine transcarbamylase (OTC), an upstream enzyme of arginine anabolism (Figure 2). In this report we demonstrate that arginine withdrawal rapidly and markedly induces autophagy in SKOV3 cells (Figures 3 and 4). Under starvation, the biosynthetic pathway is impaired while basal autophagy rises up, and both these pathways are controlled by mTOR [27, 36]. Autophagy fuels the cytoplasm with the amino acids deriving from proteolysis and CQ is expected to interrupt this process by impairing the formation of autophagolysosomes and by inhibiting the acid-dependent proteolysis mediated

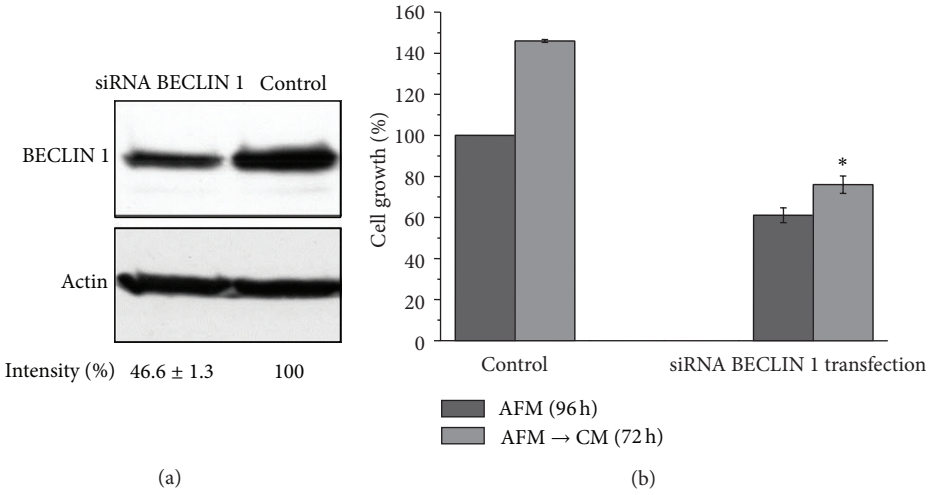

Figure 6: Transcriptional silencing of BECLIN 1 expression leads to decreased viability of SKOV3 cells under arginine starvation. (a) Western blot analysis of BECLIN 1 protein in SKOV3 cells transfected with BECLIN 1 siRNA or with nonsilencing control siRNA. β-Actin served as the loading standard. Densitometry quantification and the calculation of the relative band intensity were performed as described in Section 2. Intensity represents the mean value of three independent experiments and is expressed as means ± SD; (b) histogram represents the effect of BECLIN 1 silencing on the viability of arginine-starved cells and their proliferative potential after arginine resupplementation. $^{*}P < 0.05$.

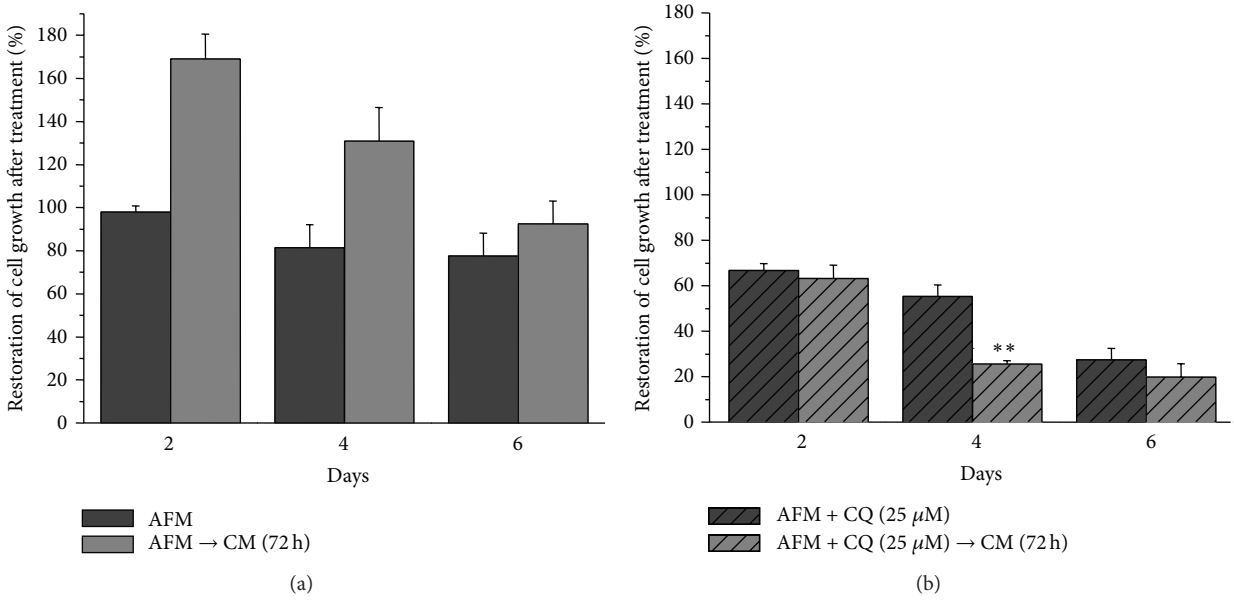

Figure 7: Effect of the autophagy inhibitor chloroquine (CQ) on SKOV3 cells viability and proliferative potential. Histograms showing cell survival and ability to resume proliferation after arginine resupplementation in cells deprived of arginine (AFM) (a) or cotreated with CQ (b). After the indicated periods of the treatment, the medium was changed to CM and cells were allowed to grow for additional 72 h. Viable cell numbers were determined by the trypan blue exclusion test. 100% is the number of cells at zero time point. $^{**}P < 0.01$.

by lysosomal cathepsins. The mTORC1 complex senses the availability of amino acids and phosphorylates downstream substrates in order to switch on and off the pathways for autophagy degradation or for protein synthesis accordingly [28]. Arginine deprivation in fact depressed the activation of the signalling kinases 4E-BP1 and p70-S6k that govern the protein synthesis pathway (Figure 5). These kinases are downstream of mTOR [29], which also negatively controls autophagy. CQ, which further reduces the availability of autophagy-derived amino acids, affected the signalling that governs the biosynthetic pathway in the cells cultivated in CM, indicating that despite the presence of amino acids in the culture medium the block of the autophagy degradation imposed by chloroquine was sensed by mTOR. By contrast, in AFM the mTOR pathway was inactive since the first 2 h of incubation and CQ did not reduce further the level of phosphorylation of 4E-BP1 and p70-S6k, indicating that arginine deprivation *per se* was sufficient to limit or inhibit the

activation of the protein synthesis pathway. We also demonstrate that autophagy process is important for maintaining cell viability under arginine deprivation. This conclusion is supported by the significant drop in viability of arginine-starved SKOV3 cells in which autophagy is inhibited. In this respect, either coadministration of CQ or transcriptional silencing of the essential autophagy protein BECLIN 1 produced similar effects (Figures 6 and 7). Strikingly, in the case of BECLIN 1 siRNA silencing, the observed decrease in viability and proliferative potential was roughly proportional to the remaining level of BECLIN 1 protein in the transfected culture (Figure 6). Preliminary data from our laboratories indicate that coapplication of taxane (taxol) at low doses may further decrease viability of ovarian carcinoma SKOV3 cells under arginine deprivation (to be published elsewhere). In this context, it is to be noted that taxol is a disruptor of the cytoskeleton and negatively impacts on the autophagosome-lysosome fusion step. In conclusion, our data support the conception that combinational treatment based on arginine deprivation and an autophagy inhibitor (e.g., chloroquine, a known nontoxic antimalarial drug) can potentially be applied as a second line treatment for a subset of ovarian carcinomas deficient in ASS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Epigenetic Control of Autophagy by MicroRNAs in Ovarian Cancer

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Autophagy is a lysosomal-driven catabolic process that contributes to the preservation of cell homeostasis through the regular elimination of cellular damaged, aged, and redundant molecules and organelles. Autophagy plays dual opposite roles in cancer: on one hand it prevents carcinogenesis; on the other hand it confers an advantage to cancer cells to survive under prohibitive conditions. Autophagy has been implicated in ovarian cancer aggressiveness and in ovarian cancer cell chemoresistance and dormancy. Small noncoding microRNAs (miRNAs) regulate gene expression at posttranscriptional level, thus playing an important role in many aspects of cell pathophysiology, including cancerogenesis and cancer progression. Certain miRNAs have recently emerged as important epigenetic modulators of autophagy in cancer cells. The mRNA of several autophagy-related genes contains, in fact, the target sequence for miRNAs belonging to different families, with either oncosuppressive or oncogenic activities. MiRNA profiling studies have identified some miRNAs aberrantly expressed in ovarian cancer tissues that can impact autophagy. In addition, plasma and stroma cell-derived miRNAs in tumour-bearing patients can regulate the expression of relevant autophagy genes in cancer cells. The present review focuses on the potential implications of miRNAs regulating autophagy in ovarian cancer pathogenesis and progression.

1. Introduction

The research fields of autophagy and microRNAs (miRNAs) are relatively new (less than 20 years from their definition and discovery) and our knowledge of these fields is in tremendous expansion; on the other hand, the ovary cancer remains a deadly disease since no significant improvement in overall survival was achieved in the last three decades [1]. Here we focus on the involvement of macroautophagy in the pathogenesis of cancer and on the molecular significance of miRNAs that potentially regulate this process. Targeting of the autophagy pathway is being under evaluation as a new anticancer therapeutic option [2, 3]. Therefore, unravelling the clinical implications of autophagy-miRNA interaction in ovary cancer might hopefully open the way to new diagnostic and molecular therapeutic approaches for this highly malignant disease.

2. MicroRNAs and Cancer

Over the last decade, several classes of molecules that form a complex transcriptional regulatory network are being identified and still their complete characterization is ongoing [4]. The most well-known small noncoding RNAs, discovered nearly 20 years ago, are the miRNAs, which posttranscriptionally regulate gene expression through base pairing with the 3′-untranslated region of target mRNAs [5]. MiRNA-mediated repression of gene expression occurs

through complex mechanisms not fully understood, including translational inhibition and mRNA degradation [6]. MiRNAs, as master regulators of gene expression, are among the major players in development, cell biology, and disease onset; in fact, it has been estimated that miRNAs can regulate the expression of more than half of protein-coding sequences in mammalian genomes. Accumulating evidence shows that miRNA expression is dysregulated in many types of cancer and that they can act either as oncogenes or tumour suppressors, depending on the cellular context and the expression of the miRNA targets in the particular tissue (reviewed in [7]). The effects of miRNA deregulation in cancer progression, diagnosis, and therapy have been extensively reviewed [8, 9].

3. Autophagy and Cancer

3.1. Morphological Aspects and Biochemical Regulation of Autophagy in Brief. Autophagy refers to a cellular process committed to the lysosomal degradation of self-constituents [10, 11]. Depending on the mechanism through which the substrate is delivered into the lysosome, autophagy is classified as macroautophagy, microautophagy, and chaperon-mediated autophagy [12–15]. However, macroautophagy (now simply referred to as autophagy) is the process mainly subjected to fluctuations to comply the needs for keeping cell homeostasis in response to stressful injuries. Autophagy, in fact, is the only pathway allowing the degradation of macromolecular aggregates, portion of cytoplasm, membranes, and entire organelles [16]. In this process, the autophagy substrate is sequestered within a newly formed vesicle (named autophagosome) that subsequently fuses with several endosomes and lysosomes to form autophagolysosomes (or autolysosomes) in which the autophagy substrate is fully degraded by the lysosomal acid hydrolases [17]. The substrates are selectively incorporated within the nascent autophagosomes through the intervention of proteins, such as p62/SQSTM1 (sequestosome), NBR1 (neighbour of BRCA1 gene 1), and Nix/BNIP3, that bridge the substrate and the membrane-bound LC3 [18–20]. LC3 (light chain 3; the mammalian orthologue of yeast atg8) derives from the posttranslational modification of MAP-LC3 (microtubule-associated protein-LC3) and is specifically associated with autophagosomal membranes [21]. The autophagosome originates from the nucleation and expansion of a preautophagosomal structure, a double-layered omega-shaped semicircle originating from the smooth endoplasmic reticulum [22]. Eventually, this structure closes up to form the autophagosome, which entraps the cargo. While being on formation, the lipidated isoform LC3-II is inserted onto the internal and external membranes of the autophagosome. The autophagosomes then move toward the microtubular organizing center, where they meet and fuse with the lysosomes [23]. The cargo is then completely degraded, along with the internal membrane of the autophagosome, within the acidic lumen of the autophagolysosome [24]. LC3-II present on the internal membrane of the autophagosome is also degraded, so that its consumption serves as readout of the autophagy flux [21]. Finally, the monomeric substrates are then pumped out in the cytosol for recycling purposes [25].

The autophagy pathway is controlled by a variety of signalling molecules [26, 27]. The ULK1 (Unc51-like kinase 1, the homolog of the yeast Atg1) kinase is believed to master the induction of autophagy [28]. Its function is under the control of two upstream kinases, AMPk and mTOR. Schematically, the class I PI3-k-AKT signalling pathway negatively impinges on autophagy through the activation of mTOR complex 1 (mTORC1), which inhibits the ULK1 complex, while the LKB-AMPk signalling pathway positively regulates autophagy through the inactivation of mTORC1 and the direct activation of ULK1 [29]. The activation of these pathways is influenced by intracellular and extracellular factors. The availability of nutrients (essentially, glucose and amino acids) and of growth factors activates the class I PI3k-AKT-mTORC1 pathway, thus repressing autophagy, whereas starvation strongly induces autophagy [30, 31]. On the other hand, energy depletion (i.e., shortage of ATP), oxidative stress, and DNA damage activate the LKB-AMPk pathway and therefore trigger autophagy [32–35]. The ULK1 complex signals to (also known as Vps34), which forms an active complex with Beclin-1 (also known as ATG6 or Vps30) [31]. This complex is recruited at the level of the preautophagosomal structure and locally produces PI3P (phosphatidyl -3-phosphate), the starting platform for the recruitments of membranes necessary for the biogenesis of the autophagosome [12].

3.2. The Pathophysiological Role of Autophagy in Cancer. The role of autophagy in cancer biology is not unequivocal. While basal (constitutive) autophagy prevents carcinogenesis through the constant elimination of damaged molecules and organelles that may increase the probability of oxidative stress mediated DNA mutation [36], induced autophagy can help cancer cells to face adverse situations such as the metabolic stress due to hypoxia and hyponutrition or the damaged provoked by anticancer treatments [37, 38]. In addition, the upregulation of autophagy may switch cancer cells into a dormant state, thus posing the basis for tumour relapse [39–41].

Many oncogenes and oncosuppressors regulate autophagy [42]. In general, oncogenes (e.g., *AKT, BCL2*) tend to repress autophagy, though for some of them (e.g., *RAS*) the final effect is cell context dependent [43–46]. It has been proposed that the abnormal expression of oncogenes favours the induction of prosurvival autophagy in cancer cells experiencing a metabolic stress. By contrast, oncosuppressors (e.g., PTEN, TSC1/TSC2, and DAPk) positively regulate autophagy and thus their lack reduces or abrogates the level of basal and inducible autophagy. Consistently, loss of function of the oncosuppressors Beclin-1 [47, 48] or PTEN [49, 50] predisposes to spontaneous cancers. The role of the oncosuppressor p53 in the regulation of autophagy in cancer cells appears ambiguous: while nuclear DNA-binding proficient p53 promotes the transcription of certain autophagy genes [51], p53 mutants that reside in the cytoplasm hamper autophagy [52, 53].

Besides, microenvironmental factors (hypoxia, pH, oxidative stress, nutrient availability, cytokines, hormones, and growth factors) and the physical-metabolic interaction of tumour cells with surrounding cells (inflammatory cells, fibroblasts) in the matrix greatly influence the actual level of autophagy in the cancer cells [54–56].

4. Ovarian Cancer Genesis and Progression: The Potential Role of MicroRNAs and of Autophagy

4.1. Involvement of Autophagy in the Pathogenesis of Ovarian Cancer. Based on the traditional view, ovarian tumours arise from subsequent metaplastic changes in the ovarian surface epithelium that lead to the development of four main histologic types: serous, endometrioid, mucinous, and clear cell (for a review see, [57]). More recently, the correlation of clinicopathological features with genetic studies has suggested a new paradigm for the pathogenesis and origin of epithelial ovarian cancer based on a dualistic model of carcinogenesis that classifies ovarian cancer in two types [58]. Type I tumours comprise low grade serous and endometrioid carcinomas, clear cells, and mucinous carcinomas which develop in a stepwise fashion from well-defined precursor lesions. They are indolent and relatively genetically stable, being characterized by a variety of somatic mutations or amplification/deletion of oncogenes or oncosuppressors including *K-RAS*, *B-RAF*, and *PTEN* [59, 60]. In contrast, type II tumours comprise high-grade serous and endometrioid carcinomas, malignant mixed mesodermal carcinomas, and undifferentiated carcinomas; they are rapidly growing and highly aggressive. Type II tumours are chromosomally unstable and express mutated *TP53* in more than 95% of the cases and *BRCA* inactivation in up to 50% of high-grade serous tumours (for a review see [61]). Besides these genetic abnormalities, also epigenetic alterations in the expression of critical genes may occur during cancer progression, and these changes are reflected in the signalling pathways that govern cell proliferation, cell migration, dormancy, and chemoresistance. At least 15 oncogenes and 16 oncosuppressor genes have been found deregulated in ovarian cancers because of genetic or epigenetic alterations [62–64]. Many of these oncogenes and oncosuppressors have also been involved in the regulation of autophagy [65]. Indeed, there is experimental evidence linking autophagy to ovarian cancer genesis. For instance, poorly differentiated and highly malignant ovarian cancer cells were shown to express very low level of the autophagosomal marker LC3, compared to benign hyperplastic tissues and borderline ovarian tumours [66]. The expression of the oncosuppressor *BECN 1*, which activates PI3k III-dependent autophagy (see above), was found downregulated in ovarian cancers, compared to benign lesions [66]. Also *DRAM* (damage-regulated autophagy regulator) 2, a p53-transcribed gene that positively regulates autophagy [67], was found to be expressed at very low level in aggressive ovarian tumours [68]. As many as 60 to 80% of both sporadic and familial ovarian cancers have been shown to bear mutations and deletions of the oncosuppressor *TP53* gene [64, 69, 70]. Deletion of *TP53* could favour high level of basal autophagy [71], whereas DNA-binding deficient p53 mutants, which are found in human ovarian carcinomas [72], are unable to sequester BCL-2 or BCL-XL and indirectly could inhibit autophagy [53]. On the other hand, the hyperactivation of mTOR, which results in suppression of basal autophagy, was associated with a poor prognosis in ovarian carcinoma patients [73]. Taken together, it seems that ovarian carcinogenesis associates with insufficient autophagy. Another interesting gene linking autophagy and ovarian cancer is the aplasia ras-homolog member 1 (*ARH1*; also known as *DIRAS3*), which codes for a ras-homolog 26 kDa GTPase. The expression of *ARHI* correlates with prolonged progression-free survival and has been found downregulated in more than 60% of ovarian cancers [74, 75]. *ARH1* is an imprinted oncosuppressor gene (one allele is inherited in a hypermethylated form), and therefore one single event (deletion, mutation, or epigenetic silencing) affecting the functioning allele is sufficient to cause the loss of function [76, 77]. ARH1 protein has recently been shown to modulate autophagy and dormancy in ovarian cancer cells [40]. It was shown that reactivation of *ARHI* by stromal factors could rescue dormant ovarian cancer cells through modulation of autophagy [40].

4.2. Modulation of Autophagy by MicroRNAs. Considering the implications of both miRNAs and autophagy in cancer-related processes and given the lack of current evidence linking these two rapidly growing fields of research, we prompted to review miRNAs regulating autophagy.

Recently, Jegga et al. used a system biology-based approach to define the complex regulatory and functional networks of genes controlling the autophagy-lysosomal pathway and found miR-130, miR-98, miR-124, miR-204, and miR-142 as putative posttranscriptional regulators of this pathway at various levels [78].

In principle, autophagy could be regulated by miRNAs targeting the mRNA of key molecules that indirectly induce or suppress autophagy, as, for instances, miR-504 that negatively regulates p53 [79] or miR-20b that negatively regulates the expression of HIF-1α [80] or any miRNA implicated in the regulation of the PI3k-(PTEN)-AKT-mTOR pathway as is, for instance, the case of miRNAs targeting PTEN [81]. More recently, miRNAs specifically targeting the mRNA of autophagy proteins are being identified [82]. For instance, members of the miR30 family can target Beclin-1, ATG2, ATG5, and ATG12 [83, 84]; miR-130a targets ATG2B [85]; mi-R181a-1 targets ATG5 [86, 87]; miR-290-295 targets ATG7 and ULK1 [88]; miRNA-17 and miR-119a-5p target ATG7 [89, 90]; miR376b targets ATG4 and BECLIN-1 [91]; miR-630 targets ATG12 [86]; and miR-519 targets Beclin-1, ATG10, and ATG16L1 [86].

Here, we will focus on those miRNAs that are either up- or downexpressed in ovarian cancers and that potentially regulate autophagy.

4.3. MicroRNAs Aberrantly Expressed in Ovarian Cancer. Comparative miRNAs expression profiling of ovarian cancer

Table 1: (a) Autophagy-related genes and their function identified as targets of the microRNA involved in ovarian cancer development and progression (miRanda release, August 2010; TargetScan release 6.2.). (b) Genes coding for autophagy-regulating molecules identified as targets of the microRNA involved in ovarian cancer development and progression (miRanda release, August 2010; TargetScan release 6.2.).

(a)

miRNAs (involved in ovarian cancer progression)	Predicted autophagy Target Genes	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-141 hsa-miR 200a	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
hsa-miR-199a -5p	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
	ATG4D	Processing of MAP1-LC3	Yes	Yes
	BECN1	BCL-2 interacting myosin/moesin-like coiled-coil protein 1, part of the class III PtdIns 3-kinase complex (activating macroautophagy)	Yes	Yes
hsa-miR-214	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
hsa-miR-182	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
	ATG16L1	A component of the ATG12-ATG5-ATG16 complex for the formation of autophagosome	Yes	Yes
	MAP1LC3B	Microtubule-associated protein 1 light chain 3, precursor of LC3-II inserted in autophagosomal membranes	Yes	Yes
hsa-miR-140-5p	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
hsa-miR-125b	*UVRAG*	Interacting with Beclin-1 and Bif-1 (activation and stimulation of macroautophagy)	Yes	Yes
hsa-miR-34a	*ATG4B*	Processing of MAP1-LC3	Yes	Yes
	ATG9A	A transmembrane protein involved in lipid transport for phagophore expansion	Yes	Yes
hsa-let-7a	*ATG4B*	Processing of MAP1-LC3	Yes	Yes
	ATG9A	A transmembrane protein involved in lipid transport for phagophore expansion	Yes	Yes
	ATG16L1	A component of the ATG12-ATG5-ATG16 complex for the formation of autophagosome	Yes	Yes
hsa-miR-15a hsa-miR-15b	*ATG13*	A component of the ULK1 complex needed for ULK1 kinase activity	No	Yes
	ATG9A	A transmembrane protein involved in lipid transport for phagophore expansion	Yes	Yes
	ATG14L	A component of the class III PtdIns 3-kinase complex	No	Yes
hsa-miR-210	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
hsa-miR-449b	*ATG4B*	Processing of MAP1-LC3	Yes	Yes

(b)

miRNAs (involved in ovarian cancer progression)	Predicted autophagy Target genes	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-141 hsa-miR 200a	*PTEN*	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR 200b hsa-miR 200c	*PTEN*	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
hsa-miR 21	*TSC1*	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
	BCL2	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes

(b) Continued.

miRNAs (involved in ovarian cancer progression)	Predicted autophagy Target genes	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-125b	*UVRAG*	Interacting with Beclin-1 and Bif-1 (activation and stimulation macroautophagy)	Yes	Yes
	BCL2	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes
hsa-miR-101	*MTOR*	Mammalian target of rapamycin (kinase) component of MTORC1 (that inhibits autophagy) and of MTORC2 (that phosphorylates Akt)	No	Yes
	RAB5A	Endocytic vesicle associated ras-homolog GTPase (involved in autophagosome formation)	Yes	Yes
hsa-miR-31	*RAB1B*	Endocytic vesicle associated ras-homolog GTPase (involved in autophagosome formation)	Yes	Yes
hsa-miR-34a	*BCL2*	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes
has-let-7a	*TSC1*	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR-15a hsa-miR-15b	*BCL2*	Interactor of BECLIN 1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
	FKBP1A	An immunophilin that forms a complex with rapamycin and inhibits mTOR activity	Yes	Yes
hsa-miR-155	*PDK1*	Kinase that phosphorylates AKT in Thr308	Yes	Yes
	RPTOR	Regulatory associated protein of mTOR (component of MTORC1)	No	Yes
hsa-miR-99a hsa-miR-100	*MTOR*	Mammalian target of rapamycin (kinase) component of MTORC1 (that inhibits autophagy) and of MTORC2 (that phosphorylates Akt)	No	Yes
hsa-miR-449b	*BCL2*	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes

and normal ovary epithelium specimens has been performed in several laboratories and the readers can refer to some excellent comprehensive reviews [92, 93]. The laboratory of Carlo Croce first reported on the differential expression of some miRNAs between normal and cancer ovary epithelial tissues, showing an upregulation of miR-200a/b/c, miR141, miR-21, miR-203, and miR-205 and a downregulation of miR-199a, miR-140, miR-145, miR-222, and miR-125b1 [94]. In another study, miR-21 was found as the most upregulated and miR-125b as the most downregulated miRNA in ovary cancer versus normal ovary epithelium tissues [95]. However, a clear consensus on the diagnostic and prognostic value of a miRNA signature has not been reached yet. One study reported the complete downregulation of 44 miRNAs (including the oncosuppressive miR-15a, miR-34a, and miR-34b) and the upregulation of miR-182 in late-stage ovary cancers [96]. Another group found miR-199a, miR-214, and miR-200a as the ones most upregulated and miR-100 as the most downregulated miRNA in high-grade and late-stage ovary cancers [97]. Also miR-200a, miR-34a, and miR-449b were found downregulated in late-stage ovary cancers [98]. Late-stage ovary cancers are associated with the acquisition of chemotherapy resistance and metastasis formation, with the latter resulting from the phenotypic transformation known as epithelial-to-mesenchymal transition (EMT). A miRNA signature of the mesenchymal-like phenotype of epithelial ovary cancer was shown to include miR-141, miR-200, miR-29c, miR-101, miR-506, and miR-128 [99]. Further, the response to chemotherapeutics (e.g., Platinum) was found to be associated with a particular miRNA signature that includes let-7i [100], hsa-miR-27a, hsa-miR-23a, and miR-378 [98, 101].

In searching for the molecular pathways responsible for the metabolic and phenotypic alterations associated with a certain miRNA signature, it must be taken into account that one single miRNA can target the mRNA of multiple genes and that one single mRNA can have multiple target sequences for different miRNAs. Recently, another level of complexity in the global regulation of gene expression by miRNAs has emerged. It was in fact shown that the overexpression of certain miRNAs could indirectly regulate the level of other miRNAs in ovarian cancer cells [102].

4.4. Regulation of Autophagy by MicroRNA Aberrantly Expressed in Ovarian Cancer. As stated above, the modulation of autophagy by environmental stressful conditions (nutrient depletion, hypoxia, oxidative stress, and

Table 2: Genes coding for proteins involved in the autophagy pathway identified as targets of microRNA involved in the cytotoxic response to cis-Platinum in ovarian cancer (miRanda release, August 2010; TargetScan release 6.2.).

miRNAs (involved in cis-Pt response)	Predicted gene(s) involved in autophagy	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-27a	*PDK1*	Kinase for the phosphorylation of AKT in Thr308	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR-23a	*UVRAG*	Interacts with Beclin-1 and Bif-1 (activation and stimulation macroautophagy)	Yes	Yes
	ATG12	A ubiquitin-like protein that modifies (autophagosome expansion)	Yes	Yes
	BCL2	Interactor of Beclin-1 (represses autophagy) and of Bax (represses apoptosis)	Yes	Yes
	PTEN	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
	RAPTOR	Regulatory associated protein of mTOR (component of MTORC1)	No	Yes
hsa-miR-378	—			
hsa-let-7i	*ATG4B*	Processing of MAP1-LC3	Yes	Yes
	ATG16L1	A component of the ATG12-ATG5-ATG16 complex for the formation of autophagosome	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes

chemotherapeutic drugs) and/or by genetic and epigenetic hints may confer resistance to the chemotherapeutic treatments in cancer cells and may also favour the EMT and metastasization of cancer cells [103]. MiRNAs could contribute to the modulation of autophagy in these situations. For instance, the treatment with cisplatin could induce chemoresistance-promoting autophagy through the downregulation of certain miRNAs targeting ATG proteins or the pathways that control autophagy. As an example, miR-214 was shown to confer cisplatin resistance in ovarian cancer cells by targeting PTEN [97], and PTEN is known to positively regulate autophagy [104]. PTEN expression is posttranscriptionally regulated by a set of miRNAs [81, 97, 105]. In ovarian cancers, overexpression of miR-21 correlated with late stage and metastasis and significantly decreased the expression of PTEN [106].

We have made an "in silico" search of the *ATG* genes that are potential target candidates of the most relevant miRNAs found aberrantly expressed in ovary cancers. In Table 1 we report the results obtained using two algorithms for the prediction of microRNA gene targets, namely, the "TargetScanHuman" [107] and the miRanda [108] software. We have considered three different sets of miRNAs: in Tables 1(a) and 1(b) are reported the miRNAs that were found aberrantly expressed (either up- or downregulated with respect to the normal ovary epithelium) in ovarian cancers and that are possibly involved in ovarian tumorigenesis and progression; in Table 2 are reported the miRNAs that apparently play a role in chemoresistance; in Table 3 are reported the miRNAs that were found involved in the epithelial-to-mesenchymal transition of the phenotype. For clarity, in Table 1 we have separately described the *ATG* genes coding for ATG proteins (a) and the genes coding for signalling molecules that directly or indirectly control the induction and progression of autophagy. (b) In the tables, we also describe the function of the proteins coded by the genes predictably targeted by the miRNAs. In general, the two algorithms agreed in the identification of *ATG* target genes for most of the miRNAs of interest. The main discordances between miRanda and TargetScan were relative to the recognition of ATG14L as target of miR-21, miR-214, miR-140, miR-15a, and miR15b, and of ATG13 as a target of miR-15a and miR15b.

For some of these miRNAs the *ATG* gene target has been validated in tumours other than ovarian cancer. Although these data should be considered with caution due to the possible context and tissue specificity of miRNA regulation, we can assume that some available information can be applied also to ovarian cancer. For instance miR-101, reported to act as inhibitor of autophagy in breast cancer by targeting STMN1, RAB5A, and ATG4D mRNAs [109], has been found downregulated also in ovarian cancer compared to normal tissue, and its reexpression exerted tumour-suppressive effects in ovarian carcinogenesis [110]. Of note, stathmin overexpression showed a significant association with poor prognosis in ovarian cancer patients [111]. In keeping with the potential of miR-101 to regulate autophagy and ovarian cancer progression, it is to be mentioned that its target RAB5A was shown to be upregulated and to promote cell

Table 3: Genes coding for proteins involved in the autophagy pathway identified as targets of the microRNA involved in the epithelial-to-mesenchymal transition process in ovarian cancer (miRanda release, August 2010; TargetScan release 6.2.).

miRNAs (involved in EMT)	Predicted gene(s) involved in autophagy	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-141 hsa-miR 200a	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
	PTEN	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR 29c	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
	PTEN	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
hsa-miR-101	*MTOR*	Mammalian target of rapamycin (kinase) component of MTORC1 (that inhibits autophagy) and of MTORC2 (that phosphorylates Akt)	No	Yes
	RAB5A	Endocytic vesicle associated ras-homolog GTPase (involved in autophagosome formation)	Yes	Yes
hsa-miR-506	—			
hsa-miR-128	*PDK1*	Kinase that phosphorylates AKT in Thr308	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes

proliferation in ovarian cancer [112]. Also, miR-30a, which negatively regulates the expression of Beclin-1 in ovarian cancer cells [113], was found deregulated in stage I ovarian cancer patients together with other miRNAs; in particular, it was downregulated in samples from relapsing patients [114, 115]. This result is in line with possible involvement of miR-30a in autophagy-dependent chemoresistance in ovarian cancers.

5. Conclusion: Clinical Implications and Future Perspectives

Modulation of autophagy has a great impact on the carcinogenesis process. In fact, depending on whether it is considered at the precancerous or at the advanced stage, up- or downregulation of autophagy may elicit either tumour-promoting or tumour-suppressive effects [116, 117]. The actual level of ongoing autophagy in the tumour cells is dictated by genetic mutations but also influenced by the epigenetic regulation of gene expression [65, 103]. In the context of the intricate involvement of autophagy in cancer progression, emerging data point to the role of miRNAs as regulators of autophagy gene expression. The immediate and acute modulation of protein expression mediated by miRNAs plays a fundamental role in the adaptive response of the cell metabolism to environmental stresses such as nutrient shortage, hypoxia, and genotoxic stress. Autophagy is one of the main stress response pathways. Therefore, the modulation of ATG proteins and/or of signalling molecules that regulate autophagy by miRNAs finally impacts the capability of the cell to overcome the stress. This aspect is of particular relevance when considering the cytotoxic response of cancer cells to a chemotherapeutic drug. Chemosensitivity could be rescued by manipulating the level of miRNAs targeting autophagy. In fact, certain miRNAs can target both the autophagy and the apoptosis pathways and therefore can influence the cross-talk between these two processes and determine whether the cancer cell will resist or succumb to the toxic drug. For instance, miR-199a-5p was shown to increase chemoresistance by simultaneously promoting autophagy and suppressing apoptosis. By downregulating Beclin-1 expression, miR-30a and miR-376b downregulate not only autophagy but also apoptosis since the level of free antiapoptotic BCL-2 protein in the cell will increase. Thus, miRNAs can act as molecular switches to turn on or off either or both of the autophagy and apoptosis processes. These findings provide the rationale for designing novel therapeutic approaches combining the conventional anticancer drugs with miRNAs targeting the autophagy process.

Autophagy is clearly deregulated in ovarian cancer (reviewed in [65]), and here we have highlighted the possibility that the miRNAs aberrantly expressed in ovarian cancer could be involved in such deregulation.

The miRNA landscape of ovarian cancer is in rapid progress [118] and advance in detection and functional evaluation of miRNAs is expected to strongly contribute to unravelling the network of apoptosis and autophagy regulation in this complex disease. In the near future, studies ongoing in our and other laboratories will likely identify the miRNA signatures associated with autophagy in ovarian cancer, thus posing the basis for the possible harnessing of these miRNAs as therapeutic targets, as well as possible diagnostic-prognostic tools.

Conflict of Interests

The authors declare that the present study was performed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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Review Article

Recent Concepts of Ovarian Carcinogenesis: Type I and Type II

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Type I ovarian tumors, where precursor lesions in the ovary have clearly been described, include endometrioid, clear cell, mucinous, low grade serous, and transitional cell carcinomas, while type II tumors, where such lesions have not been described clearly and tumors may develop *de novo* from the tubal and/or ovarian surface epithelium, comprise high grade serous carcinomas, undifferentiated carcinomas, and carcinosarcomas. The carcinogenesis of endometrioid and clear cell carcinoma (CCC) arising from endometriotic cysts is significantly influenced by the free iron concentration, which is associated with cancer development through the induction of persistent oxidative stress. A subset of mucinous carcinomas develop in association with ovarian teratomas; however, the majority of these tumors do not harbor any teratomatous component. Other theories of their origin include mucinous metaplasia of surface epithelial inclusions, endometriosis, and Brenner tumors. Low grade serous carcinomas are thought to evolve in a stepwise fashion from benign serous cystadenoma to a serous borderline tumor (SBT). With regard to high grade serous carcinoma, the serous tubal intraepithelial carcinomas (STICs) of the junction of the fallopian tube epithelium with the mesothelium of the tubal serosa, termed the "tubal peritoneal junction" (TPJ), undergo malignant transformation due to their location, and metastasize to the nearby ovary and surrounding pelvic peritoneum. Other theories of their origin include the ovarian hilum cells.

1. Introduction

Ovarian carcinoma is the most lethal gynecological malignancy. It is estimated that there will be >140000 deaths per year worldwide [1]. Although many surgical techniques and chemotherapies have been developed for ovarian carcinoma, the prognosis remains poor, with a five-year survival rate of 45% [2]. Although the prognosis is more favorable in patients with stage I/II tumors, the majority of patients present with advanced stage disease (III/IV).

Most ovarian carcinomas have been suggested to originate from the ovarian surface epithelium or postovulatory inclusion cysts formed after follicular rupture and repair [3, 4]. Factors associated with a lower risk of developing ovarian cancer include pregnancies, the use of oral contraceptives, breast feeding, tubal ligation, and hysterectomy [5]. As of these factors are all associated with reduced numbers of ovulations, and it is believed that an increased lifetime number of ovulations play a significant role in the development of ovarian cancer [6].

According to the incessant ovulation hypothesis, every ovulation creates a wound, and the surface ovarian epithelial cells are then repaired by increased proliferation. This may increase the likelihood for DNA damage and carcinogenic mutations [5]. However, this hypothesis is inconsistent with the observation that patients with polycystic ovarian syndrome who have decreased ovulatory cycles appear to have an increased ovarian cancer risk [7]. The increased risk of ovarian cancer after the use of infertility drugs supports the fact that stimulation of the ovarian surface epithelium with gonadotropins increases the risk of ovarian cancer development [3, 8, 9]. The hypothesis regarding the gonadotropin-based stimulation is supported by the fact that the ovarian cancer incidence increases after menopause, when the gonadotropin levels rise [3, 10, 11].

Ovarian carcinomas have been classified according to the different epithelia of the reproductive female tract. The tumors are divided into serous, mucinous, endometrioid, clear cell, and transitional cell carcinomas. It has traditionally been thought that ovarian carcinomas are derived from

the ovarian surface epithelium and that subsequent metaplasia leads to the development of the various cell types (serous, mucinous, endometrioid, clear cell, and transitional) which constitute the morphological subtypes of ovarian epithelial carcinomas. However, new histopathological, molecular, and genetic studies have recently provided a better model for ovarian carcinogenesis, showing two broad categories, which are designated as type I, where precursor lesions in the ovary have clearly been described, and type II, where such lesions have not been described clearly and tumors may develop *de novo* from the tubal and/or ovarian surface epithelium [4, 12]. Type I tumors include low grade serous, mucinous, endometrioid, clear cell, and transitional cell carcinomas, while type II tumors comprise high grade serous carcinomas, undifferentiated carcinomas, and carcinosarcomas.

Type I tumors are suggested to behave in an indolent behavior and appear to form part of a morphological and molecular continuum starting with cystadenoma/adenofibroma benign tumors that subsequently develop towards atypical proliferative or borderline tumors and then finally towards invasive tumors. They are often confined to the ovary at the time of diagnosis, with a stable genome and without TP53 mutations, although somatic mutations are frequently detected in a number of genes [13]. Each morphological subtype exhibits a distinctive molecular profile characterized by mutations targeting specific cell signaling pathways. Even though clear cell carcinoma is listed as a type I tumor, it may actually belong to an intermediate category because of its mutations and behavior.

Type II tumors are suggested to be more aggressive, are found at advanced stage, and are genetically highly unstable; the majority have TP53 mutations, and almost half of the cases have mutation, hypermethylation, or dysfunction of breast cancer gene (BRCA) 1/2 [14–17]. Several lines of evidence now indicate that these tumors may originate from the epithelium of the fimbrial portion of the fallopian tube [18–21] and/or the ovarian surface epithelium.

2. Type I

2.1. Endometrioid Carcinoma and Clear Cell Carcinoma. The recent study followed a cohort of 6398 women with clinically documented endometrioma and evaluated the risk of ovarian cancer based on the varying time periods from diagnosis of endometriosis [22]. During the follow-up of up to 17 years, 46 (0.7%) incidental ovarian cancers were identified, translating into a standardized incidence ratios of 13.2. This risk increased with age, with an incidence ratio of 13.2 in patients over 50 years old. In the malignant transformation cases of endometriotic cyst, serial transvaginal ultrasonography (USG) examinations revealed an increase in its size [23]. A review of 29 studies published from 1973 to 2002 on the prevalence of endometriosis in epithelial ovarian cancers organized by location of disease examined the different histologic subtypes with endometriosis in the same ovary. It was found that there was a prevalence of 4.5% in serous, 1.4% in mucinous, 35.9% in clear cell, and 19% in endometrioid carcinomas [24]. There is increasing evidence that clear cell and endometrioid carcinomas can arise from endometriosis. The specific correlation between endometriosis and ovarian malignancy and their epidemiological patterns have been studied. For both endometriosis and ovarian carcinoma, increased risks were associated with infertility, early menarche, late menopause, and nulliparity, and the protective factors were tubal ligation, hysterectomy, the use of oral contraceptives, and pregnancy [25].

Molecular aberrations that are characteristics of inflammatory processes in endometriosis may contribute a number of survival and growth signals to the malignant transformation of the ovarian surface epithelium. Endometriosis at the ovary confers an imbalance in the cytokine milieu (interleukin-1, interleukin-6, and interleukin-8) inducing surges of immunomodulatory and growth-stimulating cytokines (e.g., tumor necrosis factor- (TNF-) alpha) similar to those observed in ovarian malignancy. Endometriosis also drastically changes the hormonal milieu and generates growth factor (such as insulin-like growth factor (IGF)) to which ovarian cancer cells have demonstrated dependency [24]. The propensity of endometriotic cells to expand clonally, as a result of intrinsic anomalies and advanced inflammation in endometriosis, generates a constitutive abundant flux of several stimulatory signals, which induces progressive transcriptional changes that drive sustained proliferation. This also increases the rate of DNA repair and the likelihood of accumulation of mutations in these cells.

Mutations of the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) tumor suppressor are frequently found in ovarian endometrioid carcinomas [26]. The identification of PTEN mutations in endometriotic lesions (20.6%) adjacent to ovarian endometrioid (20%) and clear cell carcinomas (8.3%) supports the notion that endometriosis is a precursor lesion for endometrioid and clear cell carcinomas [27]. In a mouse model of endometrioid ovarian carcinoma, PTEN deletion on the background of oncogenic K-RAS activation within the ovarian surface epithelium gave rise to endometriotic-like precursor lesions which developed into invasive endometrioid ovarian carcinoma within seven to twelve weeks [28]. These results indicate that expression of oncogenic K-RAS and inactivation of the PTEN tumor suppressor gene is an early event in the development of endometrioid carcinoma. Pathologically, the coexistence of ovarian carcinoma and endometriosis is frequently observed, with the latter called "atypical endometriosis," which is a putative precursor lesion including atypia of cell nucleus [29].

The overexpression of hepatocyte nuclear factor-1 (HNF-1) beta [30] and mutations of the ARID1A gene [31] are also found in some atypical endometriosis adjacent to the carcinoma. Furthermore, the histogenesis of endometrioid carcinoma could arise from endometriosis, which originates from HNF-1 beta-negative inclusion cyst cells. In contrast, the expression of HNF-1 beta could be associated with the late secretory or menstrual phase endometrial-endometriosis—clear cell carcinoma (CCC) lineage, which means that CCC could arise from the HNF-1 beta—positive epithelial cells associated with endometriosis [32]. HNF-1 beta promotes aerobic glycolysis, which may contribute to cell survival under hypoxic conditions. The overexpression of HNF-1 beta

may also play a role in the occurrence of CCC in stressful environment [33].

It has recently been recognized that carcinogenesis of endometrioid and clear cell carcinomas arising from endometriotic cysts is significantly influenced by the microenvironment in which the tumor arises [34]. As the content of an endometriotic cyst includes highly concentrated old blood, the concentration of iron is markedly high in endometriotic cysts [35]. Free iron is associated with cancer development through the induction of persistent oxidative stress. The epithelial cells within the endometriotic cyst are exposed to extensive oxidative stress (reactive oxygen species (ROS)) and hypoxia, and, as a result, they are subjected to more cellular and DNA damage and have less efficient DNA repair [36, 37].

Ovarian clear cell carcinomas (OCCCs) are rare tumors in Europe (4%) [38] and the United States (5%) [39]; however, they are common in Japan (20%). OCCCs are commonly considered to be chemoresistant tumors. Even though OCCC is listed as a type I tumor, it may actually belong to an intermediate category. Using a microarray dataset of ovarian cancers, the OCCC signature comprising 437 genes was identified [40]. Such OCCC signature genes contain many oxidative stress-related genes, which are actually upregulated by epigenetic mechanism in OCCCs. Therefore, it is estimated that the OCCC signature is first induced by the stressful environment in the endometriotic cyst and then becomes fixed during the course of development of the OCCC. It has been shown that there is similarity in the gene expression profile between renal cell carcinomas (RCCCs) and OCCC [41]. Sorafenib, which has recently been approved for RCCC, also showed significant antitumor activity in both of the two OCCC patients [42] and may thus represent a novel therapeutic agent for OCCC.

Mutations in the beta-catenin gene, CTNNB1, which is involved in cell proliferation and the Wnt pathway, have been found in up to 30% of endometrioid carcinomas but are uncommon in other subtypes [43, 44]. This finding suggests that beta-catenin and dysregulation of the wnt pathway are important in the development of endometrioid carcinomas. Furthermore, somatic mutation of the PI3K gene (PIK3CA) has been reported in 20% of endometrioid and clear cell carcinomas [45]. A more recent study has reported that PIK3CA mutations occur only in high grade endometrioid or high grade CCCs [46]. Microsatellite instability is present in endometrioid carcinoma and CCC but is only rarely detected in serous and mucinous tumors [47, 48].

2.2. Mucinous Carcinoma. There are several theories accounting for the origin of ovarian mucinous carcinoma. It is well recognized that a subset of mucinous carcinomas can develop in association with ovarian teratomas; however, the majority of these tumors do not harbor any teratomatous component [49, 50]. Other theories regarding the origin of these tumors include mucinous metaplasia of surface epithelial inclusions, endometriosis, and Brenner tumors [23, 50]. However, it is exceedingly rare to find mucinous metaplasia of the ovarian surface epithelium or within the lining of cortical inclusion cysts [51, 52], and there have only been a few case reports of such findings [53, 54]. Mucinous carcinomas can develop from endometriosis; however, this observation appears to be relatively uncommon, except for müllerian endocervical mucinous or mixed (seromucinous) borderline tumors [55, 56].

The association between Brenner and mucinous tumors has been known [57–59]. Amplification of 12q14-21 in both a mucinous carcinoma and an associated Brenner tumor was reported recently [60]. Mucinous carcinomas (intestinal type) and Brenner tumors may share similar histogenesis from transitional cell (Walthard) nests at the tubal peritoneal junction (TPJ) [61, 62]. Small mucinous tumors are rarely diagnosed, possibly because they are thought to be Brenner tumors with foci of mucinous differentiation [62].

Regardless of the origin of ovarian mucinous carcinoma, morphological transitions from cystadenoma to a mucinous borderline tumor (MBT) to intraepithelial carcinoma and invasive carcinoma have been recognized for some time, and an increasing frequency of KRAS mutations at codons 12 and 13 has been reported in cystadenomas, MBTs and mucinous carcinomas, respectively [63–66]. Similar to low grade serous carcinomas, mucinous carcinoma and adjacent MBT and mucinous cystadenoma show the same KRAS mutation, supporting the hypothesis of the "adenoma-carcinoma sequence" [67, 68] and the view that mucinous carcinomas develop in a stepwise fashion from mucinous cystadenomas and MBTs. These findings suggest that KRAS mutation is an early occurrence in the pathogenesis of ovarian mucinous tumors. Unlike low grade serous carcinomas, BRAF mutations are not a feature of ovarian mucinous neoplasms of intestinal type.

Most primary ovarian mucinous carcinomas (and borderline tumors) are of the so-called intestinal (enteric or nonspecific) type, unilateral, and stage 1. Advanced stage neoplasms (stage 3 or 4) are extremely uncommon. It is now clear that ovarian mucinous neoplasms associated with pseudomyxoma peritonei are almost always of vermiform appendix origin [69, 70]. While many primary ovarian mucinous carcinomas contain goblet cells and even occasionally Paneth or neuroendocrine cells, the presence of goblet cells is not a prerequisite for an intestinal type mucinous tumor. In fact, many of these more closely resemble gastric or pancreaticobiliary (upper gastrointestinal) mucinous neoplasms [71].

A much more uncommon müllerian (endocervical) type of ovarian mucinous carcinoma and borderline tumor also exists [72, 73]. While borderline mucinous neoplasms of müllerian type have been well described, malignant müllerian mucinous tumors are extremely uncommon.

According to several studies, smoking has been found to be a risk factor associated with benign, borderline, and mucinous carcinomas [74–76]. It has been speculated that the relationship between cigarette smoking and the development of mucinous tumors could be due to the similarity of mucinous tumors to the gastrointestinal mucosa. The latter tumors, such as the stomach and pancreas carcinomas, have consistently been associated with cigarette smoking [75].

2.3. Low Grade Serous Carcinoma. The low grade serous carcinomas are genetically stable and are characterized by their low number of genetic mutations. Therefore, they develop slowly from well-recognized precursors and behave in an indolent fashion. They are much less common than high grade serous carcinomas and are thought to evolve in a stepwise fashion from benign serous cystadenoma to serous borderline tumors (SBTs) (also referred to as atypical proliferative serous tumor) and finally to low grade serous carcinoma. Some authors have also suggested that serous tumors with micropapillary architecture may represent an intermediate step between SBTs and low grade serous carcinomas [77–80]. KRAS mutations at codons 12 and 13 occur in one-third of SBTs [81] and in 33% of low grade serous carcinomas [82]. Similarly, BRAF mutations at codon 599 occur in 28% of SBTs and 30% of low grade serous carcinomas [81, 83, 84]. Mutations in ERBB2 occur in less than 5% of these tumors. Mutations of KRAS and BRAF are detected in both SBTs and cystadenoma epithelium adjacent to SBTs [85]. These findings suggest that mutations of KRAS and BRAF are very early events in tumorigenesis, preceding the development of SBTs. The KRAS, BRAF, and ERBB2 oncogenes are upstream regulators of mitogen-activated protein kinase (MAPK), and mutations in these genes result in constitutive activation of the MAPK signal transduction pathway, which in turn leads to uncontrolled cell proliferation [17].

In contrast to high grade serous carcinoma, p53 mutations are uncommon in low grade serous carcinoma and are identified in <10% of these tumors [86]. A methylation profile distinct from that of high grade serous carcinoma has been identified in epigenetic studies [87]. Low grade serous carcinomas have a DNA content and level of copy number alterations which more closely resembles SBTs than high grade serous carcinomas and are intermediate between the two [88, 89]. A recent study involving a whole exome analysis of low grade serous carcinomas of the ovary identified an average of only 10 somatic mutations per tumor [90, 91]. In contrast, high grade serous carcinomas are generally aneuploidy, with a high level of copy number alterations [89]. These carcinomas typically sustain 50–70 somatic mutations, with TP53 representing a clear driver mutation [92].

Several theories exist to explain the origin of serous tumors. The traditional concept has been that they were derived from ovarian epithelial inclusions formed by invaginated ovarian surface epithelium that has undergone müllerian metaplasia [52]. It has been postulated that the native ovarian surface mesothelium possesses the potential to transform into an epithelial or mesenchymal phenotype in response to signals such as those associated with ovulation. The exposure of the mesothelial cells of an inclusion cyst to the ovarian stromal microenvironment may result in transformation to müllerian epithelium. However, well-documented examples of a transition of these cysts to serous carcinomas are rare.

Another theory is that tumors may be derived from a secondary müllerian system, thought to represent embryological remnants of the proximal müllerian ducts, located within the ovarian hilum [93, 94]. These müllerian epithelial cysts form the serous tumors, and their proliferation induces subsequent obliteration of the adjacent ovarian parenchyma. However, SBTs are only rarely reported to occur in the ovarian hilum [95, 96]. There was a recent theory which suggested that low grade serous carcinoma may be derived from the fallopian tube. The theory suggests that shed tubal epithelial cells can implant on the ovarian surface epithelium, be taken into inclusion cysts, and transform serous neoplasms, while implants on other peritoneal surfaces may account for extraovarian endosalpingiosis and noninvasive tumor implants [97, 98]. It is thought that chronic inflammation may induce the proliferation of the tubal epithelium, from which clusters of cells can then shed and implant on the ovarian and peritoneal surfaces, resulting in SBTs, noninvasive implants, and endosalpingiosis.

3. Type II

3.1. High Grade Serous Carcinoma. These tumors are high grade from the start, evolve quickly, and are frequently found at an advanced stage. At the molecular level, high grade serous carcinomas show TP53 gene mutations in nearly 80% of cases [14–17] and a high Ki67 proliferation index (between 50% and 75%). Overexpression of HER2/neu is also found in 20–67%, AKT activation in 12–30% and inactivation of p16 in 15% of cases. In addition, the overexpression of the human leukocyte antigen-G (HLA-G) system is found in 61% of cases, and there is overexpression of apolipoprotein E (apoE) in 66% of cases, but these are rarely found in low grade serous carcinomas. Chromosomal rearrangements are far more common in these types of tumors, probably reflecting the high degree of associated gene instability. Currently, up to 10–15% of ovarian carcinomas are believed to be hereditary [99]. Mutations in the high penetrance gene, BRCA1 and BRCA2, are associated with 90% of hereditary ovarian carcinoma cases. The lifetime risk of developing ovarian cancer is approximately 40–50% for BRCA1 mutation carriers and 20–30% for BRCA2 mutation carriers. Inherited BRCA1 and BRCA2 mutations predispose females to high grade serous carcinoma of the ovary.

In 2001, Piek et al. found new transformations on tubal segments removed from females who had either BRCA mutations or a strong family history of ovarian carcinoma who underwent a risk-reducing bilateral salpingo-oophorectomy (BSO) [18]. Of 12 tubal specimens, six had areas of cellular dysplasia noted in the tubal epithelium and five additional specimens had hyperplastic lesions in the microscopic findings. These dysplastic and hyperplastic lesions resembled high grade serous carcinoma but without stromal invasion. These malignancies were found in the distal tube in 4–17% of females with BRCA mutations at the time of their risk-reducing surgery, 57% to 100% of which were located in the distal portion of the tubes [100–104]. Dysplastic lesions within the tubal epithelium are termed "serous tubal intraepithelial carcinomas (STIC)." In 2003, Piek et al. hypothesized that hereditary serous carcinomas might originate from the epithelium of the fallopian tube which has spilled onto the surface of the ovary or peritoneum [105].

A very early abnormality termed "secretory cell outgrowths" (SCOUTs) has recently been reported [106]. This consists of a succession of at least 30 almost exclusively secretory epithelial cells with a pseudostratified appearance [107]. An immunohistochemical analysis can confirm the diagnosis, which is characterized by a low PTEN and Ki67 index, and, in most cases, there are no TP53 mutations [106, 108–110]. The TP53 signatures are the next earlier entities, which have an immunohistochemical definition of at least 12 consecutive secretory cells that are p53 positive and have a low proliferative index (Ki67 < 10%). In the next place, "serous tubal intraepithelial lesions" (STILs) [111] also called "transitional intraepithelial lesions of the tube" (TILTs) by some authors have proliferative p53 signatures, tubal dysplasia, and even tubal epithelial atypia [18, 112]. These have also been described as a group of tubal anomalies with different p53 signatures compared to STICs [113]. In the serous carcinogenic sequence, SCOUTs may be able to evoke in benign lesions expressing p53 (p53 signature) with a low proliferation index and little genetic instability. Then, benign lesions expressing p53 would appear (STILs/TILTs), corresponding to tubal dysplasia lesions, finally culminating in the appearance of STICs [114]. Rate of the STICs was 59% in patients with serous tumors [21] and the former was clonally related to the latter [115]. There were no STICs identified in mucinous, endometrioid, or carcinosarcoma histology. Thus, STICs seem to be associated with the development of serous carcinoma.

It has recently been reported that the junction of the fallopian tube epithelium with the mesothelium of the tubal serosa, termed the "TPJ," might be a potential site of carcinogenesis, as the role of epithelial junctions, notably the uterine cervical squamocolumnar, gastroesophageal, and anorectal junctions, in neoplasia is well recognized [116]. This junction is highly tortuous with tongues of mesothelium extending from the infundibular peritoneal-fimbrial junction at the outer edges of the fimbriae, with irregular tongues of peritoneum extending onto some of the plicae. The extensive and elaborate lymphovascular system is in almost direct contact with the basement membrane of the tubal epithelium, suggesting that even a minimally invasive carcinoma could easily invade this system and rapidly spread throughout the abdominal cavity.

Given that STICs have shorter telomeres than high grade serous ovarian carcinoma and also have gamma H2AX overexpression, these results seem to suggest that DNA repair mechanisms are activated in the early conditions [117]. Telomere shortening appears to take place in most human preinvasive epithelial lesions [118]. As a result, some authors have hypothesized that STICs are not metastases from ovarian carcinoma (different telomere lengths between STICs and ovarian carcinomas) but tubal precursor lesions of ovarian carcinoma [119]. In brief, the small areas of STICs undergo malignant transformation and, due to their location, metastasize to the nearby ovary and surrounding pelvic peritoneum.

On the other hand, serous carcinoma may have a truly ovarian origin. Our group reviewed the clinical charts of 543 patients with epithelial ovarian carcinomas who underwent laparotomy and collected patients whose clinical and transvaginal ultrasonography (USG) findings for adnexal regions 12 months or fewer prior to the surgery were available [23]. The data of 35 patients were available (11 serous, 6 mucinous, 8 clear cell, and 10 endometrioid). In their series of serous carcinomas, there had been no apparent abnormalities in the adnexal regions, 2 to 12 months prior to the diagnosis in 9 of the 11 (82%) patients. Strikingly, 8 of 9 serous carcinoma patients with no apparent abnormalities at the last visit presented with stage III tumors and the final pathological findings after surgery showed that there were no malignancies in adjacent lesions, indicating that serous carcinomas might develop from the ovaries suddenly and progress very rapidly. However, their report raises the possibility that there might be the small malignancy lesion of the fallopian tubes which was not able to be detected in some cases.

In 1994, Bell and Scully reported 14 cases of incidentally found microcarcinoma in normal-appearing ovary [120]. Most of them were serous carcinoma, suggesting *de novo* carcinogenesis, whereas there were no cases of mucinous carcinoma. Furthermore, a notable study has recently been reported, in which the ovarian hilum cells show increased transformation potential after inactivation of tumor suppressor genes transformation-related protein 53 (Trp53) and retinoblastoma 1 (Rb1) in mice [121]. These pathways are altered frequently in the high grade serous carcinoma. In brief, the ovarian surface epithelium (OSE) at the TPJ contains a novel stem cell niche that is responsible for OSE regeneration and is prone to malignant transformation. These stem cells in the hilum may have increased transformation potential after inactivation of Trp 53 and Rb 1 and be the origin of high grade serous carcinoma.

4. Conclusions

The new model of assigning ovarian epithelial carcinomas into two groups demands a radical change of current clinical management. Type I ovarian carcinomas are considered to arise via a well-defined adenoma-carcinoma sequence from a benign precursor lesion, such as a borderline tumor or endometriosis, and to evolve in a stepwise fashion. Type I carcinomas are, in general, slow growing, indolent neoplasms, and like type I endometrial carcinomas. In contrast, type II carcinomas are high grade clinically aggressive neoplasms. Most represent high grade serous carcinoma. Carcinosarcoma and undifferentiated carcinoma, which are both predominantly variants of high grade serous carcinoma, are also included in this category. Type II carcinomas are often associated with TP53 mutations and are like type II endometrial carcinomas. There is emerging evidence that many arise from the epithelium of the distal fallopian tube and/or ovary.

Clinicopathological features and molecular genetic alterations of two types of ovarian carcinoma are summarized in Table 1.

We study the carcinogenesis of ovarian carcinoma to determine the characteristics of each subtype carcinoma and to optimize the treatment of the disease. For example, there

Table 1: Summary of clinicopathological features and molecular genetic alterations of two types of ovarian carcinoma.

	Type I tumors		Type II tumors	
Behavior	Indolent		Aggressive	
At the time of the diagnosis	Early stage		Advanced stage	
Survival rate at 5 years	About 55%		About 30%	
Histological type/Precursors				
	Endometrioid carcinoma/	Endometriosis	High grade serous/	Probably *de novo* starting at the tubo-
	Clear cell carcinoma/	Endometriosis		ovarian surface epithelium; SCOUT →
	Mucinous carcinoma/	Mucinous Cystadenoma, Endometriosis		P53 signature → STIL/TILT → STIC
		Teratoma, Brenner Tumor, and MBT		or ovarian hilum stem cell
	Low grade serous carcinoma/	Serous cystadenoma, Adenofibroma	Undifferentiated carcinoma/	?
		Atypical proliferative serous tumor (SBT)	Carcinosarcoma/	?
		Müllerian epithelial cyst		
	Transitional cell carcinoma/	Brenner tumor		
Gene expression profile				
Genetic instability	Not very unstable		Very unstable	
PTEN mutation	15–20%		Low	
HNF-1 beta overexpression	90%		Low	
ARID1A mutation	40–50%		Not found	
CTNNB1 mutation	30%		Low	
PIK3CA	20%		Low	
Microsatellite instability	50%		8–28%	
KRAS mutation	30–65%		Low	
BRAF mutation	30–65%		Low	
TP53 mutation	Low		50–80%	
HER2/neu overexpression	Low		20–67%	
AKT overexpression	Low		12–30%	
p16 inactivation	Low		15%	
HLA-G overexpression	Low		61%	
APO E overexpression	12%		66%	
BRCA 1/BRCA2 mutation	Low		High	
Ki67 proliferation index	10–15%		50–75%	

are similarities in the gene expression between RCCC and OCCC, as determined using a microarray analysis. Sorafenib, which has recently been approved for RCCC, also showed significant antitumor activity in the patients with recurrent chemoresistant OCCC. With regard to benign cysts of the ovary (e.g., endometriotic, serous, and mucinous cysts), there need to be a unification of the preventive treatment strategy and to detect an early cancer by careful follow-up with USG at every 6 months and some bioindices (countermeasures against type I carcinomas). Complete bilateral salpingo-oophorectomy as a risk-reducing strategy in patients with BRCA mutations in an approach is worthy of further investigation and it may be reasonable to consider bilateral salpingectomy for all patients undergoing hysterectomy for benign disease (countermeasures against type II carcinomas). In other words, we are entering a period of individualized therapies including preventive therapies, where it is necessary to know the characteristics of each carcinoma using biomarkers and gene profiling. We hope that all type I and type II carcinomas of the ovary will be able to be prevented and/or cured completely in the near future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Application of MicroRNA in Diagnosis and Treatment of Ovarian Cancer

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Ovarian cancer has a poor prognosis because early detection is difficult and recurrent ovarian cancer is usually drug-resistant. The morbidity and mortality of ovarian cancer are high worldwide and new methods of diagnosis and therapy are needed. MicroRNAs (miRNAs) are posttranscriptional regulators of gene expression that are involved in carcinogenesis, metastasis, and invasion. Thus, miRNAs are likely to be useful as diagnostic and prognostic biomarkers and for cancer therapy. Many miRNAs have altered expression in ovarian cancer compared to normal ovarian tissues and these changes may be useful for diagnosis and treatment. For example, deficiencies of enzymes including Dicer and Drosha that are required for miRNA biogenesis may be adverse prognostic factors; miRNAs such as miR-214 and miR-31, which are involved in drug resistance, and the miR-200 family, which is implicated in metastasis, may serve as biomarkers; and transfection of downregulated miRNAs and inhibition of upregulated miRNAs may be effective for treatment of ovarian cancer. Chemotherapy targeting epigenetic mechanisms associated with miRNAs may also be effective to reverse gene silencing.

1. Introduction

Ovarian cancer is the eighth most common female cancer worldwide and ranks seventh in mortality. About 220,000 women are diagnosed with ovarian cancer each year and the disease causes about 140,000 deaths annually [1]. In Japan, the incidence and mortality of ovarian cancer have increased over the past 10 years [2, 3]. The 5-year survival rate for patients with advanced ovarian cancer is only 30%, despite the development of chemotherapy with platinum-based drugs and taxanes [4]. The high mortality is associated with difficulties in early detection because ovarian cancer rarely causes subjective symptoms and safe and minimally invasive procedures for early detection have not been established. Consequently, 40% to 50% of cases are detected in advanced stages III and IV. Another cause of the high mortality is resistance to chemotherapy. Ovarian cancer is highly responsive to initial anticancer treatment, but about half of the advanced cases recur within two years and have a decreased response to chemotherapy, resulting in a poor prognosis [5]. For these reasons, there is an urgent need to develop new therapies, find clinically useful biomarkers, and identify new targets for treatment of ovarian cancer.

Many studies of ovarian cancer have focused on protein-coding genes. However, RNA molecules transcribed from noncoding genes also have biological functions. These noncoding RNAs include microRNAs (miRNAs) that cleave a target mRNA and repress translation of proteins, and some miRNAs show site- and stage-specific differences in expression in ovarian cancers. Many recent studies have shown that miRNAs are involved in suppression or progression of ovarian cancer. Therefore, miRNAs may be useful as diagnostic and prognostic biomarkers and also for therapy. Epigenetic therapy related to miRNAs may be particularly effective for resensitization of ovarian cancer cells to chemotherapy after development of resistance and recurrence. In this paper, we describe the possible use of miRNAs in diagnosis and treatment of ovarian cancer.

2. miRNAs Implicated in Ovarian Function

Ovarian function, particularly follicular development, is controlled by hormones such as gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The ovary itself also produces sex hormones such as progesterone and estrogen, as well as cytokines of the transforming growth factor beta (TGF-β) superfamily [6]. A recent study suggested cross talk between the signals of local ovarian factors and endocrine system hormones including gonadotropins [7]. Thus, ovarian function is controlled by complex molecular signaling that maintains normal follicular development and atresia, in which protein expression is regulated quantitatively and temporally. Failure of the regulatory mechanisms is likely to lead to various diseases, including infertility.

Follicular cells are roughly classified into theca cells, granulosa cells, and oocytes. Granulosa cells proliferate in a FSH-dependent manner during follicular maturation and are involved in estrogen synthesis. Mase et al. found that many miRNAs in the let-7 family are expressed in human ovarian granulosa KGN cells, which maintain expression of FSH receptors. Genes targeted by the let-7 family include those involved in follicular maturation and atresia, suggesting involvement of miRNAs in these phenomena [8]. Murchison et al. produced Dicer 1 knockout mice with oocyte-specific deletion of Dicer, an important enzyme for miRNA biogenesis. In these mice, functional expression of miRNAs was completely deleted. Dicer deletion had no effect on early folliculogenesis but arrested the first meiotic division in oocytes associated with spindle and chromosomal aggregation hypoplasia. More than 2,000 mRNAs had significantly changed expression associated with these abnormalities. During oocyte maturation, including meioses, gene transcription was completely repressed and only mRNAs inherited before maturation remained in cells. Expression of many mRNAs was affected in Dicer-deleted oocytes, suggesting direct or indirect posttranscriptional regulation by miRNAs [9].

3. Changes in miRNA Expression in Ovarian Cancer

Recent studies have identified many oncogenic miRNAs (oncomiRs) and tumor suppressor miRNAs (tumor suppressor miRs) (Table 1) [10–16]. Iorio et al. found several miRNAs with altered expression in ovarian cancer tissues compared with normal tissues, with miR-199a, miR-200a, miR-200b, and miR-200c having significantly increased expression and miR-140, miR-145, and miR125b1 showing markedly decreased expression in the cancer tissues. miR-140 is located at 6q22, a common defective chromosomal site in ovarian tumors, and this miRNA is thought to target genes associated with invasion, including matrix metalloproteinase 13, fibroblast growth factor 2, and angiogenic VEGFA [10]. Bracken et al. showed that miR-429, miR-200a, and miR-200b are regulated by ZEB1 and SIP1, which are inhibitors of the epithelial-mesenchymal transition (EMT), and that miR-200a and miR-200b negatively regulate expression of ZEB1 and SIP1, providing a negative feedback loop [11].

Table 1: Changes in miRNA expression in ovarian cancer.

Upregulated (oncomiRs)	Downregulated (tumor suppressor miRs)
miR-199a	miR-140
miR-200a	miR-145
miR-200b	Let-7i
miR-200c	miR-15
miR-429	miR-16
	miR-373
	miR-520c
	miR-125b1

With regard to miRNA processing of mRNA, an interesting study of the relationship of ovarian cancer with miRNAs by Merritt et al. [12] showed that the mRNA levels of Dicer and Ribonuclease 3 (Drosha) decreased in 60% and 51% of tissue samples from 111 patients with invasive epithelial ovarian cancer. Downregulation of Dicer was significantly related to tumor stage progression and downregulation of Drosha was significantly related to a suboptimal residual tumor size >1 cm after cytoreductive surgery. Conversely, patients with high levels of Dicer and Drosha in cancer tissues had significantly prolonged median survival times. Cells with downregulation of Dicer and Drosha are likely to have lower levels of mature miRNAs, which suggests that certain miRNAs are involved in progression of ovarian cancer [12].

4. miRNAs Associated with Drug Resistance

A total of 27 miRNAs have been associated with responsiveness to chemotherapy [13]. Yang et al. found that miR-214, which targets PTEN, is frequently expressed in ovarian cancer tissues and that let-7i, which enhances resensitization to platinum resistance, is expressed less in the same tissues [14]. Mitamura et al. showed that control of MET expression by miR-31 is involved in drug-resistance mechanism in paclitaxel-resistant ovarian cancer cells [15]. Aqeilan et al. found that miR-15 and miR-16 cause cellular resistance to many drugs through targeting the BCL2 gene [16]. Leskelä et al. showed that the miR-200 family (miR-141, miR-200a, miR-200b, miR-200c, and miR-42) is implicated in the response to paclitaxel treatment and progression-free survival via β tubulin III regulation. In particular, miR-200c is significantly associated with recurrence of ovarian cancer and miR-429 is associated with progression-free and overall survival rates [17].

Key drugs against ovarian cancer are taxanes and cisplatin. Boyerinas et al. found that let-7g and let-7a are involved in drug resistance [18]. Let-7g suppresses IMP-1, which is involved in multidrug resistance and increased sensitivity to taxanes. The expression level of let-7a is a potential marker for choosing chemotherapeutic agents, since patients with extremely low let-7a expression are responsive to platinum-based drugs and paclitaxel, whereas those with high levels of let-7a had increased survival only in monotherapy with a platinum-based drug [19]. Nagaraja et al. and

Table 2: Histological types and miRNA expression in ovarian cancer.

Tissue type	Upregulation	Downregulation
Serous adenocarcinoma	miR-7 miR-200a/c miR-22 miR-302b miR-373 miR-34c-5p miR-449a miR-146b-5p	miR-148b miR-211 miR-31
Endometrioid adenocarcinoma	miR-9 miR-183 miR-205 miR-96 miR-196a miR-212 miR-182 miR-196b miR-375 miR-141 miR-200a/b/c	miR-22 miR-222 miR-324-3p miR-101 miR-299-5p miR-325 miR-194 miR-302b miR-373
Clear cell adenocarcinoma	miR-29b miR-200a/c miR-30a miR-486-5p miR-30e	miR-20a
Mucinous adenocarcinoma	miR-141 miR-200b	
Undifferentiated carcinoma		miR-9 miR-18

Peng et al. showed that miR-100, a tumor suppressor miRNA, increased sensitivity to everolimus, an anticancer drug [20, 21]. miR100 is also an independent predictor of overall survival in patients with ovarian cancer. Hong et al. showed that miR-376c suppresses signaling of Nodal/activin receptor-like kinase 7 (ALK7), which is involved in drug sensitivity, and decreases the effects of cisplatin and carboplatin [13]. Fu et al. found that miR-93 targets integrin and enhances tumor growth, angiogenesis, and the resistance for cisplatin [22].

5. Utility of miRNAs in Diagnosis of Ovarian Cancer

Many miRNAs have altered expression levels in ovarian cancer compared to normal tissues. In addition, changes in miRNA levels are dependent on and related to the ovarian cancer tissue type, stage, histological type, prognosis, and drug resistance (Table 2) [8, 10, 23–30]. These findings suggest the possibility of early diagnosis of ovarian cancer using miRNAs. In the miR-200 family, Boyerinas et al. showed that miR-200a and miR-200c are expressed in serous adenocarcinoma, clear cell adenocarcinoma, and endometrioid adenocarcinoma, and miR-200b and miR-141 occur in endometrioid adenocarcinoma and mucinous adenocarcinoma [18]. Toloubeydokhti et al. found decreased expression of miR-212 in serous cystadenoma [31]. Target genes of miR-212 include those with overexpression in this histological type of ovarian cancer and mutated genes in hereditary ovarian cancer. Therefore, miR-212 may be a marker for differentiating ovarian cancer. Downregulation of miR-31, a tumor suppressor miRNA, has been shown in serous adenocarcinoma, and miR-31 suppresses expression of cell cycle regulatory factors via p53 [32]. Expression of miR-373 is variable in undifferentiated carcinoma [33], but the target genes and function of this miRNA are unknown. Overexpression of miR-21 in clear cell carcinoma has been shown to cause downregulation of PTEN [34].

6. Utility of miRNAs in Treatment of Ovarian Cancer

Treatment options for ovarian cancer include supplementation of miRNAs that are downregulated in cancer tissue for recovery of function and inhibition of the function of upregulated miRNAs by administration of complementary nucleic acids. Garzon et al. showed that the effects of upregulated oncomiRs could be suppressed using an antagomir, an oligonucleotide complementary to the miRNA administered as an antisense oligonucleotide or LNA [35]. Lu et al. developed an anti-miRNA antisense oligodeoxyribonucleotide (MTG-AMO) for suppression of many miRNAs, including miR-21, and showed that this was effective in cancer with concurrent multiple mRNA abnormalities [36]. Dai et al. established a therapy for ovarian cancer based on targeted delivery of miR-29a to cancer tissues for the purpose of reexpressing PTEN, a tumor suppressor. The potential antitumor effect of a miR-29a-transfected chimera was apparently based on expression of downstream molecules and apoptosis of ovarian cancer cells [37].

The association of miRNAs with peritoneal metastasis, the major cause of death in patients with ovarian cancer, has also been studied. Ohyagi-Hara et al. found that integrin $\alpha 5$, a fibronectin receptor, increased the adhesion of cancer cells and induced metastasis and focused on the inverse correlation of integrin $\alpha 5$ and miR-92a levels. Transfection of ovarian cancer cells with miR-92a reduced expression of integrin $\alpha 5$ and suppressed peritoneal metastasis [38]. Cittelly et al. found that recovery of the level of miR-200c, which is known to increase sensitivity to platinum-based anticancer drugs, by transfection suppressed carcinogenesis and decreased the number of cancer cells. Recovery of miR-200c in combination with paclitaxel also decreased the cancer cells in established tumors. These results suggest that recovery of miR-200c immediately before highly cytotoxic chemotherapy improves the treatment response or reduces the effective dose of the anticancer drugs [39]. These outcomes show

that miRNA transfection has an antitumor effect. Transfected miRNAs are synthetic nucleic acids that require specific modes of administration [40–42]. These approaches include intravenous administration of a complex with atelocollagen, nanoparticles with cell-specific targeting, and conjugation with RVG peptide for crossing the blood-brain barrier. Gene therapy for introduction of miRNAs may also be useful if safety can be confirmed.

Epigenetic therapy has attracted attention as an alternative to classical approaches such as miRNA transfection. Acquisition of drug resistance reduces the survival rate in cancer and many cases of ovarian cancer are resistant to platinum-based anticancer drugs. This resistance is associated with miRNAs and various drug-resistance genes induced by methylation and signaling gene silencing. However, epigenetic changes are reversible, in contrast to gene mutations, and there is a potential to reverse gene silencing using DNA methyltransferase (DNMT) inhibitors, which are drugs that prevent hypermethylation by irreversibly binding to the active site of DNMT [43]. These drugs are effective as monotherapy for hematologic malignancy [44, 45], but not in solid cancer. However, effects on solid cancer are likely to be found in combination with other drugs. In ovarian cancer cells, DNMT inhibitors induce hypomethylation and reverse resistance to platinum-based anticancer drugs. Phases I and II clinical trials of decitabine, a DNMT inhibitor, are ongoing in ovarian cancer [46, 47]. Matei et al. found that decitabine in combination with carboplatin restored the expression of silenced tumor suppressor genes and may contribute to resensitization of platinum-resistant endometrial cancer. A phase I clinical trial of decitabine has shown that combined administration with carboplatin is safe and decreases methylation of multiple genes *in vivo* [48].

Malignant tumors, including ovarian cancer, include cancer initiation cells and cancer stem cells, which are referred to as cancer progenitor cells and are involved in development of drug resistance [49–51]. Chemotherapy targeting mitosis cannot eliminate all cancer stem cells during cell cycle arrest or low activity conditions, and residual cells promote regrowth of the tumor. Epigenetic therapy stabilizes differentiation and may target undifferentiated cancer stem cells. Thus, targeting of epigenetic mechanisms is likely to improve outcomes in ovarian cancer.

7. Conclusion

miRNAs have attracted significant interest, but the history of this field is relatively short and many issues remain to be resolved. Clinical studies of miRNAs have just started, but functional genomic analyses have produced results that may lead to clinical applications in the near future. Early diagnosis of ovarian cancer is important to improve treatment outcomes, and profiling using miRNA arrays may contribute to the detection of tissue type, stage, and prognosis. Induction of apoptosis of cancer cells using miRNAs may be a basic treatment strategy for reduction of metastasis, including peritoneal metastasis, and decreasing resistance to platinum-based anticancer drugs. Recovery of tumor suppression effects may be possible by transfection of miRNAs downregulated in cancer tissues or by suppression of upregulated miRNAs. miRNA expression may also be modified by targeting epigenetic mechanisms such as through reversal of hypermethylation. These potential treatment approaches will require further basic studies to facilitate drug discovery.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Expression Profiles of Epithelial-Mesenchymal Transition-Associated Proteins in Epithelial Ovarian Carcinoma

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Epithelial-mesenchymal transition (EMT) has been suggested to contribute to tumor progression and acquisition of therapeutic resistance. To assess the clinical significance of EMT-associated proteins, we evaluated the expression of Snail and Slug, the key regulators of EMT, in the primary ovarian cancer samples ($n = 103$) by immunohistochemistry. Snail was differentially expressed according to the histologic subtype ($P = 0.001$) and was predominantly expressed in serous and endometrioid types. In the serous and endometrioid adenocarcinomas, the expression of Snail remained high across the stage and grade, suggesting its role in the early phase of carcinogenesis. However, the expression of Snail and Slug was not related to chemoresistance and poor prognosis and did not serve as independent predictive or prognostic marker.

1. Introduction

Ovarian cancer is the seventh leading cause of cancer deaths in women worldwide and is the most lethal gynecologic malignancy [1]. Despite advances in surgery and chemotherapy, overall cure rate has remained approximately 30%. The poor clinical outcome mainly comes from the high percentage of cases being diagnosed at an advanced stage and the frequent emergence of chemoresistance. Recent evidence has suggested that epithelial-mesenchymal transition (EMT) may contribute to tumor invasion, metastasis, and acquisition of therapeutic resistance [2]. The term EMT refers to a complex molecular and cellular program that involves the loss of cell adhesion and acquisition of migratory and mesenchymal features. EMT plays a key role in normal physiologic processes during embryogenesis and wound healings, but it has also been recognized in the pathogenesis of cancer. During carcinogenesis, EMT is not only responsible for acquiring and maintaining mesenchymal phenotypes such as invasiveness and resistance to apoptosis but also confers stem cell-like characteristics upon cancer cells [3]. In addition, the expression of EMT signaling pathways has been associated with poor prognosis in various epithelial cancers, including breast, pancreas, prostate, and ovarian cancer [4].

The Snail family members, Snail (*SNAI1*) and Slug (*SNAI2*), are key regulators of EMT and directly repress the transcription of E-cadherin, a cell adhesion molecule. In epithelial ovarian cancer, the expression of these two transcriptional repressors along with the loss of E-cadherin has been shown to be related to tumor progression and sometimes poor prognosis [5–7]. In most of these studies, however, the expression of Snail and Slug has not been evaluated

and compared between different subtypes of ovarian cancer which are now considered as different disease entities with distinct biomarker expression profiles [8, 9]. In addition, it has recently been proposed that ovarian cancer can be divided into two groups based on genetic changes: low-grade (type I) and high-grade (type II) ovarian cancer [10, 11]. Type I cancers progress through a stepwise mutation process and frequently harbor *PTEN*, *KRAS*, and *BRAF* mutations. In contrast, type II cancers are characterized by frequent *TP53* mutation and progress through genetic instability without identifiable precursor lesions.

Therefore, in this study, we analyzed the differential expression of Snail and Slug according to the histologic subtype by immunohistochemistry. The p53 expression, which has been shown to be frequently aberrant in serous type, was also assessed to evaluate the correlation between p53 and EMT-related proteins. In addition, we explored the predictive and prognostic significance of Snail and Slug in epithelial ovarian cancer.

2. Materials and Methods

2.1. Patients. A total of 103 patients who had undergone primary debulking surgery for stages I–IV epithelial ovarian cancer between 2003 and 2009 at Seoul National University Bundang Hospital were included in the study analysis after obtaining approval from the institutional review board. Exclusion criteria included patients who received neoadjuvant chemotherapy before surgery because chemotherapy might be able to affect the proportion of chemoresistant tumor cells and change the expression level of EMT proteins [12, 13]. Patients with recurrent or nonepithelial ovarian cancer were also excluded. Clinicopathologic data, including age, the international federation of gynecology and obstetrics (FIGO) stage, surgical procedures, the extent of residual disease, histologic subtype, grade, adjuvant chemotherapy, and survival outcomes, were evaluated by reviewing medical charts and pathologic records.

2.2. Tissue Samples. Tissue microarrays (TMAs) were constructed from core biopsies (diameter 2 mm) of formalin-fixed paraffin-embedded primary ovarian cancer specimens using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea). Three core biopsies were taken from each individual specimen [14].

2.3. Immunohistochemistry. To detect Snail and Slug-specific immunoreactivity, sections (4 μm) from array blocks were treated as follows: after standard pressure-cooker-based antigen retrieval with citric acid (pH 6.0) pretreatment, sections were incubated with 1% horse serum in Tris-buffered saline for 3 minutes. The sections were incubated with either a rabbit polyclonal anti-Snail antibody (1 : 800) (ab17732; Abcam, Cambridge, UK) or a rabbit polyclonal anti-Slug antibody (1 : 100) (ab27568; Abcam). Both antibodies were detected using the polymer for 8 minutes and DAB substrate for 10 minutes (Leica Bond-Max Autostainer; Leica, Wetzlar, Germany). For p53 immunoreactivity, similar techniques were applied using a mouse monoclonal anti-p53 antibody (1 : 100) (M7001; DAKO, Carpinteria, USA) as a primary antibody.

Immunostaining of Snail and Slug was evaluated by two independent observers (K.M.K. and K.M.A.) for both the percentage of positive cells and staining intensity from 1 to 3 (1 weak, 2 moderate, and 3 strong). Since three cores were taken from each tumor, the average value was used for the study analysis [14]. Snail expression was mainly localized to the nucleus with weak cytoplasmic staining, and Slug was expressed in cytoplasm of tumor cells. In general, staining for Snail was more intense than that for Slug. For further statistical analysis, Snail and Slug expression was categorized into two groups: high expression, when >50% of tumor cells showed moderate-to-strong intense staining, and low expression, when ≤50% of tumor cells were positive [15, 16].

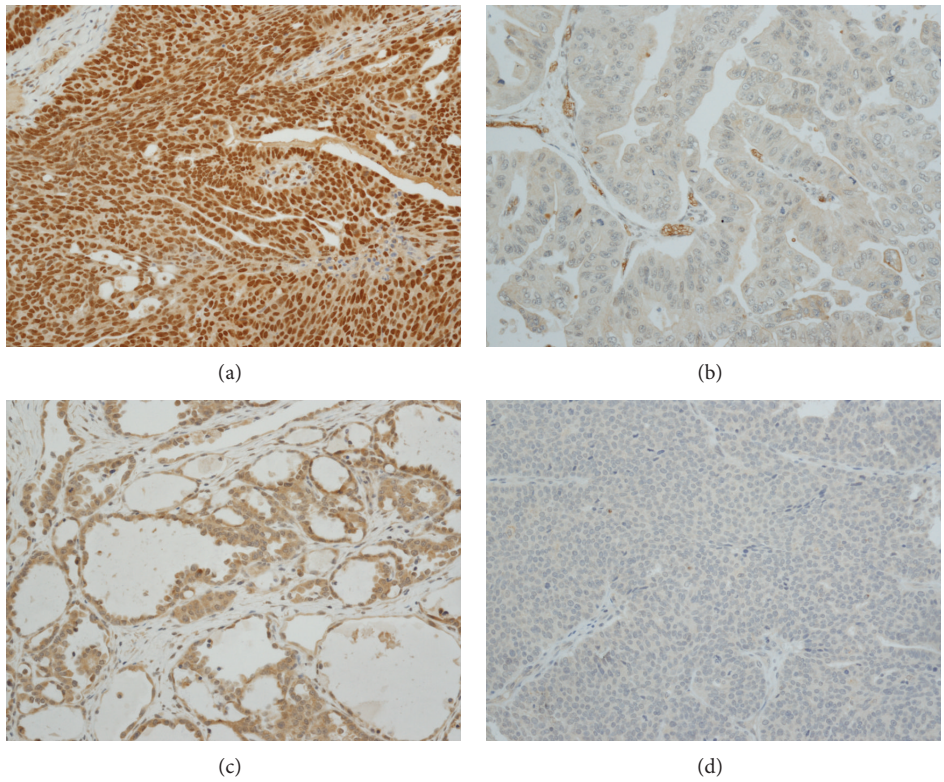
Nuclear expression of p53 was recorded as follows: completely negative, any staining in ≤50% of tumor cells, or moderate-to-intense staining in >50% of tumor cells [17].

2.4. Statistical Analysis. The differences in clinicopathologic variables according to the immunoreactivity for Snail and Slug were evaluated using chi-square test or Student's *t*-test accordingly. Survivals were also evaluated and compared using Kaplan-Meier method and log-rank test. Progression-free survival (PFS) was defined as the time interval from surgery to the first evidence of recurrence or death from any cause, whichever occurred first. Overall survival (OS) was defined as the time from surgery to death from any cause. A P value of less than 0.05 was considered to indicate statistical significance, and all tests were two-sided. The statistical analysis was performed using SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL).

3. Results

3.1. Snail/Slug Expression and Clinicopathologic Variables. Of the 103 cases with epithelial ovarian cancer, serous type was the most frequently diagnosed histologic subtype (59.2%), followed by mucinous (16.5%), clear cell (12.6%), and endometrioid type (9.7%). Most of the patients were diagnosed with stage I (34.0%) and stage III (46.6%) diseases. The majority of patients (88.5%) received platinum-based chemotherapy after the debulking surgery.

Snail was widely expressed (96.1%) and 81.6% of the cases showed high Snail expression. Slug was also expressed in the majority of tumors (91.3%), but high Slug expression was shown in 28.2% of the cases. Figure 1 shows the representative immunohistochemical findings. Snail expression was significantly higher in serous and endometrioid subtype than in mucinous or clear cell type ($P = 0.001$; Table 1). Snail expression also showed a tendency to correlate with high-grade lesions ($P = 0.048$). However, other clinical variables, such as FIGO stage, lymph node metastasis, peritoneal seeding, and residual disease status, were not associated with Snail expression. Slug expression was not significantly associated with Snail expression ($P = 0.058$), and it was not associated



(a) (b) (c) (d)

Figure 1: Immunohistochemical staining for Snail and Slug. (a) Snail-positive in serous carcinoma, (b) Snail-negative in mucinous carcinoma, (c) Slug-positive in clear cell carcinoma, and (d) Slug-negative in serous carcinoma. All figures are at 200× magnification.

with clinicopathologic variables, including histologic subtype (Table 1).

When the analysis of Snail expression was limited to serous adenocarcinomas, Snail expression remained high across the stage and grade (Table 2).

3.2. Differential Expression of Snail According to the p53 Expression. p53 was differentially expressed according to the histologic subtype of ovarian cancer ($P < 0.001$). The aberrant p53 expression, which was defined as completely negative or >50% expression [18], was significantly higher in the serous type compared to the mucinous or endometrioid subtype (86.9% versus 41.2% or 30.0%). When assessing the relationship between p53 and EMT-related proteins in serous adenocarcinomas, p53 expression was not significantly correlated to the Snail and Slug expression ($P = 0.537$ and $P = 0.132$, resp.; Table 3).

3.3. Snail/Slug Expression and Survival Outcomes. In serous adenocarcinomas, survival outcomes failed to show statistically significant differences between $Snail^{low}$ and $Snail^{high}$ population. Although there was a trend of worse PFS in $Snail^{high}$ patients (2-year PFS, 48.1% in $Snail^{high}$ versus 53.3% in $Snail^{low}$), the difference was not statistically significant ($P = 0.285$). Overall survivals also failed to show a significant difference according to the Snail expression ($P = 0.382$). Similarly, Slug expression was not associated with survival outcomes. In addition, when the platinum resistance was defined as recurrent within 6 months after the last chemotherapy, it was not associated with Snail expression ($P = 0.594$; Table 2).

4. Discussion

In the present study, we demonstrated that Snail was differentially expressed according to the histologic subtype and was highly expressed in the serous and endometrioid carcinomas. Although the expression profile of Snail was found to be subtype-specific, it failed to serve as an independent predictive or prognostic marker. The finding of differential expression of Snail according to the histologic subtype suggests that Snail might have different roles in tumor progression depending on the subtype of ovarian cancer. In addition, the high expression of Snail in the early stage serous carcinomas may suggest the potential role of Snail in the early phase of carcinogenesis.

Snail has been associated with poor clinical outcomes in various tumor types, including ovarian cancer, through induction of EMT which is responsible for metastasis and acquisition of therapeutic resistance. In epithelial ovarian cancer, Snail and Slug were shown to have distinct roles in metastasis and cancer cell survival [19, 20]. In addition, Snail and Slug were shown to contribute to the development of resistance to radiation and chemotherapy through

Table 1: Expression of Snail and Slug according to the clinicopathologic variables ($N = 103$).

Variables	Snail^{low}, n (%)	Snail^{high}, n (%)	P value	Slug^{low}, n (%)	Slug^{high}, n (%)	P value
Stage						
I	6 (17.1)	29 (82.9)	0.185	22 (62.9)	13 (37.1)	0.402
II	1 (9.1)	10 (90.9)		8 (72.7)	3 (27.3)	
III	8 (16.7)	40 (83.3)		36 (75)	12 (25)	
IV	4 (44.4)	5 (55.6)		8 (88.9)	1 (11.1)	
Histology						
Serous	5 (8.2)	56 (91.8)	0.001	45 (73.8)	16 (26.2)	0.097
Mucinous	7 (41.2)	10 (58.8)		8 (47.1)	9 (52.9)	
Endometrioid	1 (10)	9 (90)		9 (90)	1 (10)	
Clear cell	6 (46.2)	7 (53.8)		10 (76.9)	3 (23.1)	
Others	0 (0)	2 (100)		2 (100)	0 (0)	
Grade						
1	7 (33.3)	14 (66.7)	0.048	12 (57.1)	9 (42.9)	0.215
2	8 (21.6)	29 (78.4)		29 (78.4)	8 (21.6)	
3	4 (8.9)	41 (91.1)		33 (73.3)	12 (26.7)	
LN metastasis						
No	14 (18.4)	62 (81.6)	0.927	53 (69.7)	23 (30.3)	0.483
Yes	5 (19.2)	21 (80.8)		20 (76.9)	6 (23.1)	
Peritoneal seeding						
No	10 (16.4)	51 (83.6)	0.804	44 (72.1)	17 (27.9)	0.990
<2 cm	2 (20.0)	8 (80.0)		7 (70.0)	3 (30.0)	
>2 cm	7 (21.9)	25 (78.1)		23 (71.9)	9 (28.1)	
Residual tumor						
<1 cm	12 (17.6)	56 (82.4)	0.667	45 (66.2)	23 (33.8)	0.103
>1 cm	7 (21.2)	26 (78.8)		27 (81.8)	6 (18.2)	

LN: lymph node.

overcoming p53-mediated apoptosis and acquisition of stem-like characteristics in ovarian cancer cells [13]. Snail was also shown to be highly expressed in advanced stage and metastatic lesions [5, 21]. However, most of these studies did not evaluate the differential expression of EMT proteins according to the different subtypes. The present study included the primary ovarian cancer specimens with various histologic subtypes, which enabled the comparison of the distribution of histologic subtypes and survival outcomes according to the expression of EMT proteins more relevantly and demonstrated that the Snail and Slug were not independently related to survival outcomes as well as response to chemotherapy.

TP53 mutation, which is represented by aberrant p53 expression, is present in almost all cases of high-grade serous ovarian cancer (96%) [22]. In the present study, aberrant p53 expression was also frequently observed in serous type (86.9%). However, in serous adenocarcinomas where both p53 and Snail demonstrated aberrant expression commonly, Snail expression was not affected by p53 status. This might suggest that p53 and Snail have potentially different roles in ovarian carcinogenesis.

The immunopositivity of Snail in this study was much higher than the results of previous studies which reported the positive rate as 23–37.5% [6, 7]. In addition, some studies reported cytoplasmic staining of Snail rather than nuclear staining which is considered to be an active form [5, 21]. These discrepancies might be originated from the different antibodies used and the nonstandardized evaluation of staining. Our finding of widespread nuclear expression of Snail, however, is consistent with the previous study which demonstrated that Snail mRNA and protein expression were detected in almost all primary ovarian tumor specimens (93% and 100%, resp.) [23].

In the present study, the evaluation of the underlying mechanisms was limited due to the immunohistochemical analysis. In addition, the retrospective study design might cause selection biases. However, our finding of the differential distribution of tumor cells overexpressing Snail according to the histologic subtype may provide useful information regarding the patient selection for targeted therapy against EMT pathways.

In conclusion, we demonstrated that Snail expression was predominant in serous and endometrioid adenocarcinomas by immunohistochemistry. Snail and Slug overexpression, however, did not correlate with poor clinical outcomes. Our study set the stage for future studies investigating the differential roles of EMT according to the different histologic subtypes, which may provide potential therapeutic targets against cancer progression and metastasis.

Table 2: Expression of Snail according to the clinicopathologic variables in serous adenocarcinomas ($n = 61$).

Variables	$Snail^{low}$, n (%)	$Snail^{high}$, n (%)	P value
Stage			
I/II	1 (5.3)	18 (94.7)	0.574
III/IV	4 (9.5)	38 (90.5)	
Grade			
1	0 (0)	5 (100)	
2	3 (12)	22 (88)	0.591
3	2 (6.5)	29 (93.5)	
LN metastasis			
No	4 (9.3)	39 (90.7)	0.666
Yes	1 (5.9)	16 (94.1)	
Responses			
CR/PR	5 (10.2)	44 (89.8)	0.561
SD/PD	0 (0)	3 (100)	
Platinum sensitivity*			
Sensitive	4 (12.5)	28 (87.5)	
Intermediate	1 (7.1)	13 (92.9)	0.594
Resistant	0 (0)	6 (100)	

LN: lymph node; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

*Platinum sensitivity was defined according to the time interval from the date of last chemotherapy cycle to the first evidence of recurrence; sensitive when the interval was >12 months, intermediate when the interval was >6 months and <12 months, and resistant when the interval was <6 months.

Table 3: Expression of p53 according to Snail and Slug expression in serous adenocarcinomas ($n = 61$).

	p53 negative n (%)	p53 <50% n (%)	p53 >50% n (%)	P value
Snail				
Negative	2 (40.0)	0 (0)	3 (60.0)	0.537
Positive	13 (23.2)	8 (14.3)	35 (62.5)	
Slug				
Negative	14 (31.1)	5 (11.1)	26 (57.8)	0.132
Positive	1 (6.2)	3 (18.8)	12 (75.0)	

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Oxidatively Modified Proteins in the Serous Subtype of Ovarian Carcinoma

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Serous subtype of ovarian cancer is considered to originate from fallopian epithelium mucosa that has been exposed to physiological changes resulting from ovulation. Ovulation influences an increased in inflammation of epithelial ovarian cells as results of constant exposure of cells to ROS. The imbalance between ROS and antioxidant capacities, as well as a disruption of redox signaling, causes a wide range of damage to DNA, proteins, and lipids. This study applied spectrophotometric, dinitrophenylhydrazone (DNPH) assay, two-dimensional gel electrophoresis, and Western blot analyses to assess the levels of oxidatively modified proteins in 100 primary serous epithelial ovarian carcinoma and normal/surrounding tissues. These samples were obtained from 56 Caucasian and 44 African-American patients within the age range of 61 ± 10 years. Analyses showed that the levels of reactive protein carbonyl groups increased as stages progressed to malignancy. Additionally, the levels of protein carbonyls in serous ovarian carcinoma among African Americans are 40% ($P < 0.05$) higher relative to Caucasian at similar advanced stages. Results suggest that oxidative stress is involved in the modification of carbonyl protein groups, leading to increased aggressiveness of epithelial ovarian tumors and may contribute to the disease's invasiveness among African Americans.

1. Introduction

Epithelial ovarian cancer (EOC), with its various histological subtypes, is the fifth leading cause of cancer mortality among women in the United States [1]. Serous ovarian carcinoma is the most common and most aggressive subtype of EOCs [2, 3]. For this disease, differences in survival rates between African-American and Caucasian women are substantial, despite advances in surgical and chemotherapeutic management of the disease [1]. Older age and a family history of EOC are risk factors, but the disease etiology and the differences in survival rates in various groups of patients are far from being defined. A difficulty is that multiple genes are involved in the origin and in development of invasive types of EOCs [3, 4].

Ovarian cells, especially epithelial cells, are constantly exposed to ROS [5], which are generated during repeated ovulation and cause inflammation that is considered to be involved in ovarian carcinogenesis [6, 7]. Since most ovarian cancers appear in the surface epithelium, repetitive ovulation is thought to be a causative factor [8, 9]. ROS are involved in the development and progression of many human diseases, including cancer [10]. Oxidative stress is defined as the imbalance between ROS and cellular antioxidative capacities and is based on a disruption of redox signaling [11].

In addition to lipids [12] and DNA [13], proteins are targets for modifications resulting from oxidative stress. In ovarian cystadenocarcinoma, there is an increase in products associated with oxidative stress, such as 8-hydroxy-2′-deoxyguanosine relative to normal ovarian tissues [6]. Oxidized proteins that accumulate during aging are increased with oxidative stress and in some pathological conditions [10, 14]. The most protein modification caused by oxidative

Table 1: Characteristics of tumors and patients.

Characteristics	Category	Subcategory	*n* (%)
Ethnicity	African American		48 (48%)
	Caucasian		52 (52%)
Age (years)	≥61	African American	19 (40%)
		Caucasian	29 (60%)
		Total	**48**
	<61	African American	29 (56%)
		Caucasian	23 (44%)
		Total	**52**
Differentiation	Normal/surrounding		9 (9%)
	Cystadenoma		12 (12%)
	Borderline		8 (8%)
	Carcinoma		24 (24%)
	Papillary carcinoma		47 (47%)

stress is the carbonyl groups; the most sensitive amino acids are arginine, lysine, proline, threonine, and glutamic acid. Reactive protein carbonyls reflect the degree of oxidative damage and serve as a biomarker for oxidative stress [15, 16]. For detection of reactive protein carbonyl groups, several methods are available, including 2,4-dinitrophenylhydrazone (DNPH) assay and Western blot immunoassays [17–20]. In the present study, levels of reactive protein carbonyl groups were measured in samples of normal tissue and tissues of early and invasive stages of serous ovarian carcinomas, including samples obtained from Caucasians and African Americans. These experiments were performed to determine the role of oxidative stress during ovarian carcinogenesis and assess the relationship of reactive carbonyl levels with the extent of cancer in the tissues.

2. Materials and Methods

2.1. Study Samples. One hundred primary epithelial serous ovarian tumor tissues were obtained from the Southern Regional Cooperative Human Tissue Network and the University of Alabama at Birmingham (UAB) Ovarian Spore Center. These samples were stabilized by snap-freezing immediately after excision and dissection. The dissected tissues were placed in cryovials and immersed in liquid nitrogen. All samples were transferred to −80°C for long-term storage as recommended for measurement of proteins with reactive carbonyl groups [21]. Of the tissue samples, 44 were from African Americans and 56 were from Caucasians. The mean age of the patients was 61 ± 10 years. The breakdown of the 100 primary serous epithelial ovarian tissues were 9 control, normal surrounding, and 91 cystadenoma, borderline, carcinoma, and papillary adenocarcinomas (Table 1). All tissue samples were microdissected, diagnosed, and histopathologically confirmed by pathologists. Tumor stages were determined on the basis of criteria outlined by the International Federation of Gynecology and Obstetrics. Demographic characteristics of the patients were grouped based on the clinical diagnosis (Table 1). All studies were implemented under protocols approved by Institutional Review Boards of Morehouse School of Medicine and the University of Alabama at Birmingham.

2.2. Extraction of Cytosolic Fractions. The cytosolic fractions of the tissue samples were prepared by differential centrifugation using mitochondria/cytosol fractionation kits (BioVison, CA.). Approximately 400 mg of each sample was cut into the small pieces, placed in a 2 mL plastic tube on ice, and washed twice with ice-cold phosphate-buffered saline (PBS). Each tissue sample was mildly homogenized in an ice-cold Dounce tissue grinder and centrifuged at 700 ×g for 5 min at 4°C. The supernatant was removed, and 1 mL of homogenizing buffer containing protease inhibitors was added. The sample was incubated on ice for 10 min and then homogenized in an ice-cold Dounce tissue grinder, with about 50–60 passes. The homogenate was transferred into a 1.5 mL microcentrifuge tube and centrifuged at 700 ×g for 10 min at 4°C. The supernatant was collected, transferred to a fresh 1.5 mL microcentrifuge tube, and centrifuged at 10,000 ×g for 30 min at 4°C. The supernatant was collected as cytosolic fraction. This fraction was treated with a 1% streptomycin sulfate solution for 15 min to remove DNA, which could react with DNPH and contribute to the reactive carbonyl level of homogenates. After incubation, samples were centrifuged at 13,000 ×g for 15 min at room temperature. The supernatant which contained DNA-free cytosolic fraction was collected and saved for the DNPH assay.

2.3. Measurement of Total Protein Concentration. A microplate DC protein assay (BioRad) was used to measure the protein contents of the samples. Each sample was analyzed in duplicate, and a pooled tissue sample was included in each plate to estimate the interassay coefficient of variation and the coefficient of variation, which was determined to be 4.9%. A standard bovine serum albumin (BSA) containing 0.2 mg/mL to 2.0 mg/mL protein was prepared in the same homogenizing buffer and analyzed along with samples. A fraction of 5 μL of each sample and standard protein was added to the well of a 96-well microplate followed by adding

25 μL of reagent A and 200 μL of reagent B as recommended by BioRad protocol. The plate was placed on the plate mixer and mixed for 5 sec, and then it was incubated at room temperature for 15 min. The absorbance at 750 nm was determined spectrophotometrically, the protein concentration of each homogenate was extrapolated from a standard curve. Samples of an extract of MCF7 cells and a protein extract from a control cell line were included in each run as positive controls.

2.4. Protein Carbonyl Assay. Oxidized protein modifications in serous ovarian cancer samples were determined by measuring reactive protein carbonyl groups. ROS react with amino acid residues in protein, particularly histidine, arginine, lysine, and proline, to produce carbonyl functions that can react with DNPH, leading to formation of stable dinitrophenylhydrazone adducts [20, 22]. This reaction is used to estimate reactive carbonyl content of proteins in human tissues and body fluid [21]. The protein carbonyl content of the homogenates was determined as follows: DNA-free homogenates of serous ovarian tissue samples (0.5 mL) were placed in each microcentrifuge tube labeled as treated samples and control samples. Two mL of 10 mM DNPH (Sigma) in 2 M HCl was added to the treated sample tubes and two mL of 2 M HCl only was added to the control sample tubes which were incubated on a rotator at room temperature for 1 hr. The hydrazone derivatives were precipitated with 20% (wt/vol) trichloroacetic acid, treated with ethanol/ethyl acetate, 1 : 1 (vol/vol) to remove excess DNPH, and reprecipitated with 10% trichloroacetic acid. The pellet was dissolved in 6 M guanidine hydrochloride. Differences between optical densities of DNPH treated and untreated samples were determined spectrophotometrically at 370 nm. The results were calculated as nmole of DNPH incorporated per mg of protein, as determined from absorptivity using the Bear-Lambert equation and an extinction coefficient 22,000 μM^{-1} cm^{-1}. To determine the stability of the samples, protein carbonyls were measured in a pooled tissue sample that was repeatedly frozen and thawed. There was no significant difference in protein carbonyl levels after four freeze-thaw cycles. These results are similar to previously described results for plasma samples [23].

2.5. SDS-PAGE and Western Blot Analyses. In this study, to determine the number and relative mass of the DNPH derivatized proteins in the tissue samples, SDS-PAGE and Western blot immunoassays were performed using OxyBlot Protein Oxidation Detection Kits (Millipore). Samples of DNPH-derivatized proteins were resolved on 10% SDS-polyacrylamide gels. As a control an underivatized sample of each cytosolic fraction was run along with DNPH-derivatized samples. The proteins were transferred to PVDF membranes blotted with rabbit anti-DNPH antibody and detected with a superSingal West Pico chemiluminescent substrate. A DNPH-derivatized standard protein was used for estimation of molecular weight. Proteins that were oxidatively modified were identified by their appearance as bands in the lane containing the derivatized sample, but not in the lane containing the control. Blots were quantified using the UN-SCAN-IT automated digitizing system, version 5.1 (Silk Scientific Inc.), and the results were expressed as arbitrary units.

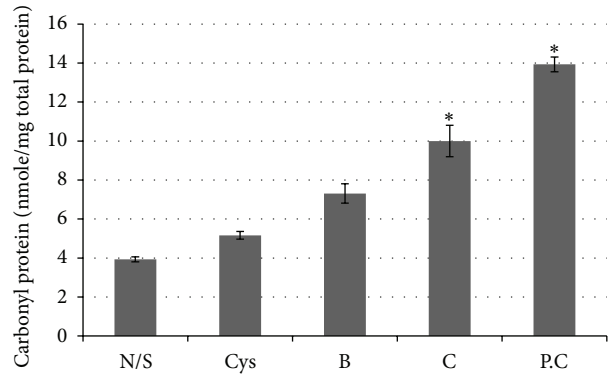

Figure 1: Levels of reactive carbonyl proteins in normal/surrounding and serous subtype ovarian cancer tissues, as measured by the spectrophotometric method. N/S: normal/surrounding; Cys: cystadenoma; B: borderline; C: carcinoma; P.C: papillary carcinoma. Significant differences between groups ($P < 0.05$)* ($N = 100$ samples).

2.6. Statistics. Data were analyzed using analysis of variance (ANOVA) and Student's t-test.

3. Results

Total Protein Reactive Carbonyls. In biological systems, free radicals generally lead to oxidative, posttranslational modifications of proteins, a process in which the degree of introduction of reactive carbonyl groups relates to the intensity of the oxidative stress [24]. Proteins from lysates of samples of normal tissues and early and invasive serous ovarian carcinomas were derivatized with DNPH to measure the levels of carbonyl groups by a spectrophotometric method. Progressively increasing levels of carbonyl groups were observed in the derivatized lysates samples from cystadenomas, borderline tissues, carcinoma, and papillary adenocarcinomas (Figure 1). The results of Western blots were similar to those obtained with the spectrophotometric techniques. Relative to normal/surrounding tissues, protein reactive carbonyls were elevated in cystadenomas, borderline tissues, and, notably, the invasive stages (Figures 2(a) and 2(b)).

Levels of carbonyl groups were also assessed to evaluate the differences between tissue samples from African Americans and Caucasians (Figure 3). For African Americans, there were, relative to similar samples from Caucasians, 40% lower levels of reactive carbonyls in proteins of borderline tissue samples ($P < 0.05$) and 40% higher levels of reactive carbonyl proteins in carcinomas and papillary carcinoma samples from African American relative to similar samples from Caucasians ($P < 0.05$ for both). In normal tissues and cystadenomas, there were no significant differences between the two groups in levels of protein carbonyls. Differences in

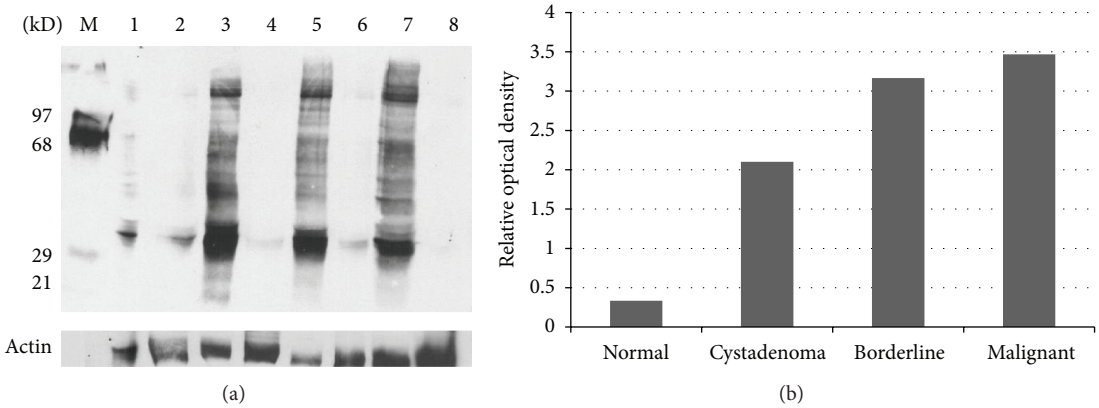

Figure 2: (a) Western blot analysis of expression of reactive carbonyl groups in the cytosolic fraction of serous ovarian carcinoma tissues. Lane M: protein marker; Lane 1: DNPH-derivatized lysate of normal tissue; Lane 2: underivatized lysate of normal tissue; Lane 3: DNPH-derivatized lysate of cystadenoma tissue: Lane 4: underivatized lysate cystadenoma tissue; Lane 5: DNPH-derivatized lysate of borderline tissue; Lane 6: underivatized lysate of borderline tissue; Lane 7: DNPH-derivatized lysate of malignant tissue; Lane 8: underivatized lysate of malignant tissue. (b) The relative optical density on the level of reactive carbonyl proteins using the UN-SCAN-IT system.

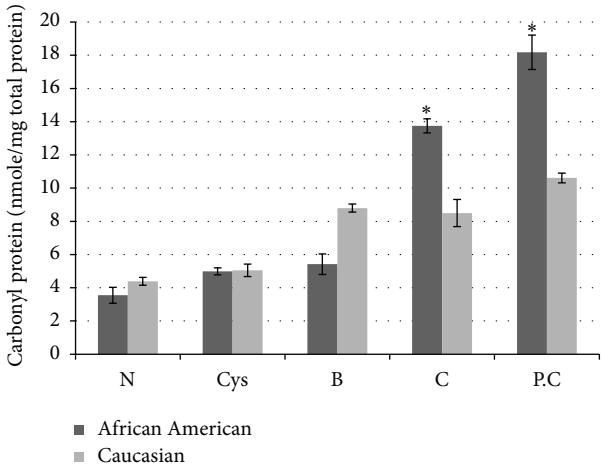

Figure 3: Levels of reactive carbonyl proteins in normal/surrounding and serous subtype ovarian cancer tissues as measured by the spectrophotometric method. N/S: normal/surrounding; Cys: cystadenoma; B: borderline; C: carcinoma; P.C: papillary carcinoma. There were significant differences between samples from African Americans ($N = 44$) and Caucasians ($N = 56$) ($P < 0.05$)*.

the levels of protein carbonyl expression within individual patient samples were also evident. Tissue samples with higher contents of tumor cells exhibited higher levels of oxidized proteins relative to normal/surrounding tissues (Figure 4(a)) and to samples with fewer tumor cells from same patient (Figure 4(b)).

4. Discussion

The accumulation of oxidized proteins (protein carbonyls) is associated with the risk of carcinogenesis [9, 14, 15] and age-related diseases [17, 20, 25]. The increase of ROS may result from altered metabolism as well as from inadequate tumor neovascularization. The levels of carbonyl content in tissues have not been determined for the process of ovarian carcinogenesis. Although protein oxidation markers are detected in cells that are already transformed, oxidative modification of proteins may be implicated in the serous ovarian carcinoma subtype, since these cancers are derived from the tubal epithelium of the ovarian surface. Repetitive ovulation is thought to be a causative factor of ovarian cancer [6, 26]. Rupture of follicles involves tissue remodeling, with high cell turnover, characteristic of inflammatory reactions. Oxidative stress is associated with inflammatory processes, resulting from stimuli, such as cytokines (tumor necrosis factor and interleukin-1) and bacterial toxins (lipopolysaccharide) [27]. Particularly in the fallopian tubes, damage to the epithelium resulting from inflammatory responses during ovulation is generally viewed as a secondary event. The primary event is the inflammatory cascade of neutrophil adherence to vascular endothelial cells, disruption of their barrier, and subsequent infiltration of inflammatory cells into the interstitial space, where oxidants and proteases are released and produce mucosal injury. A variety of chronic inflammatory conditions predispose susceptible cells to neoplastic transformation [28]. Inflammatory cytokines, such as TNF-α and ROS, activate nuclear factor kappa-B (NFκB) by phosphorylation. In its normal state, NFκB is inhibited by its inhibitory protein (IκBα), which downregulates the inflammatory response. In nuclei, NFκB induces the expression of genes involved in cell proliferation, apoptosis, and carcinogenesis [29] and also induces production of proinflammatory cytokines, which enhance the inflammatory responses.

The main effectors in the inflammatory response are ROS. These may directly or indirectly cause damage through their reactions with components of target cells [30]. ROS can also recruit other inflammatory cells, leading to additional ROS production and amplification of damage [31]. Thus, ovulation may be accompanied by inflammation that induces oxidative damage to DNA, proteins, and lipids of the ovarian

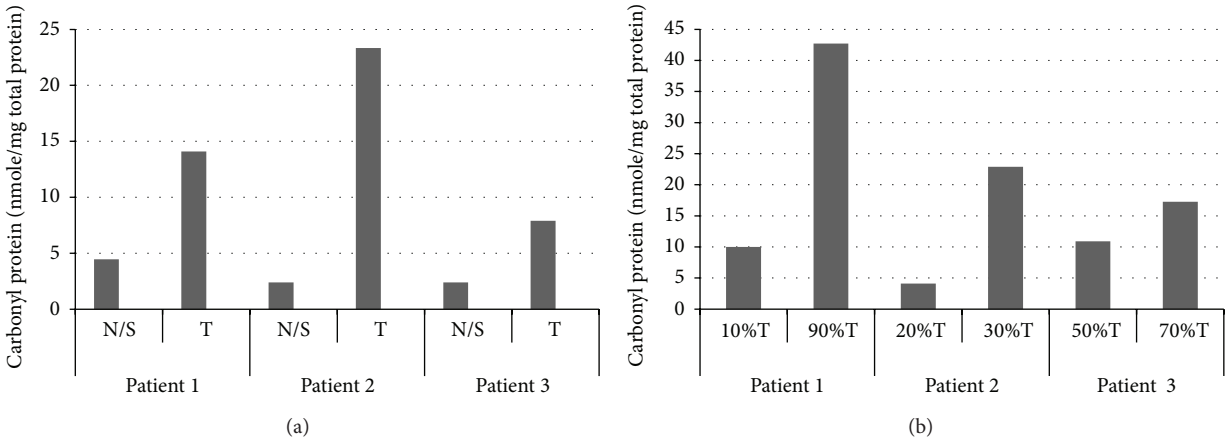

Figure 4: Levels of reactive carbonyl proteins in tumor tissue obtained from different patients as measured by the spectrophotometric method. (a) Levels of carbonyl proteins in normal/surrounding tissues relative to tumor tissues of the same patient ($n = 3$). (b) Levels of carbonyl proteins in tissues with different contents of tumor cells in individual ($n = 3$). N/S: normal/surrounding tissue; T: tumor tissue.

epithelium [32]. The reactive carbonyl content of protein is the most commonly used marker of protein oxidation [17, 25], and, in cells, oxidized proteins accumulate during aging, accompanied with oxidative stress and some pathological conditions [17–20]. Furthermore, proteins modified by oxidative stress are associated with an increased risk of cancer [17, 33]. In colorectal cancer, there is enhanced oxidative stress relative to normal intestinal tissues [34]. The present results are similar in that they show elevated expression of protein carbonyls in serous ovarian carcinomas relative to normal/surrounding tissue and to cystadenomas. This suggests that oxidative modification of proteins is involved in the formation of ovarian cancers. Carcinogenesis in general may be mediated by oxidative damage to DNA, due to mutations in critical genes, such as the tumor suppressor p^{53} [35]. Damage to the p^{53} gene may reduce the effectiveness of DNA repair mechanisms and increase the rate of cell division. Cells that are rapidly dividing cells are more prone to errors in DNA replication and repair [26] and may also be more sensitive to oxidative stress, enhancing the risk of carcinogenesis.

As demonstrated here, there are high levels of reactive protein carbonyls in tissue samples of invasive serous ovarian carcinomas from African Americans. The most likely explanation is a racial difference in the intracellular levels of oxidized proteins, reflecting the balance between the rate of protein oxidation and the rate of oxidized protein degradation [36]. This balance is a function of factors leading to the generation of ROS [37]. Various physiological and environmental processes may lead to the formation of ROS and be factors in determining the concentrations and/or activities of the proteases that degrade oxidatively damaged proteins [17]. Such degradation is also dependent upon numerous variables, including the concentrations of proteases that preferentially degrade oxidized proteins, and upon cellular components, such as metal ions, inhibitors, activators, and regulatory proteins, that affect their proteolytic activities. For example, oxidized forms of some proteins, for example, cross-linked proteins [38–40] and proteins modified by glycation [41] or by lipid peroxidation products [42], are resistant to proteolysis and could lead to production of protease inhibitors that hinder degradation of the oxidized forms [38, 42]. Therefore, inactivation of these protein inhibitors could enhance the action of proteases, such as elastase, plasminogen activator, and plasmin. This process could facilitate tumor invasion and metastasis [43], particularly in various individuals or subgroups.

A reduced dietary intake of antioxidants and an impaired mitochondrial function may render African Americans more vulnerable to diseases associated with oxidative stress [44]. This concept is based on results obtained from a study of racial differences in association of oxidative stress and insulin sensitivity in African- and European-American women. Therefore, we speculate that the effect of the elevated levels of protein carbonyls in ovarian cancer tissues from African-American patients may be involved in the aggressiveness of the disease. However, the current results do not provide evidence that an increase in protein carbonyls is solely the cause of racial differences between African Americans and Caucasians regarding ovarian cancer aggressiveness. Alterations of the redox balance within the cell, leading to oxidative damage to proteins, lipids, and nucleic acids involvement, cannot be ruled out as a cause of this difference.

To our knowledge, this is the first report to demonstrate a relationship between elevated levels of reactive protein carbonyls and the serous ovarian carcinoma subtype and to note differences in expression of reactive protein carbonyls between African-American and Caucasian women bearing the disease. This is noteworthy, since oxidative stress is considered to be triggered by ovulation-induced inflammation. Inflammation normally leads to production of oxidants to kill pathogens, but these oxidants can cause damage to DNA, proteins, and lipids and may be, therefore, involved in ovarian carcinogenesis [26].

A strength of these findings is the similarity of the results for Western blot and spectrophotometric techniques in measuring levels of protein reactive carbonyls in tissue samples of ovarian cancer. A limitation is the relatively small sample size, related to the cross-sectional nature of the study and to the limited population of African-American and Caucasian women with ovarian cancer. To fully understand the contribution of oxidative stress to ovarian carcinogenesis and racial disparities in the aggressiveness of the disease, future research should include a larger sample of women of different ethnic backgrounds and potential involvement of epigenetic regulations such as microRNAs in the regulatory circuitry underlying disparity [45–47].

In conclusion, results from this study demonstrate an association of elevated levels of reactive protein carbonyls, formed by oxidative stress, with serous ovarian carcinogenesis. The results also indicate a racial difference in levels of these carbonyl groups and invasive stages of serous ovarian carcinoma among women bearing this disease. Whether the higher prevalence of aggressiveness ovarian carcinomas in African-American women correlates with greater oxidative damage within these patients deserves further research.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Authors' Contributions

F. O. Aikhionbare and S. Mehrabi were responsible for conception and design; Development of methodology was conducted by S. Mehrabi and F. O. Aikhionbare; E. E. Partridge was responsible for acquisition of some tissue samples; S. Mehrabi and F. O. Aikhionbare conducted analyses and interpretation of data; writing was carried out by F. O. Aikhionbare and reviewed by X. Yao, W. Seffens, and F. O. Aikhionbare.

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