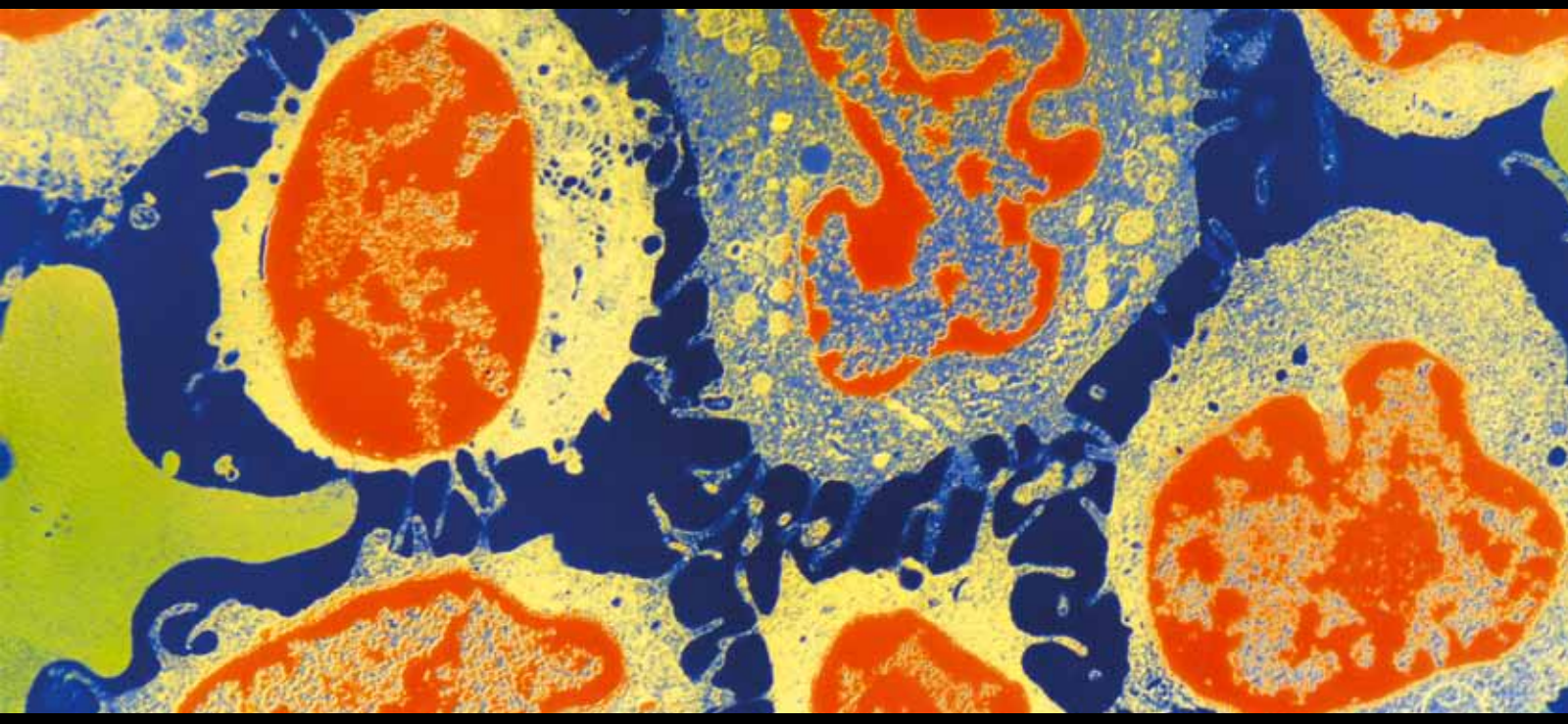
Journal of Oncology

Pathogenesis and New Therapeutic Targets of Ovarian Cancer

Guest Editors: Ie-Ming Shih, Chih-Min Ho, Kentaro Nakayama, and Ritu Salani



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Editorial Board

Contents

Pathogenesis and New Therapeutic Targets of Ovarian Cancer, Ie-Ming Shih, Chih-Ming Ho, Kentaro Nakayama, and Ritu Salani
Volume 2012, Article ID 867512, 2 pages

Early Detection of Ovarian Cancer with Conventional and Contrast-Enhanced Transvaginal Sonography: Recent Advances and Potential Improvements, Arthur C. Fleischer, Andrej Lyshchik, Makiko Hirari, Ryan D. Moore, Richard G. Abramson, and David A. Fishman
Volume 2012, Article ID 302858, 11 pages

GRP78 Protein Expression in Ovarian Cancer Patients and Perspectives for a Drug-Targeting Approach, Florence Delie, Patrick Petignat, and Marie Cohen
Volume 2012, Article ID 468615, 5 pages

Special Agents Hunting Down Women Silent Killer: The Emerging Role of the p38α Kinase, Valentina Grossi and Cristiano Simone
Volume 2012, Article ID 382159, 7 pages

Regulatory T Cells in Human Ovarian Cancer, Dong-Jun Peng, Rebecca Liu, and Weiping Zou
Volume 2012, Article ID 345164, 7 pages

Optimizing Molecular-Targeted Therapies in Ovarian Cancer: The Renewed Surge of Interest in Ovarian Cancer Biomarkers and Cell Signaling Pathways, Donavon Hiss
Volume 2012, Article ID 737981, 23 pages

Integrin Inhibitors as a Therapeutic Agent for Ovarian Cancer, Kenjiro Sawada, Chifumi Ohyagi-Hara, Tadashi Kimura, and Ken-ichirou Morishige
Volume 2012, Article ID 915140, 6 pages

Ovarian Cancer: Opportunity for Targeted Therapy, Tomoko Tagawa, Robert Morgan, Yun Yen, and Joanne Mortimer
Volume 2012, Article ID 682480, 9 pages

DNA Damage Response is Prominent in Ovarian High-Grade Serous Carcinomas, Especially Those with Rsf-1 (HBXAP) Overexpression, Malti Kshirsagar, Wei Jiang, and Ie-Ming Shih
Volume 2012, Article ID 621685, 7 pages

Hindawi Publishing Corporation
Journal of Oncology
Volume 2012, Article ID 867512, 2 pages
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Editorial

Pathogenesis and New Therapeutic Targets of Ovarian Cancer

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Ovarian cancer remains the most lethal gynecologic malignancy, largely due to the lack of early detection tools and effective therapeutic interventions. Anti-tumor agents targeting critical molecular pathways hold promise for improving survival in these patients and understanding the critical molecular pathways involved in the pathogenesis of ovarian cancer development is central to the development of such agents. For example, using genome-wide DNA copy number analysis, investigators have identified amplification in a genomic locus (ch11q13.5) harboring a chromatin remodeling gene, RSF1, encoding Rsf-1 in high-grade ovarian serous carcinomas. Recent studies have shown that excessive Rsf-1 expression attributes to genomic instability and alters gene expression profiles to favor tumor growth and survival, especially in the presence of cytotoxic agents. The article entitled "*DNA damage response is prominent in ovarian high-grade serous carcinomas, especially those with Rsf-1 (HBXAP) overexpression*" reported by M. Kushirsagar et al. in this special issue provides new evidence that Rsf-1 overexpression was correlated with DNA damages which was observed more frequently in high-grade ovarian serous carcinoma. This finding should have several biological and clinical implications for the future studies of Rsf-1 in human cancer.

The article entitled "*Regulatory T cells in human ovarian cancer*" from D.-J. Peng et al. is a succinct review of the roles of the immune system in ovarian cancer development. In this article, they focus on summarizing the functions of regulatory T cells in the pathogenesis of ovarian cancer from a perspective of immune response and regulation. The knowledge of ovarian cancer immunity is fundamental to understand the molecular interplay between cancer cells and their tumor microenvironment and serves as an important road map for future development of immune-based therapy in ovarian cancer.

Another interesting review article in this special issue is from C. Ohyagi-Hara et al. who summarized the potential to apply *integrin inhibitors as a therapeutic agent for ovarian cancer.* This is considered as a highly rational approach because the initial critical step of ovarian cancer metastasis is the attachment of cancer cells onto the peritoneum surface, and targeting integrins holds promise to inhibit ovarian cancer metastasis. Although no integrin inhibitors have shown favorable results so far, integrin-targeted therapies remain attractive for further clinical investigation.

The review article "*Optimizing molecular targeted therapies in ovarian cancer: the renewed surge of interest in ovarian cancer biomarkers and cell Signaling pathways*" from D. Hiss timely reviewed the biomarkers and signaling pathways of ovarian cancer. The author comprehensively summarized the recent advances in this highly competitive field with special emphasis on their translational implications. The number of literatures cited exceeds 400 which provide a compendium for ovarian cancer biomarker studies. The review article entitled "*Ovarian cancer: opportunity for targeted therapy*" by T. Tagawa et al. focuses on discussing exciting molecular targets including PARP, MEK, microRNAs, and molecules involved in angiogenesis. The authors keenly separate different types of ovarian cancer (type I and type II) in the discussion because it has become increasingly clear

that different histological subtypes of ovarian cancer use distinct molecular alterations for their development. Thus, it is essential to consider this important factor in studying molecular targeting and developing new therapeutics in ovarian cancer. To this end, the article "*GRP78 expression in ovarian cancer patients and perspectives for a drug-targeting approach*" proposes to use GRP78 as a drug delivery system targeting ovarian cancer cells. This is of great interest given that GRP78 upregulation is considered as a cellular response to endoplasmic reticulum stress which is common in tumor cells. The finding of abundant GRP78 molecules in ovarian cancer cell surface invites the development of novel drug delivery to specifically bring the cytotoxic drug or other antitumor agents in the future clinical test. Another very interesting article "*Special agents hunting down women silent killer: the emerging role of the p38α kinase*" described the potential to target the pathways involved in cancer-specific metabolism and drug resistance. One of the pathways that the authors highlighted is the p38alpha which has been in the cancer research spotlight in recent years. Moreover, small compound inhibitors of p38alpha have been evaluated in clinical studies and the encouraging results should hold promise for the future applications of p38 inhibitors as an emerging treatment option for ovarian cancer treatment.

Finally, as tumor imaging techniques have advanced significantly in the past several years, the article entitled "*Early detection of ovarian cancer with conventional and contrast-enhanced transvaginal sonography: recent advances and potential improvements*" is a timely review that summarizes the potential to use imaging systems to early detect ovarian cancer and distinguish benign versus malignant ovarian neoplasms. For example, it has been shown that the 3D-transvaginal sonography combined with matrix array transducers/probes can enhance the visual inspection of cystic wall anomalies in adnexal masses, promote the comfort for patients and most importantly, improve reproducibility. In summary, we hope that these articles appearing in this special issue will provide useful research information to those investigators who are devoted to ovarian cancer research in the years to come.

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Review Article

Early Detection of Ovarian Cancer with Conventional and Contrast-Enhanced Transvaginal Sonography: Recent Advances and Potential Improvements

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Recently, there have been several major technical advances in the sonographic diagnosis of ovarian cancer in its early stages. These include improved assessment of tumor morphology with transvaginal sonography (TVS), and detection and characterization of tumor neovascularity with transvaginal color Doppler sonography (TV-CDS) and contrast-enhanced transvaginal sonography (CE-TVS). This paper will discuss and illustrate these improvements and describe how they enhance detection of early-stage ovarian cancer. Our initial experience with parametric mapping of CE-TVS will also be mentioned.

1. Introduction

This year in the United States approximately 24,000 women will be diagnosed with ovarian cancer, and there will be approximately 14,000 associated deaths, predominantly from epithelial ovarian cancer (EOC). Worldwide, it is estimated that 204,449 patients with ovarian cancer will be diagnosed this year with an estimated 124,860 disease-related deaths. The incidence of ovarian cancer has been steadily increasing over the past 10 years, with an overall lifetime risk of 1.8% [3]. Despite improvements in surgical techniques and new chemotherapeutic regimens, the overall survival for women with stage III/IV EOC has remained relatively unchanged (15%) over the past 40 years [3]. In contrast, women diagnosed with disease confined to the ovary (stage I) require less morbid surgical intervention, may not require adjuvant chemotherapy, have a significantly improved quality of life, and most importantly have an overall 5-year survival approximating 90% [3]. Unfortunately, 75% of women continue to be diagnosed with advanced-stage disease. It is thought that accurate diagnosis of EOC at an earlier stage may decrease overall disease-related mortality.

Evaluation of adnexal masses can be performed with several imaging methods, including TVS, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). CT can detect large adnexal masses but has lower sensitivity for small adnexal masses, especially in thin patients in whom adnexal lesions can be misinterpreted as other pelvic structures or loops of pelvic small bowel. Furthermore, the ability of CT to characterize adnexal lesions as benign or malignant is limited by low inherent tissue contrast, with the notable exception of ovarian dermoids that can be characterized based on the presence of macroscopic fat and/or calcification. MRI offers higher spatial and contrast resolution than CT and can characterize a wider spectrum of adnexal lesions based on magnetic signal properties or enhancement behavior, but accuracy of MRI may diminish for borderline ovarian tumors and small ovarian masses. Compared to TVS, MRI is costly and has limited availability [4]. PET can identify aggressive

adnexal lesions on the basis of increased fluorodeoxyglucose (FDG) uptake, but it suffers from low specificity for small lesions, noting that normal premenopausal ovaries will demonstrate increased metabolic activity at mid cycle, and a physiological corpus luteum can therefore mimic an aggressive malignancy. The accuracy of PET is also limited secondary to false negatives in borderline ovarian neoplasms [5].

TVS is widely available and offers high-resolution imaging without the use of ionizing radiation. For these reasons, TVS is the initial diagnostic modality of choice for the evaluation of most patients with a pelvic mass. As previously mentioned, TVS has limited sensitivity and specificity for the definitive diagnosis of ovarian cancer because of overlapping morphologic features seen in benign and malignant lesions. Recently, however, significant technologic advances have yielded vast improvements in the sonographic depiction of early-stage ovarian cancer, and these improvements have translated into improved sonographic discrimination of benign from malignant disease in preliminary studies. Combined evaluation of sonographic morphology and CDS forms a set of basic "simple rules" for sonographic distinction of benign from malignant ovarian masses based on the data derived from a European multicenter study which included 1,223 adnexal tumors (sensitivity 93%; specificity 90%) [6] (Table 1).

Three-dimensional transvaginal sonography (3D TVS) has improved the morphologic depiction of ovarian cancers beyond the capabilities of traditional TVS. Improvements in transvaginal color Doppler sonography (TV-CDS) have enhanced sonographic assessment of large tumor vascular networks, and contrast-enhanced transvaginal sonography (CE-TVS) now allows for interrogation of tumor microvascularity [7–9]. This paper discusses these newer techniques, specifically CE-TVS, with emphasis on their advantages and areas for potential improvement.

1.1. 2D and 3D TVS. Conventional sonographic criteria for ovarian cancer diagnosis are based on morphological classification of ovarian masses. Ovarian malignancy is unlikely in simple cysts with smooth walls, but presence of a solid mass or solid projections (papillary excrescences) into the cyst cavity significantly increases the risk of malignancy.

Hirai has described the morphologic features on TVS associated with stage I ovarian cancer in a lay-screening population in Japan [1] (Figures 1, 2, and 3). In general, the stage IA ovarian cancers with normal CA-125 were small and had less solid components than stage IA cancers with elevated CA-125. Papillary excrescences typically occur in areas of epithelial neoplasia and can be seen borderline rather than frankly malignant lesions.

Over the last 10 years, the diagnostic accuracy for conventional 2D TVS has been improving [10]. A 1997 study reported that gray-scale sonography identified malignant tumors with a sensitivity of 91% and a specificity of 84% [11], while a 2008 study found a sensitivity of 93% and specificity of 90% [12]. As the result of these studies, several morphological scoring systems have been developed for sonography, including features such as the presence of papillary projections or irregular and/or thick septae. Results of a meta-analysis provide evidence that sonographic techniques that combine gray-scale morphologic assessment with tumor vascularity mapping are significantly better in ovarian lesion characterization than Doppler arterial resistance measurements, color Doppler flow imaging, or gray-scale morphologic information alone [13].

Table 1: "Simple rules" for sonographic diagnosis of ovarian cancer*.

Benign	Malignant
(1) Unilocular cyst	(1) Irregular solid tumor
(2) Solid components <7 mm	(2) Ascites
(3) Acoustic shadows	(3) Papillary excrescences
(4) Smooth multilocular	(4) Irregular multiloculated/solid tumor
<10 cm	>10 cm
(5) No color Doppler flow	(5) Very high color content

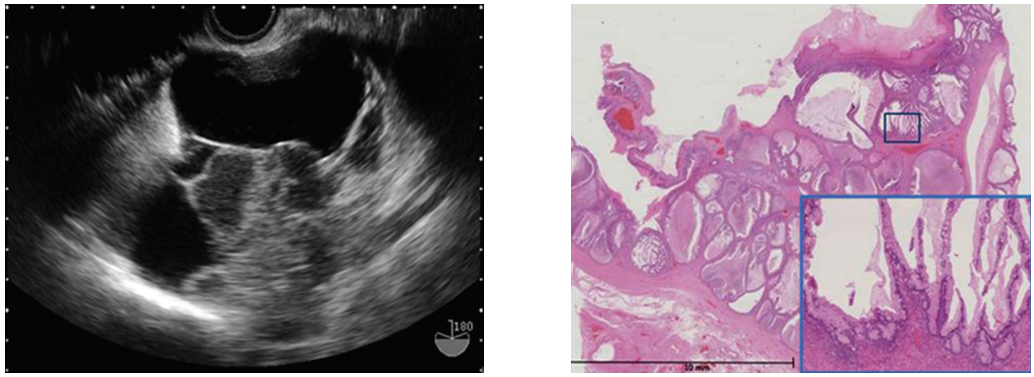
* Timmerman, D, US O/G 31 : 681, 2008.

The recent development of 3D-TVS improves the detection of morphologic abnormalities indicative of neoplastic ovarian masses. In particular, small papillary excrescences or focal wall (mural) irregularities can be detected which are associated with epithelial malignant growth in ovarian masses [8]. The recent advent of matrix array transducers/probes may improve visualization of both internal and external wall (capsular) abnormalities, increase comfort for the patient, and increase reproducibility.

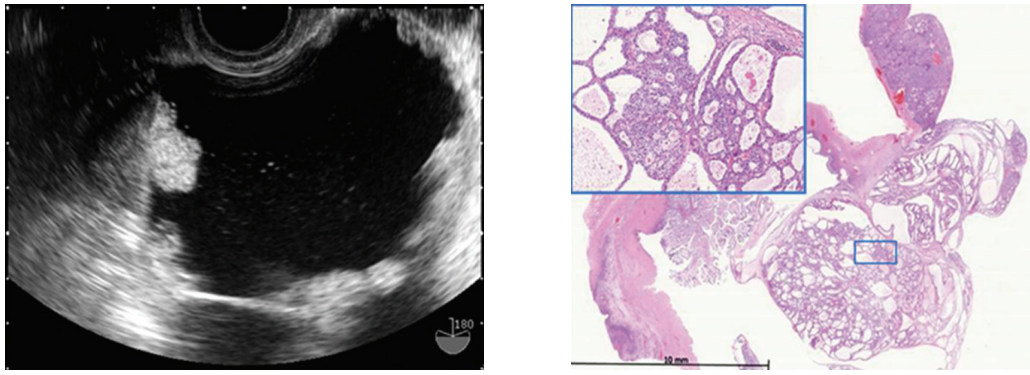
1.2. Transvaginal Color Doppler Sonography (TV-CDS). TV-CDS provides depiction of the macrovascularity (over 200 μ) of tumors but does not delineate microscopic (capillary) tumor neovascularity. The vascular network in tumors can be further interrogated using Doppler techniques to indicate the impedance within vessels [7, 8]. This in turn roughly reflects pressure gradients.

Combining morphologic assessment with TVS with color Doppler features has allowed accurate assessments of whether a mass is benign or malignant by following "simple rules" [6]. Using color Doppler techniques, the overall vascularity was classified as high, low, or intermediate, rather than determining vascular indices such as resistance or pulsatility. In a European multicentered study, it was shown that this paradigm resulted in 90% sensitivity and 92% sensitivity [6].

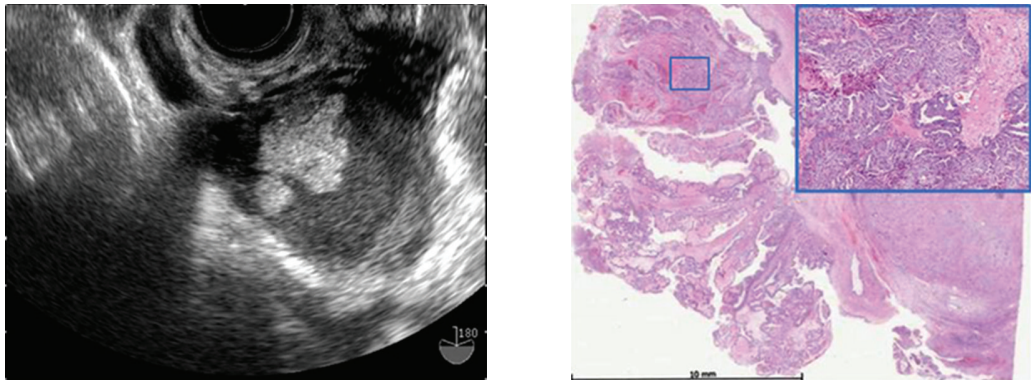
1.3. Contrast-Enhanced Transvaginal Sonography (CE-TVS). Both micro- and macroscopically, tumor neovascularity is characterized as vessels that demonstrate irregular caliber and branching. TV-CDS can only detect flow in relatively large vessels. Microvascular (i.e., capillary) tumor neovascularity can be depicted using microbubble contrast (Figures 4, 5, and 6). On dynamic CE-TVS malignant tumor neovascularity usually demonstrates a higher peak



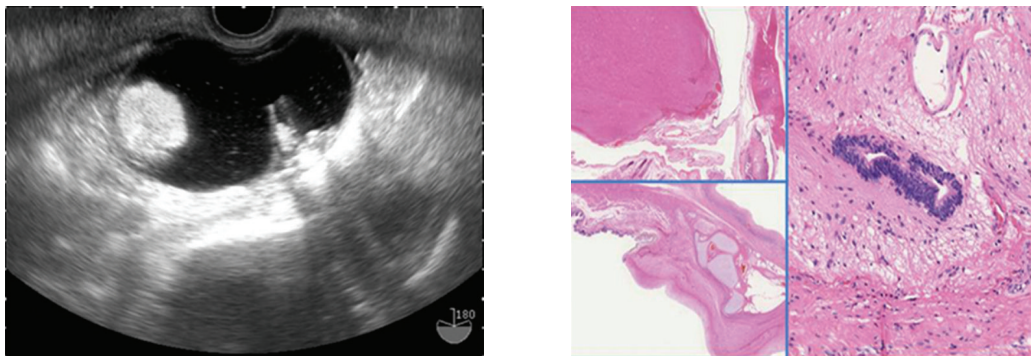
(a) Irregular solid area in a mucinous cystadenocarcinoma



(b) Papillary excrescences in a clear cell carcinoma

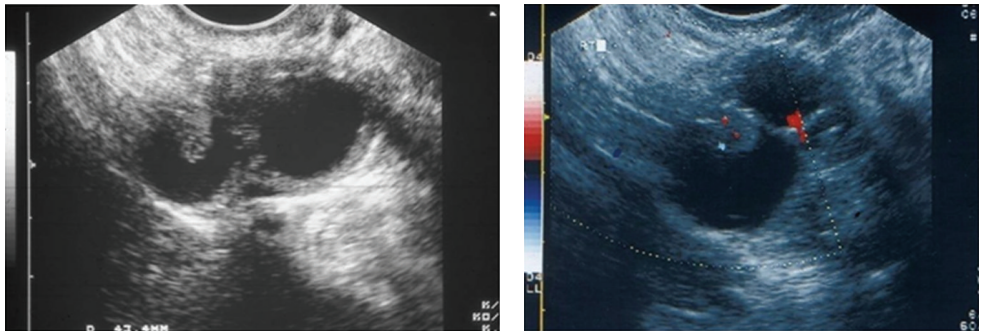


(c) Mural nodules in an endometrioid cancer

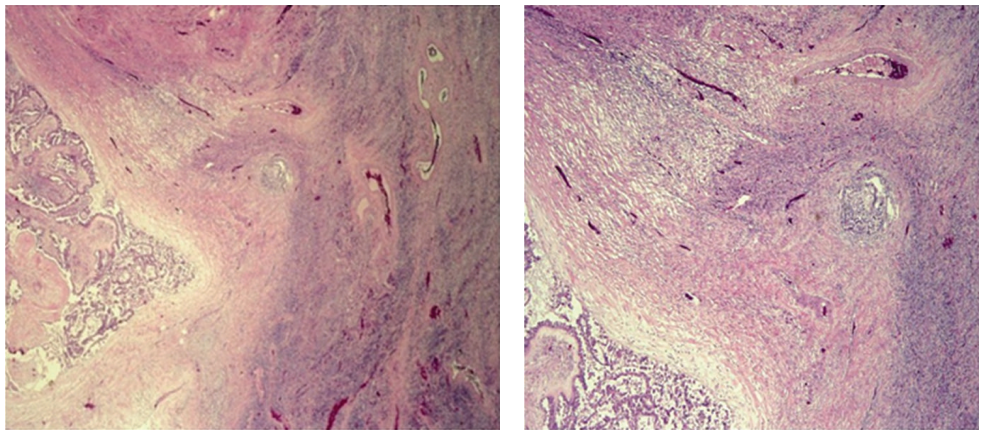


(d) Irregular septa and echogenic solid foci within immature teratoma

Figure 1: Morphologic signs of malignancy with histopathologic correlation on TVS in various histologic types of stage 1A ovarian cancer.

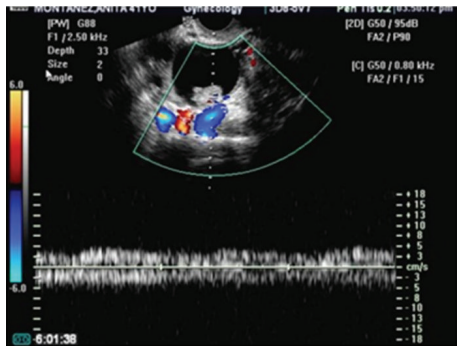


(a) (L) TVS of bilobed cystic ovarian mass containing a papillary excrescence in one locule. (R) CDS showing flow within papillary excrescence

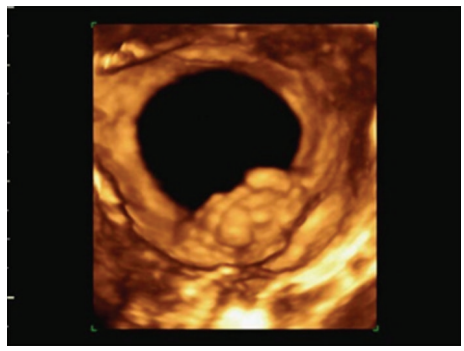


(b) Photomicrographs of histology showing vessels in (L) low power, (R) high power

Figure 2: 2D CDS of showing flow within papillary excrescence within a papillary cystadenofibroma.



(a) 2D TV-CDS of papillary cystadenoma showing low-impedance flow within a papillary excrescence



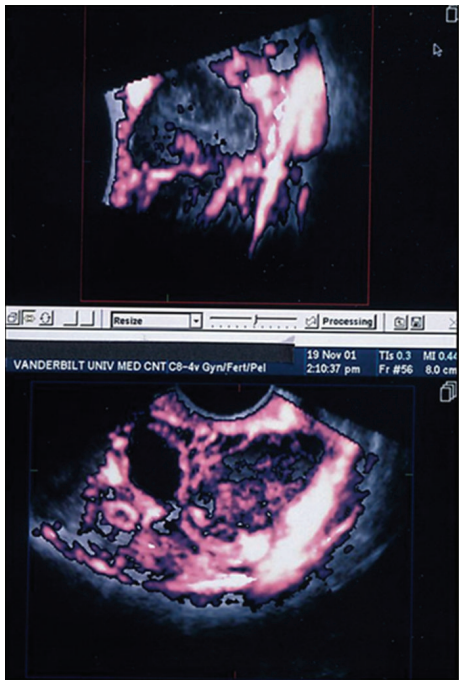
(b) 3D TVS (surface rendition) showing papillary excrescences

Figure 3: 3D TV-CDS of papillary excrescences within a papillary serous cystadenoma.

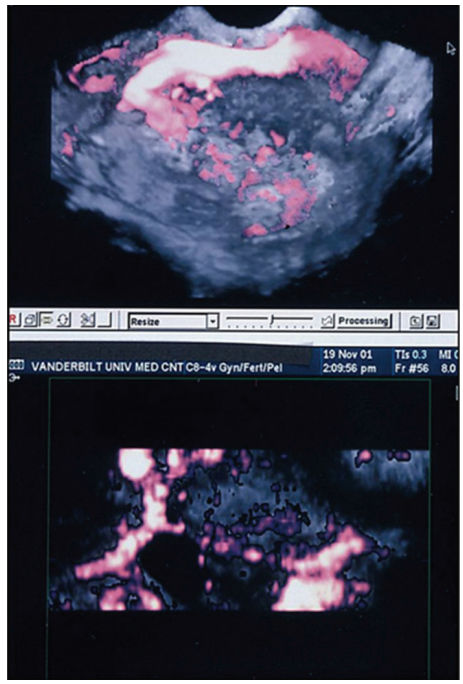
of contrast enhancement and prolonged contrast washout when compared to benign tumors [1, 9, 14, 15] (Figure 9).

In our previous study, all malignant tumors and 50% of benign tumors showed detectable contrast enhancement (image intensity > 10% above the baseline) after microbubble injection [9]. When contrast enhancement dynamics were assessed, we found that malignant lesions had a similar time to peak (T_p; 26.2 ± 5.9 versus 29.8 ± 13.4 seconds; $P = .4$), greater peak enhancement (PE; 21.3 ± 4.7 versus 8.3 ± 5.7 dB; $P < .001$), a longer half wash-out time ($((1/2)T_{wo}$; 104.2 ± 48.1 versus 32.2 ± 18.9 seconds; $P < .001$), and a greater area under the curve (AUC; 1807.2 ± 588.3 versus 413.8 ± 294.8 seconds^{-1}; $P < .001$) when compared with enhancing benign lesions (Figure 9).

AUC greater than 787 seconds^{-1} was the most accurate diagnostic criterion for ovarian cancer, with 100.0% sensitivity and 96.2% specificity. Additionally, PE greater than 17.2 dB (90.0% sensitivity and 98.3% specificity) and

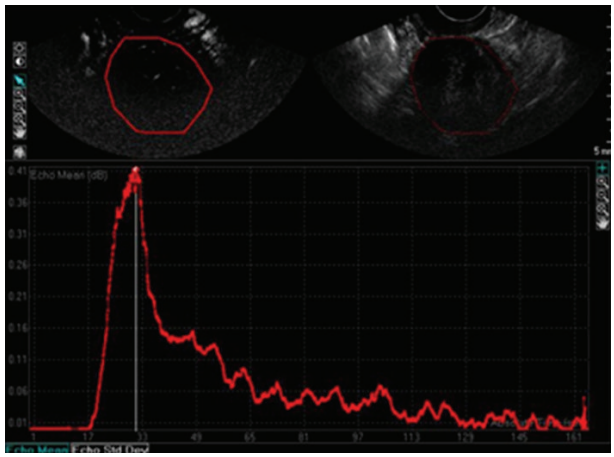


(a) Top: long axis showing central flow with an irregularly shaped solid adnexal mass. Bottom: same as top in short axis

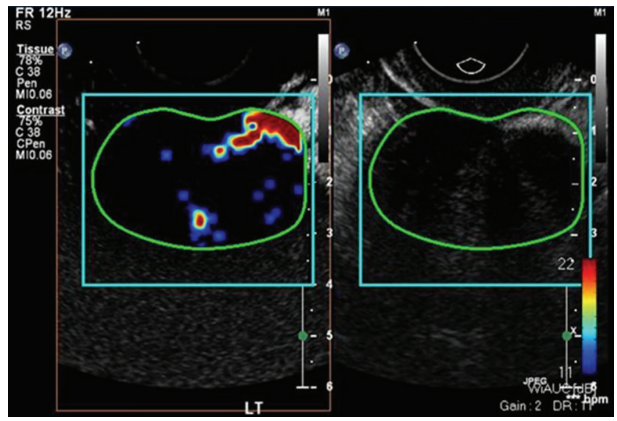


(b) Top: 3D TV-CDS showing (combined volume redition) cluster of vessels within morphologically abnormal area. Bottom: coronal

Figure 4: 3D TV-CDS of papillary cystadenocarcinoma showing multiplanar reconstruction (MPR) images.



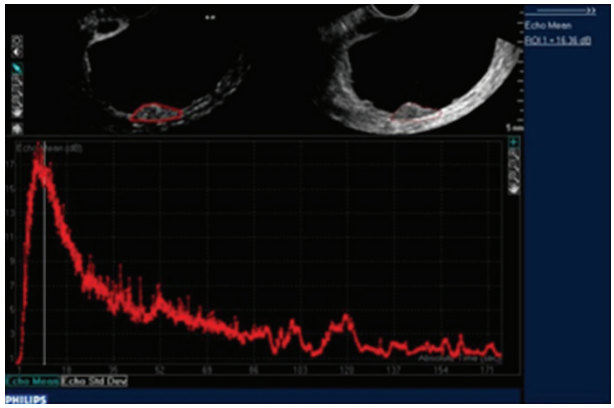
(a) Solid mass with no internal flow with fundamental (top right of image) and harmonic (top left) images. Time intensity curve shows relatively high peak enhancement and short wash-out time



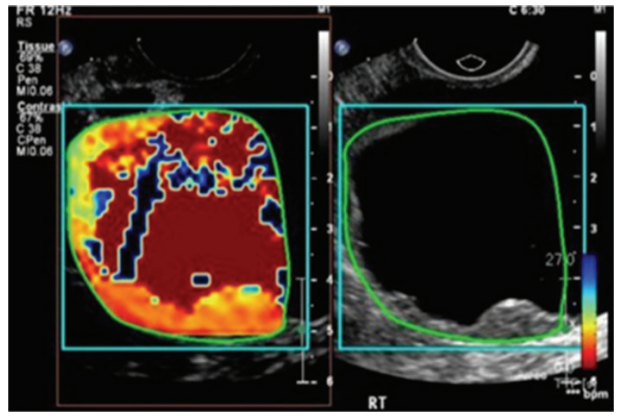
(b) Parametric images showing little internal flow

Figure 5: CE-TVS of a benign fibroma.

a $(1/2)T_{wo}$ of greater than 41.0 seconds (100.0% sensitivity and 92.3% specificity) proved to be useful. Initial analysis of contrast-enhanced kinetic was done using time intensity curves for mean, standard deviation, and P value (Figures 9 and 11) and subsequently by receive operator characteristic curves for vascular index (VI); flow index (FI); vascular flow index (VFI) (Figure 12). The receiver operator characteristics of each parameter is shown in Figure 13 with predetermined cutoff values as established with receive operation curves and compared to VI, FI, and VFI values. These results show that contrast-enhanced nonlinear pulse inversion sonography is a more appropriate method for characterizing blood flow dynamics in ovarian tumors than by TV-CDS and can provide an important tool to aid differential diagnoses between benign and malignant ovarian tumors.

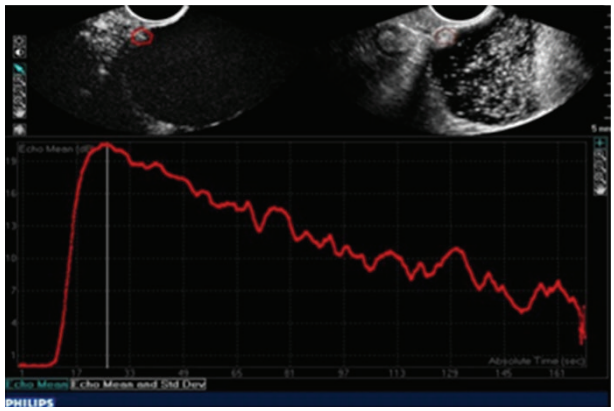


(a) (R) Fundamental and (L) harmonic image showing mural nodule. There is quick wash-in and wash-out within the mural nodule indicating benignancy

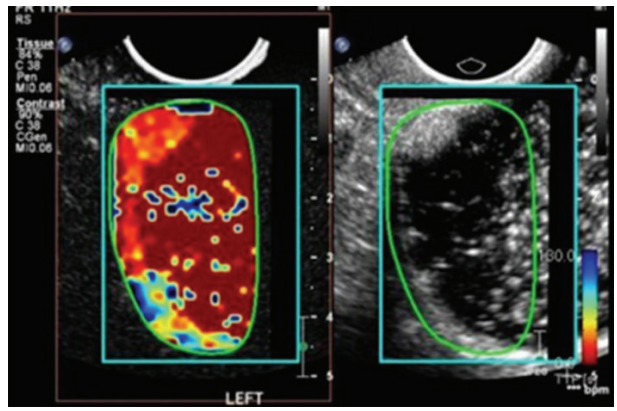


(b) Parametric image showing different time of arrivals within mural nodule, wall, and septum

Figure 6: CE-TVS of serous cystadenoma with mural nodules.

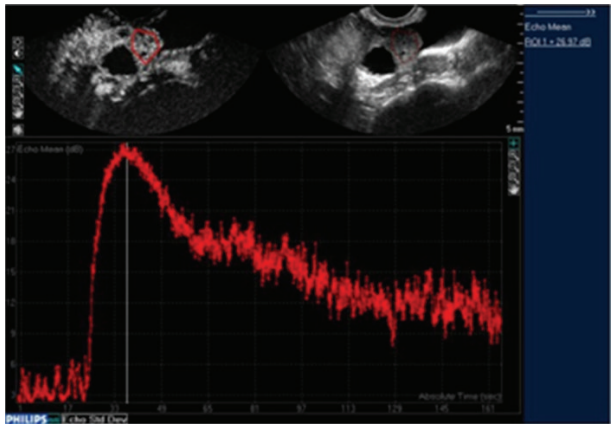


(a) (R) Fundamental image showing mural nodule and mobile echogenic material (L), same using harmonic imaging. There is quick wash-in and long washout within the mural nodule

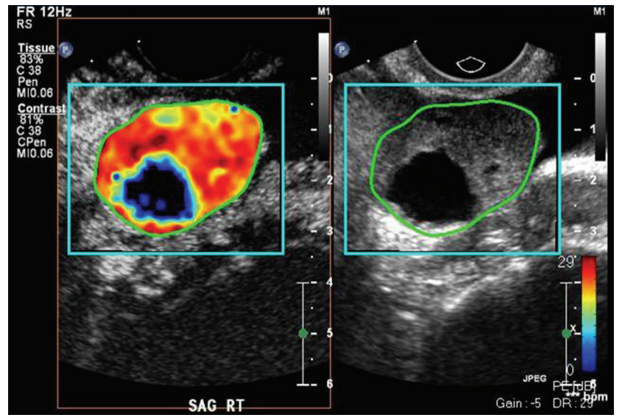


(b) Parametric image different time of arrival for mural module versus wall

Figure 7: CE-TVS of borderline mucinous (intestinal) cystadenocarcinoma.



(a) (R) TVS shows normal sized ovary with small cystic area. (L) CDS shows marked vascularity within ovary. Time-intensity curve shows high peak intensity and long washout



(b) Parametric image showing diffuse vascularity

Figure 8: CE-TVS of stage I papillary serous cystadenocarcinoma.

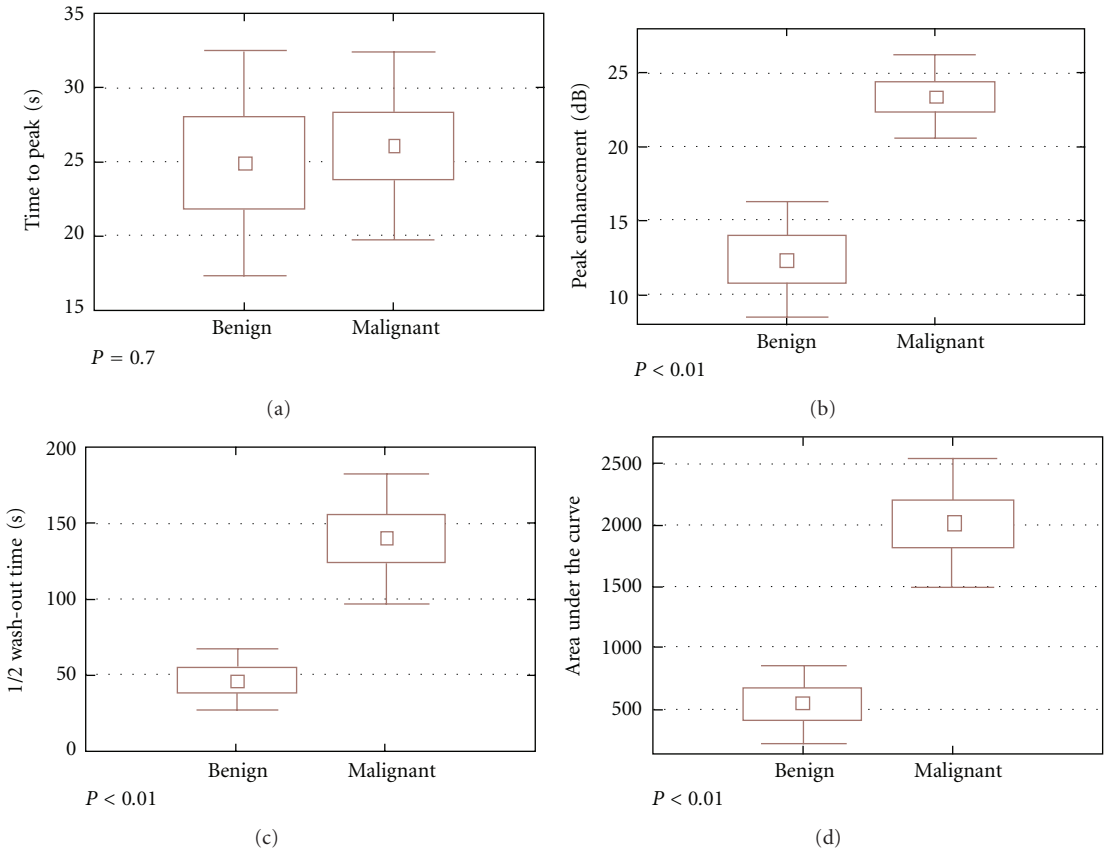


Figure 9: Box graph of contrast-enhanced parameters. While there is no difference in time of peak (T wash-in), there are significant differences in peak enhancement, wash-out time and vascularity ((b), (c); (d)) from [1].

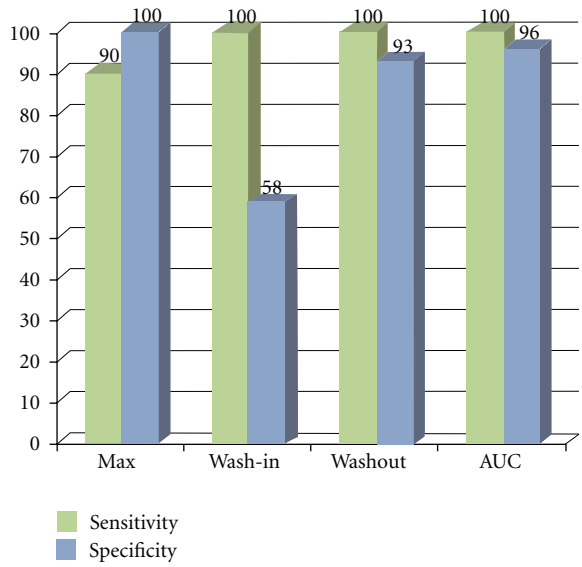


Figure 10: Sensitivities and specificities of maximum enhancement, wash-in, wash-out and area under curve (AUC). Maximum enhancement, wash-out and AUC had greatest accuracy.

1.4. Parametric Mapping of CE-TVS. While the time-intensity curve has traditionally been calculated from mean signal intensities over a region of interest, parametric mapping of time-intensity curve variables on a pixel-by-pixel basis allows for more global visualization of tumor hemodynamics. The use of this technique in ovarian cancer has been limited to selecting the pixel with greatest peak enhancement (PE) and using that pixel's time-intensity curve for further analysis [15]. However, parametric maps of CE-TVS have recently been used with limited success for differentiation of benign and malignant focal liver lesions [16] and breast lesions [17, 18]. Preliminary results for using parametric mapping of ovarian tumors seem to indicate significant potential for improving diagnostic accuracy.

Our preliminary results from a subset of 29 out of the 57 subjects analyzed in our previous region of interest (ROI) study show potential for this technique to differentiate benign and malignant ovarian masses [19]. The methods of data acquisition are outlined in the previously described study [9]. Analysis with a quantification software prototype (Bracco Suisse SA, Geneva, Switzerland) utilized parametric maps of T_p (sec), PE (dB), and wash-in AUC (wiAUC; arbitrary units, a.u.). The region of interest was kept constant in size between subjects and was corrected for motion. The map color scales were adjusted such that abnormal hemodynamics were represented by red for PE > 24 a.u, $T_p < 11\,s$, and wiAUC > 35 a.u. (cutoffs chosen at optimum points on receiver operator characteristics curve), and the presence of any red color was used to differentiate benign and malignant tumors.

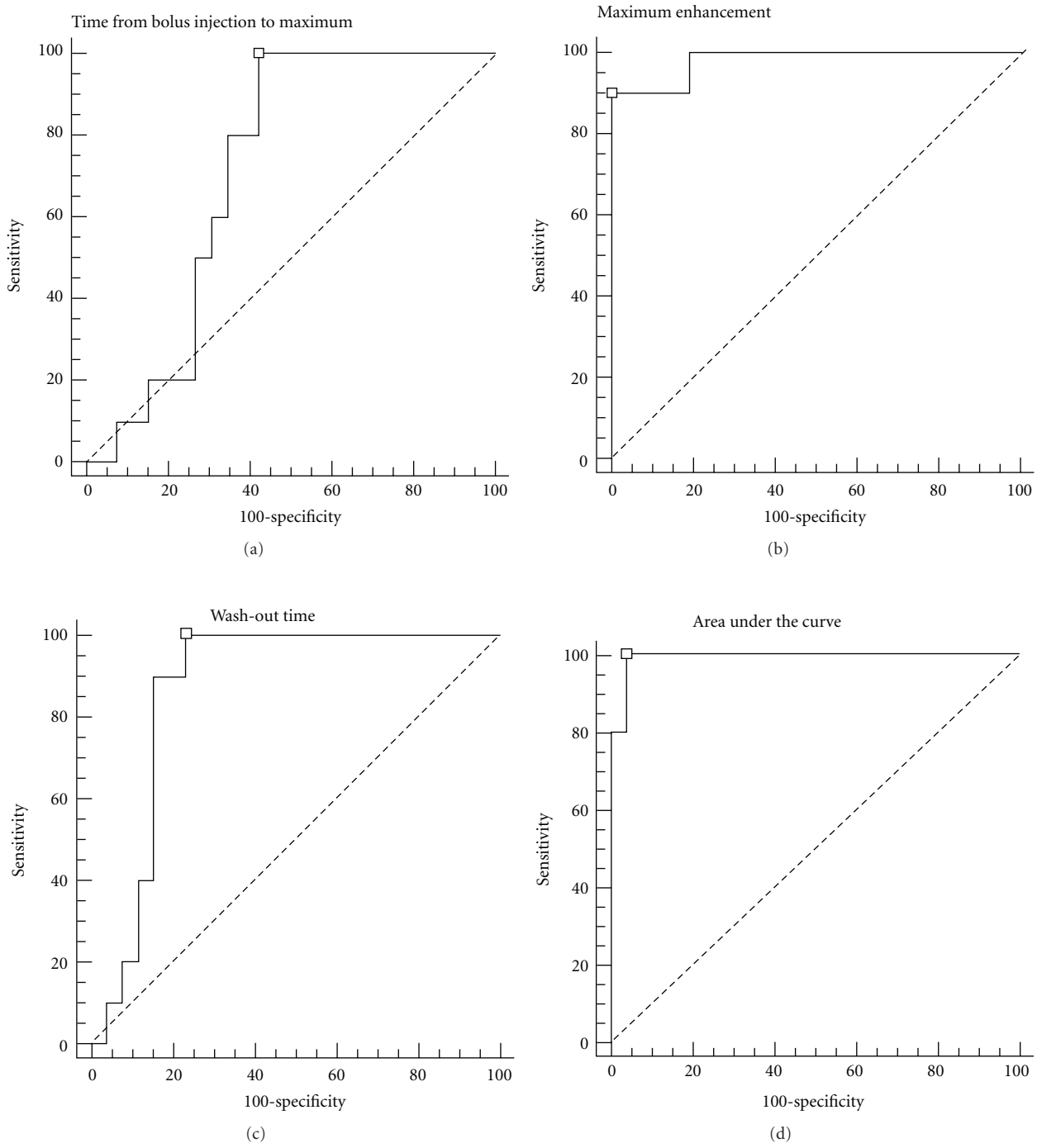


Figure 11: Receiver operator characteristerics for (a) wash-in, (b) maximum enhancement, (c) wash-out, and (d) area under curve.

The preliminary results from the subanalysis of 18 benign and 11 histologically proven malignant ovarian masses showed greatest diagnostic accuracy for maps of PE (sensitivity 100%, specificity 67%) and wiAUC (sensitivity 73%, specificity 94%), while maps of T_p were least accurate (sensitivity 100%, specificity 17%). Final analysis of all 57 subjects is needed to determine the ultimate utility of these methods, but preliminary results are promising.

2. Discussion

CE-TVS can significantly improve the diagnostic ability of transvaginal sonography alone to identify early microvascular changes that are known to be associated with early-stage ovarian cancer [1, 9, 14, 15, 20]. Currently, contrast agents play a pivotal role in the imaging modalities of CT and MRI by increasing lesion conspicuity, accentuating morphologic

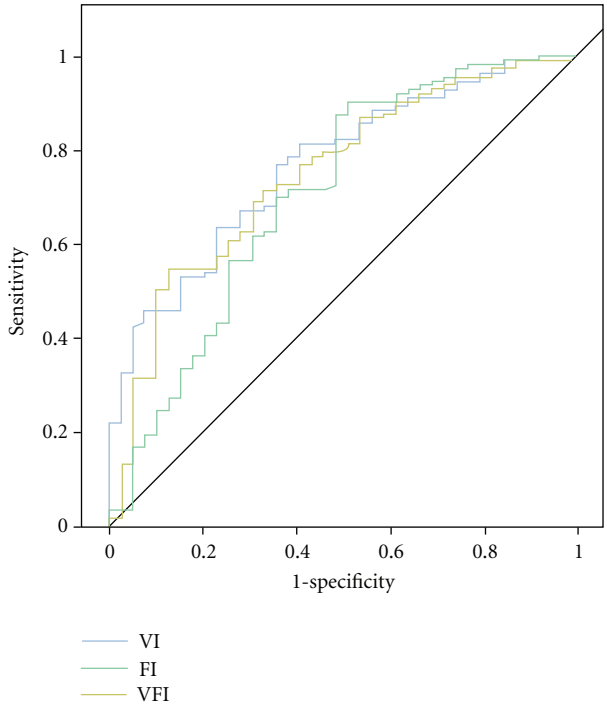


Figure 12: Receiver operator characteristic of various parameters showing cutoff points for vascular index (VI), flow index (FI), and vascular flow index (VFI) (from [2]).

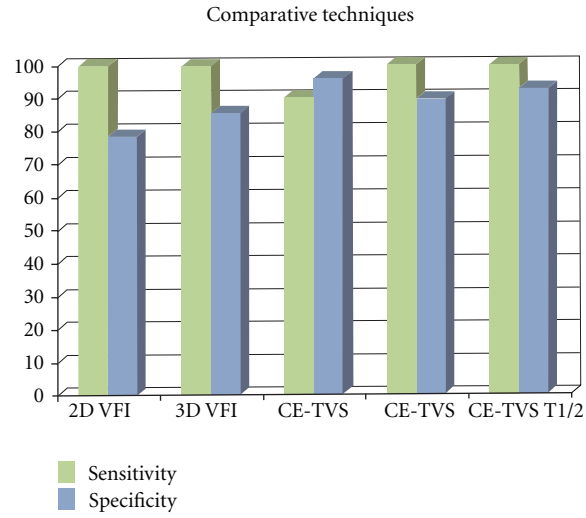


Figure 13: Relative accuracy (sensitivity and specificity of enhancement kinetic parameters) of various techniques using predetermined cutoff points of: 2D VFI (>0.4), 3D VFI (>0.5), CE-TVS (max >17.2 dB), CE-TVS ($(1/2)T_{wo}$ > 41 sec), CE-TVS (AUC > 787 s^{-1}).

features within a lesion, and defining time-resolved lesion enhancement patterns that serve as additional imaging parameters by which a lesion may be characterized. Indeed, contrast agents have received such widespread acceptance that a CT exam performed without intravenous contrast or an MRI without contrast for many indications is now considered limited. Preclinical studies demonstrated that the intravenous contrast agents for sonography hold great promise in a multitude of potential clinical applications, especially in identifying aberrant vascular changes associated with malignancy [19, 21].

Previous studies have addressed the use of CE-TVS for benign and malignant tumors by showing greater enhancement of malignant tumors on Doppler imaging. According to the initial work reported by Kupesic and Kurjak, the use of a contrast agent with 3D power Doppler sonography showed very high diagnostic efficiency (95.6%) that was superior to that of nonenhanced 3D power Doppler sonography (86.7%) [22]. However, simple documentation of tumor enhancement may not be sufficient because some benign tumors show detectable contrast enhancement. This limitation can be addressed by assessment of the contrast enhancement kinetics. Only a few studies have been published that used kinetic parameters of the contrast agent to compare benign with malignant tumors in the power Doppler mode. Orden et al. demonstrated that after microbubble contrast agent injection, malignant and benign adnexal lesions behave differently in degree, onset, and duration of Doppler US enhancement. Doppler contrast-enhanced parameters in that study had 79–100% sensitivity and 77–92% specificity [23]. Marret et al. reported that wash-out times and AUC were significantly greater in ovarian malignancies than in other benign tumors ($P < .001$), leading to sensitivity estimates between 96% and 100% and specificity estimates between 83 and 98% [14]. They concluded that Doppler contrast-enhanced parameters had slightly higher sensitivity and slightly lower specificity when compared with transvaginal sonographic variables of the resistive index and serum CA-125 levels [15].

Our preliminary clinical studies explored differences in enhancement parameters in benign versus malignant ovarian masses using a new method of CE-TVS termed pulse inversion nonlinear imaging [9]. This method produces more reliable estimates of tumor microvascular perfusion and provides more consistent results compared to Doppler-based contrast-enhanced ultrasound. Our data suggest that, except for the T_p, contrast enhancement parameters are significantly different in benign versus malignant ovarian masses. The T_p probably reflects intrinsic circulation depending on cardiac contraction, blood pressure, and overall vascular tone. Once blood circulates through the tumor, however, differences may reflect the unique branching patterns and vessel morphologic characteristics in the microvascularity of the tumors.

As a general statement, contrast enhancement patterns significantly differ between benign and malignant ovarian masses. The addition of a vascular sonographic contrast agent allows a more complete delineation of the vascular anatomy through enhancement of the signal strength from small vessels (capillaries) and provides an entirely new opportunity to time the transit of an injected bolus. CE-TVS has higher sensitivity and specificity to differentiate between benign and malignant lesions than conventional TVS and for detecting occult stage I disease.

2.1. Future Improvements in CE-TVS. CE-TVS can detect tumor neovascularity (Figures 4, 5, 6, 7, and 8). Tumor neovascularity is characterized by vessels with abnormal endothelial structure that are irregular in caliber and branching patterns. In order to recognize these features, contrast enhancement kinetics show relatively high vascular volume (AUC) and PE. These parameters seem to be best depicted using time-intensity curves. These parameters may also be shown in a parametric map, which allows for stricter cutoff criterion than ROI analysis, as peak values are visualized on a much finer scale than typical ROIs. The traditional approach of calculating the time-intensity curve over ROIs chosen on morphology alone allows small areas of neovascularity to be missed, as they are averaged over larger heterogeneous areas. The primary utility of parametric mapping of ovarian tumors may be guiding selection of ROIs to the area of greatest malignant potential.

With improved imaging technology comes the potential for enhanced therapeutic measures. Specifically, this includes directed therapeutic measures after labeled microbubbles are used [2]. In a murine model this method has been shown to accurately detect sites of active angiogenesis [24]. It is possible that labeled microbubbles could provide directed therapy.

In conclusion, it is hoped that this paper may contribute to the development of new methods for diagnosing, enhancing therapy, and detecting tumor response for this dreaded gynecologic malignancy, possibly with the use of targeted microbubbles [2, 24–28].

Acknowledgment

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Review Article

GRP78 Protein Expression in Ovarian Cancer Patients and Perspectives for a Drug-Targeting Approach

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Glucose-regulated protein of 78 kD (GRP78) is a chaperone protein mainly located in the endoplasmic reticulum (ER). This protein is normally present at low levels in adult cells but its expression is triggered by ER stress including glucose deprivation and hypoxia. In tumor cells, it is overexpressed with fraction of protein found at the cell surface. This paper presents the physiology of GRP78 in the context of ovarian cancer and its potential use as drug delivery systems targeting ovarian cancer cell.

1. Introduction

Glucose-regulated protein 78 (GRP78) is an endoplasmic reticulum (ER) chaperone protein belonging to the heat shock protein 70 family. It consists of two functional domains, a 44 kDa N-terminal ATPase and a 20 kDa C-terminal polypeptide-binding domain, and a variable 10 kDa C-terminal tail of unknown function.

This protein, as other members of this family, plays an essential role in protein biosynthesis (for review, see [1]). It facilitates folding and assembly of newly synthesized proteins and prevents intra- or intermolecular aggregation during stress conditions [2, 3]. GRP78 expression is induced by a variety of environmental and physiological stress conditions leading to impairment of essential ER functions and homeostasis in order to protect organs and tissues against apoptosis [4]. Its expression also varies with developmental stages and tissue specificity. A low basal level is identified in most adult tissues whereas it is highly induced in cancer [5, 6]. GRP78 expression is induced under such conditions as hypoxia and nutrient deprivation, partially explaining its high level in tumour cells [7].

GRP78 generally resides inside the ER lumen. However, GRP78 is also found at the cell surface in a wide variety of cancer cells, including neuroblastoma, lung adenocarcinoma, colon adenocarcinoma, ovarian tumour cells [8], prostate cancer [9], proliferating endothelial cells, and, more generally, stressed tumour cells [10]. It is still unknown how GRP78 localizes to the various cellular compartments, and its physiological role at the cell surface membrane is still not fully understood. A hypothesis is that upon GRP78 overexpression, it escapes to ER retention and reaches cell surface. Some proteins are involved in GRP78 relocation, as MTJ-1 and Par-4 [11, 12]. Through its binding to other proteins at the cell surface, GRP78 mediates cell-signalling pathways. For example, cell surface GRP78 acts as a receptor for alpha-2-macroglobulin, leading to activation of PAK-2, to induction of cell motility [12, 13], and to activation of MAPK and PI3K pathways which promote proliferation and survival in a variety of tumours [14, 15]. Other proteins have been identified as partners of cell surface GRP78 such as Cripto I [16], angiogenesis inhibitor plasminogen kringle 5 [17], Par-4 [18], or MHC-I molecule [19].

2. GRP78 and Its Role in Cancer

In a variety of cancer cells and solid tumours (breast, lung, prostate and ovarian cancers, melanoma, and glioma cells),

the level of GRP78 expression is highly induced and could be essential for the survival of stressed cells such as cancer cells. Its expression correlates with malignancy, metastasis development, and drug resistance [9, 20–24]. It was shown that knockdown of GRP78 inhibits tumour cell invasion *in vitro* as well as tumour growth and metastasis aggressiveness in xenograft models [25, 26], suggesting an important role of GRP78 in cancer progression. However, the mechanism whereby GRP78 promotes growth and metastasis is just emerging. The presence of GRP78 at the cell surface of highly metastatic cancer cells tends to suggest that it might mediate signal transduction pathways inducing proliferation and invasion [14].

In xenograft models treated with antivascular and anti-angiogenic agents, GRP78 induction is most important in tumour cells bordering necrotic regions induced by the treatment [5]. Chemoresistance of various cancer cells correlates with GRP78 expression and apoptosis inhibition [26–28]. This could be due to the fact that GRP78 can interact and inhibit the activation of apoptosis pathway components as described with caspase-7 [10] or p53 [29]. It can also bind to and inhibit the activation of BIK, BAX, and prevent cytochrome c release from mitochondria [30–32]. Furthermore, GRP78 forms a complex with other proteins and may indirectly decrease the activity of proapoptotic components.

It was recently found that GRP78 could play another important role in cancer progression in regulating VEGF-induced endothelial cell proliferation through the VEGF-MAPK signal cascade [33].

3. GRP78 Autoantibodies

GRP78 is overexpressed and relocated at cell surface of various cancer cells. It represents a potent biomarker of cell invasion, but its level may be too low to be detected in serum of women diagnosed with cancer. Mintz et al. have demonstrated the presence of GRP78 autoantibodies in patients with prostate cancer and suggested that GRP78 could act as a target of antibodies in these patients [9]. A strong and specific positive correlation was observed between serum reactivity to GRP78, development of metastatic androgen-independent disease, and shorter overall survival. Moreover, these antibodies do not seem to be increased in serum of patients with lung, breast, and ovarian cancer compared to control, suggesting specificity towards prostate cancer [9]. However, GRP78 autoantibodies were also identified in sera of mice bearing lung tumour as a model, and the titer was associated with the detection of primary tumour and metastases earlier than clinical identification. These observations suggest their potential utility in cancer detection and prognosis [34]. GRP78 autoantibodies were detected in serum of patients with gastric cancer, melanoma, and ovarian cancer but it is not clear if their level increases with stage of disease [35–38].

Circulating autoantibodies against GRP78 purified from prostate cancer patients were able to bind to GRP78 expressed at the surface of tumour cells, to the same site as the one recognized by its physiological agonist, the alpha2-macroglobulin [39]. This binding promoted proliferation of prostate cancer lines. Moreover, these antibodies protected cells from apoptosis induced by tumour necrosis factor alpha [39], suggesting that they could facilitate the emergence of more aggressive prostate cancer cell phenotype. In contrast, commercial antibodies against the C-terminus of GRP78 could act as antagonists and inhibit cellular proliferation and promote apoptosis [15, 40]. This contradicting observation suggests that the role of anti-GRP78 antibodies in tumour development may be dependent on the nature of the GRP78 epitope recognized by the antibodies. Autoantibodies against GRP78 isolated from serum of melanoma patients were also found to promote tumour growth [41]. This activity could depend on glycosylation of antibodies [36].

4. GRP78 in Ovarian Cancer

Epithelial ovarian cancer (EOC) accounts for the vast majority of ovarian malignancies. EOC survival rate is dependent on disease stage at the time of diagnosis. When diagnosed at stage III or more advanced stage, the 5-year mortality rate is close to 70% [42]. Due to the lack of specific symptoms and reliable ovarian cancer biomarkers, 75% of EOC patients have advanced stage disease at presentation, making EOC the most lethal gynecologic malignancy. The excellent survival rates for women with early stage disease provide a strong rationale to support research effort in developing strategies to identify the disease before it spreads outside the pelvis. Currently, it remains a big challenge.

Recently, researches have moved away from cytotoxic drugs to new targeted therapies, such as vascular endothelial growth factor (VEGF) inhibition and other agents that inhibit angiogenesis or cell-signaling pathways. Targeted therapy is now coming to the forefront of research and clinical trials in order to overcome resistance to cytotoxic drugs. The most promising at this time are angiogenesis inhibitors. Deeper understanding of cell-signaling pathways in ovarian cancer is needed to develop innovative strategies to improve outcome of EOC patients.

Despite the great interest of GRP78 in cancer development and progression, few data is available on GRP78 and ovarian cancer. First evidence of GRP78 as antigen associated with ovarian cancer was brought in 1997 by the detection of humoral immune response to GRP78 in ovarian cancer patients [43]. This presence is certainly associated to the expression of membrane GRP78 in ovarian cancer cells [8, 44]. Sera from ovarian cancer patients failed to recognize GRP78 on normal ovarian tissue suggesting that this antigen is unique to cancer [43]. However, Mintz et al. described the presence of GRP78 autoantibodies in serum of control female and the lack of difference between the level in ovarian cancer and control patients [9]. The same observation was recently reported by Lu et al. [44]. The level of GRP78 autoantibodies remains controversial since it was recently suggested that GRP78 autoantibodies increased with ovarian cancer stage [37] whereas Cohen and Petignat described the opposite [35]. There might be a variety of reasons why results are different, such as the methods of GRP78 autoantibodies detection (ELISA or immunoblot) or different sample size.

If some authors focused on GRP78 autoantibodies as prognostic marker of ovarian cancer, none reported a possible correlation between GRP78 level in ovarian cancer tissue and disease stage and chemotherapy resistance as suggested in lung and breast cancer, melanoma, and glioma cells [27, 28, 45–47].

5. GRP78 as a Recognition Element for Drug Targeting in Ovarian Cancer

Targeted therapy consists in the design and application of drugs specifically directed against well-defined targets that are critical for tumor survival and not compromising for normal organs and tissues. It may also involve the recognition of a molecular entity specific to the organ or cells of interest. This strategy takes all its sense when cancer mass is spread as micrometastasis as encountered in ovarian cancer. Specific localisation to the cancer cells will moreover provide safer therapy by reducing the high toxicity of chemotherapeutic drugs and the adverse effects related to the unfavourable biodistribution to both cancerous and healthy tissues. Targeted therapy is usually mediated via a ligand specific to a molecular target overexpressed or ideally exclusively expressed at the cells of interest.

GRP78 represents a very interesting target to be associated with drug delivery systems, as it is specifically expressed at cancer cell membranes.

Arap et al. designed two ligand peptides to specifically bind to the GRP78 expressed at cell surface [48]. After intravenous administration, these peptides were shown to be able to target prostate or breast cancer cells implanted in mice models whereas they were not detected in healthy tissues. The ligand peptides were linked to a proapoptotic peptide before systemic administration to tumour-bearing mice weekly for 4 weeks. Tumour volume was significantly smaller in chimera-treated groups compared to animals receiving either the vehicle or unconjugated mixture of the ligand peptides with the proapoptotic peptide.

In another study, GRP78 was identified as the receptor for the best candidate of a cohort of cyclic peptides screened for internalisation in melanoma cell lines, the "Pep42" [49]. After combination with quantum dots, cellular uptake and ER localisation of the conjugate were observed *in vitro*. Furthermore, direct combination of the cyclic peptide with taxol leads to an increased apoptosis rate *in vitro* in melanoma cells compared to free taxol. A control construct where taxol was conjugated to a linear peptide analogue had a weaker effect compared to taxol alone. The ability of Pep42 to selectively bind to human cancer cells was further tested with melanoma cells (Me6652/4), lung adenocarcinoma cells (A549), osteosarcoma cells (SJSA-1), hepatoma cells (HepG2), and two normal fibroblast cell lines [50]. The peptide bound to cancerous cells whereas only limited recognition was observed with normal cells. Selective apoptosis was induced in cancer cells and not in normal cells when Pep42 was linked to a proapoptotic peptide. Pep42 conjugated with quantum dots were administered to tumour-bearing animals. The conjugates concentrated in the tumour tissue without accumulation in other organs, demonstrating the specificity of the targeting.

These data support that cancer cells expressing the GRP78 at their surface are more sensitive to cytotoxic drugs when these are conjugated to recognition elements targeting the GRP78. Direct ligation of a ligand to a drug molecule is a common mean to achieve targeting. However, it needs chemical modification of the active compound and the further release of the drug that may compromise the pharmacological efficiency. Therefore, other approaches involving synthetic drug carriers have been developed. The drug is encapsulated or associated with a polymer or lipid core that is decorated at its surface with a recognition moiety.

Pegylated liposomes were surface-modified by Katanasaka et al. [33] with the peptide developed by Arap et al. [48]. It was shown *in vitro* that the liposomes were able to target VEGF-activated HUVEC cells as well as DU145, a prostate cancer cell line [48]. Biodistribution studies in mice demonstrated a preferable localisation of the targeted liposomes to the vasculature and the tumour tissue with no accumulation in normal tissues except for the spleen. Furthermore, the targeted liposomes were loaded with doxorubicin. In the dorsal air pouch model in mice implanted with C26 tumours, these liposomes suppressed the tumour-induced vascularisation angiogenesis whereas untargeted liposomes were not as efficient. Survival rate was also significantly increased in mice treated with targeted liposomes compared to sucrose solution or untargeted liposomes. This study shows the interest of using GRP78 as a target element to bring large amount of drug to a tumour via colloidal carriers. It also shows that GRP78 can be a target for cancer antineovascularisation therapy.

A more recent study reports the development of paclitaxel-loaded polyester nanoparticles conjugated with a GRP78-recognising peptide [51]. This approach proved to increase paclitaxel concentration and apoptosis in irradiated breast carcinoma in mice for up to 3 weeks. No significant tumour growth delay was observed when free paclitaxel or untargeted nanoparticles were used after irradiation compared to irradiation alone.

These studies strengthen the significance of using GRP78 as a targeting moiety for the development of more efficient anticancer treatments. So far, to our knowledge, only peptides have been used as recognising entity, and no studies on ovarian cancer has been reported. Antibodies against GRP78 may be used for their targeting capacities; however, they can also be of advantage as antitumoral agent as was demonstrated by Cohen and Petignat [35]. As proof of principle, we recently combined paclitaxel-loaded nanoparticles with anti-KDEL (NPs-Tx-KDEL) antibodies (unpublished data). Briefly, the results showed that antibody-coated particles presented a higher binding to the cells even though the internalization rate appeared limited. Moreover, despite the fact that KDEL antibodies exhibit unforeseen antiapoptotic properties (in the opposite to purified GRP78 autoantibodies), NPs-Tx-KDEL significantly increased sensitivity of Bg-1, an ovarian cancer cell line, to the drug compared to other treatments (free paclitaxel, unloaded carrier, or untargeted nanoparticles).

In summary, GRP78 appears of great interest as prognostic marker and therapeutic target for various types of cancer. Exciting data have been gathered regarding its role in the development of cancer, and a few studies have shown interesting prospective in using this antigen as a receptor for targeted therapy. Nevertheless, very few studies investigated GRP78 and anti-GRP78 autoantibodies in ovarian cancer. If potentiality for this protein has been suggested, no data is so far available to show its interest as marker or therapeutic target of ovarian cancer. Development of targeted drug delivery systems offers the possibility to deliver a high concentration of chemotherapeutic agents to the right place. Furthermore, if chosen wisely, the antibodies against the GRP78 may have anticancer activity by themselves.

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Review Article

Special Agents Hunting Down Women Silent Killer: The Emerging Role of the p38α Kinase

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Ovarian cancer is sensitive to chemotherapy with platinum compounds; however, the therapy success rate is significantly lowered by a high incidence of recurrence and by the acquisition of drug resistance. These negative outcomes mainly depend on altered apoptotic and drug resistance pathways, determining the need for the design of new therapeutic strategies to improve patient survival. This challenge has become even more critical because it has been recognized that hindering uncontrolled cell growth is not sufficient as the only curative approach. In fact, while current therapies are mostly conceived to impair survival of highly proliferating cells, several lines of research are now focusing on cancer-specific features to specifically target malignant cells with the aim of avoiding drug resistance and reducing adverse effects. Recently, great interest has been generated by the identification of metabolic reprogramming mechanisms occurring in cancer cells, such as the increase in glycolysis levels. In this light, pharmacologic manipulation of relevant pathways involved in cancer-specific metabolism and drug resistance could prove an effective approach to treat ovarian cancer patients.

1. Introduction

Ovarian cancer has historically been called the "silent killer," even if around 80% of patients do actually have symptoms. Indeed, only 20% of ovarian cancers are currently diagnosed while still limited to the ovaries, when up to 90% of patients can be cured using available therapies. Its poor prognosis is related to late diagnosis, which usually occurs at advanced stages, and to acquisition of chemoresistance [1]. To date, more than 30 oncogenes and tumor suppressor genes have been identified that are involved in ovarian oncogenesis inducing modifications in proliferation, apoptosis, anoikis, motility, adhesion, and invasion [2].

2. Genetic Alterations in Ovarian Cancer

Although ovarian cancer risk is, at least in part, influenced by hormonal, environmental, and racial factors, a major role is played by genetic factors. Indeed, a key advance in the study of ovarian cancer etiology has been the identification of mutations in the BRCA genes. BRCA1 and BRCA2 genes act as tumor suppressor genes and, when mutated, are associated with the accumulation of chromosomal abnormalities and thus with a higher risk of developing cancer. Inheritance of mutations in BRCA genes is associated with a 27% to 44% lifetime risk of ovarian cancer. A higher incidence of carcinomas of the ovary has also been detected in families affected by the HNPCC syndrome (hereditary nonpolyposis colorectal cancer) [3], which is caused by mutations in DNA mismatch repair genes. HNPCC carriers account for approximately 1% of ovarian cancer patients, and their estimated lifetime risk of ovarian cancer is 9% to 12% [4].

Mutations in BRAF, KRAS, and erbB2 oncogenes and in the tumor suppressor PTEN have been found in a large subset of ovarian cancers [5, 6]. The inactivation of PTEN and an activating mutation of KRAS are sufficient to induce ovarian endometrioid carcinoma in a mouse model [7]. Furthermore, mutations of beta-catenin have been detected both in ovarian carcinomas and in their precursor lesions [8]. Indeed, inactivation of the Wnt/beta-catenin and

the PI3K/PTEN pathways has been shown to induce the development of endometrioid carcinoma in an engineered mouse model [9]. The small G-protein RAB25, which regulates motility, aggressiveness, apoptosis, and autophagy and mediates survival in response to stress, has also been found upregulated in the majority of ovarian cancers [10].

The Aurora-A kinase (Aurora-A) is associated with tumor initiation and progression and is overexpressed in various malignancies. Inhibition of Aurora-A induces cell cycle arrest and decreases proliferation of epithelial ovarian cancer stem cells, which represent the chemoresistant population and act as a source of recurrence [11]. All of these and several other amplified oncogenes are potential targets for ovarian cancer therapy.

2.1. Chromatin Remodeling and Ovarian Cancer. Molecular genetic changes in chromatin remodeling genes have been identified as a new mechanism in cancer pathogenesis. ARID1A (BAF250a), which promotes the formation of SWI/SNF chromatin remodeling complexes containing BRG1 or BRM, has emerged as a candidate tumor suppressor gene based on its frequent mutations in gynecological cancers. 46%–57% of ovarian clear cell carcinomas, 40% of uterine endometrioid carcinomas, and 30% of ovarian endometrioid carcinomas display somatic sequence mutations in ARID1A [12–14]. Guan and colleagues recently reported that restoring wild-type ARID1A expression in ovarian cancer cells that harbor ARID1A mutations is sufficient to suppress cell proliferation and tumor growth in mice. Moreover, they showed that ARID1A/BRG1 complexes directly interact with p53 and that mutations in the ARID1A and TP53 genes were mutually exclusive in tumor specimens. The regulation of p53-related genes by ARID1A raises the possibility that ARID1A cooperates at the molecular level with p53 to inhibit tumor growth. In non-transformed cells, ARID1A and p53 act as a pair of gatekeepers that prevent tumorigenesis by transcriptional activation of tumor-inhibiting downstream genes, such as CDKN1A and SMAD3. The authors found that all tumors with mutated ARID1A contained wild-type TP53 and tumors with mutated TP53 harbored wild-type ARID1A. Mutations in either ARID1A or TP53 were sufficient to inactivate the ARID1A/BRG1/p53 complex and silence transcription of CDKN1A and SMAD3. This recent study suggests a close collaboration between genetic and epigenetic alterations in cancer pathogenesis [15].

2.2. Imprinting and Ovarian Cancer. Genomic imprinting is a molecular mechanism that plays an important role in development, growth, and cell differentiation in mammals. However, only 74 genes have been identified as imprinted among the over 30,000 that can be expressed in human cells. Several of these imprinted genes have been implicated in human oncogenesis. Indeed, while functional inactivation of non-imprinted genes usually requires two genetic alterations, loss of function of imprinted genes may occur following a single genetic or epigenetic event (including loss of heterozygosity (LOH), hypermethylation, and altered transcriptional regulation) occurring on the single functional allele. Moreover, in the case of ovarian oncogenesis, spontaneous mutations may occur during the proliferation of ovarian epithelium to repair ovulatory defects. In this light, downregulation of the imprinted growth-inhibitory genes Aplasia Ras homologue member I (ARHI) and paternally expressed 3 (PEG3) may be particularly important in the pathogenesis of ovarian cancer [16].

ARHI, also known as DIRAS3, is a maternally imprinted tumor suppressor gene encoding a 26 kDa GTPase with 55%–62% homology to Ras and Rap, which inhibits cancer cell growth, motility, and invasion. It is expressed by ovarian epithelial cells and is lost or markedly downregulated in 60%–70% of ovarian cancers [17–19]. Loss of ARHI expression is associated with tumor progression and poor prognosis, while its re-expression in cancer cells inhibits signaling through the Ras/MAPK pathway, induces p21WAF1/CIP1, and downregulates cyclin D1 [19]. Besides, Lu et al. [16] demonstrated that ARHI re-expression causes autophagic death of ovarian cancer cells in culture and participates directly in autophagosome formation by upregulating the ATG4 enzyme that processes the microtubule-associated protein LC3I to LC3II. Autophagy is a process of "self-eating" that involves enzymatic digestion and recycling of cellular constituents in response to stress. While it can contribute to cancer cell death in response to chemotherapeutic agents [20], its role in oncogenesis remains ambiguous as it may also permit survival of cancer cells in response to environmental stress or cytotoxic drugs [21–23]. Indeed, induction of ARHI in xenografts does not kill ovarian cancer cells but instead induces tumor dormancy [24], and its subsequent downregulation rapidly resumes cancer growth.

PEG3 is an imprinted gene encoding a 140 kD Kruppel-type (C2H2) zinc-finger protein that plays an important role in the p53/c-myc-mediated apoptotic pathway. It is significantly downregulated in the majority of ovarian cancers due to promoter hypermethylation and LOH, and its re-expression markedly inhibits ovarian cancer growth. Of note, a high degree of correlation has been found between ARHI and PEG3 in terms of mRNA levels and promoter methylation [25].

3. Current Therapies and New Therapeutic Targets

The platinum compounds cisplatin and carboplatin are the most effective chemotherapy agents currently used in ovarian cancer. The antitumor activity of cisplatin (cis-diamminedichloroplatinum (II)) was discovered by Rosenberg and colleagues in 1961 [26]. Cisplatin has been the most active drug used for the treatment of ovarian cancer for the last 4 decades, and response to cisplatin is considered a prognostic factor for patients with ovarian cancer [27]. A high percentage of women with ovarian cancer respond to front-line platinum combination chemotherapy, but in most of them the disease will become resistant to cisplatin, ultimately leading to death [27]. Thus, methods of preventing resistance to cisplatin could prove very useful against ovarian cancer.

The classical therapeutic sequence combines maximal debulking surgery followed by adjuvant platinum- and paclitaxel-based chemotherapy [28, 29]. Unfortunately, 20% of patients do not respond to chemotherapy and recurrent disease occur in >50% of those who initially achieve complete remission, with a 5-year overall survival of only 30%–40% for all stages [30].

New therapeutic approaches based on targeted biologic agents have generated great interest and are currently being investigated in several clinical trials focused on treatments for recurrent ovarian cancer (Figure 1). As is the case for other cancers, angiogenesis is a key process implicated in the metastatization of ovarian cancer. Several growth factors, including vascular endothelial growth factor A (VEGFA), lysophosphatidic acid (LPA), interleukin 6 (IL6), interleukin 8 (IL8), and fibroblast growth factor 1 (FGF1) and 2 (FGF2) are involved in this process [31, 32]. To date, agents that target the VEGF pathway have proven the most effective against the disease.

VEGFA activity has been inhibited by various mechanisms. Bevacizumab, a VEGFA-specific antibody, induced an objective response rate in 16% of patients with recurrent ovarian cancer and stabilized disease for 5.5 months in 50% of patients [33], while improved response rates have been observed in platinum-resistant disease when it was used in combination with cytotoxic chemotherapy [34]. The VEGF Trap is based on a different approach [35]: it is a fusion protein that acts as a soluble VEGF receptor and binds with high affinity to VEGF. Several small molecule inhibitors have been used in ovarian cancer to target VEGF and other pathways. Sorafenib, an oral multikinase inhibitor with activity against Raf and other receptor kinases (including the VEGF receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR), and c-Kit) may have antiangiogenic effects through inhibition of VEGFR. This inhibitor has also been used with promising results in combination with bevacizumab and in combination with chemotherapy, both in recurrent disease and as initial therapy in newly diagnosed patients. Sunitinib is an oral agent that inhibits a number of receptor tyrosine kinases implicated in epithelial ovarian cancer (EOC) growth and metastasis, including VEGFR and PDGFR. It has been assessed in phase II studies for the treatment of advanced or metastatic recurrent EOC [36]. Cediranib (AZD2171) is an oral tyrosine kinase inhibitor with selective activity against VEGFR1, VEGFR2, VEGFR3, and c-Kit. Recent clinical trials showed that cediranib has anticancer activity in recurrent EOC [37]. Pazopanib is an oral angiogenesis inhibitor targeting VEGFR, PDGFR, and c-Kit, which is currently being tested in clinical trials on ovarian cancer.

The epidermal growth factor receptor (EGFR) family is commonly overexpressed in ovarian cancer and has been associated with a negative prognosis; however, limited efficacy has been observed with molecules targeted to the EGFR pathway. Gefitinib and erlotinib, which are inhibitors of EGFR, stabilized disease in 11%–44% of patients with ovarian cancer but produced objective regression in only 4%–6% of cases [38, 39]. The effect of EGFR inhibitors might be reduced by activation of the RAS-MAPK signalling pathway, as happens in colorectal cancers [40]. ErbB2 (also known as HER2) expression in ovarian cancer is associated with advanced stage, higher recurrence frequency, shorter survival time, and lower sensitivity to platinum-based chemotherapy. Trastuzumab and pertuzumab are humanized antibodies targeted against HER2, which act through different mechanisms [41, 42]. In phase II monotherapy clinical studies, trastuzumab has shown activity in certain ovarian cancers overexpressing HER2, while pertuzumab is currently undergoing ovarian cancer trials in combination with cytotoxic agents including gemcitabine [43] and carboplatin [44].

The estrogen receptor α (ERα) has also been targeted for the treatment of ovarian cancer. Phase II trials of aromatase inhibitors (AIs) have shown modest response but rather better disease stabilization rates, especially when patients are selected on the basis of ERα expression [45].

Activation of the PI3K pathway, which occurs in approximately 70% of ovarian cancers, is associated with resistance to cytotoxic chemotherapy. Inhibitors of PI3K and Akt prevent the growth of ovarian cancer xenografts and potentiate the cytotoxic effects of paclitaxel and cisplatin [46]. Perifosine is an alkylphospholipid compound that inhibits Akt and is currently being tested in combination with docetaxel. Development of more specific Akt inhibitors is currently underway and PI3K inhibitors are entering phase I-II trials [47].

Overexpression of IL6 has been detected in the majority of ovarian cancers. It induces a signaling pathway that ultimately stimulates proliferation, inhibits apoptosis, and promotes angiogenesis. Antibodies against IL6 and inhibitors of proteins involved in its pathway, such as JAK2 and STAT3, are currently in development for use in ovarian cancer [48].

Upregulation of the LPA receptors LPAR2 and LPAR3 has been described during the malignant transformation of ovarian surface epithelial cells. An approach targeting this pathway in ovarian cancer cells through antibodies capable of neutralizing LPA and through inhibitors of LPA receptors is currently being studied [49].

Constitutive activation of the NFkB transcription factor has been observed in the majority of ovarian cancers [50, 51]. Activated NFKB induces upregulation of anti-apoptotic genes, growth regulatory cytokines (IL6 or growth regulated α (Gro1)), and angiogenic factors (IL8) [52]. A clinical trial is currently underway to study the efficacy of liposomal adenoviral E1A, which interferes with NFKB signaling, in combination with paclitaxel in patients with recurrent ovarian cancer.

The use of poly(ADP-ribose) polymerase (PARP) inhibitors in ovarian cancer is being evaluated in various preclinical, and clinical studies. By interfering with PARP single-strand DNA repair activity, this strategy is aimed at increasing the cytotoxicity associated with DNA damage induced by chemotherapy and takes advantage of the fact that loss of function of BRCA genes, which are also involved in DNA strand breaks repair, is a common feature of this type of cancer [53]. Response to treatment has been observed in 46% of ovarian cancer patients with a BRCA mutation administered with the oral PARP inhibitor AZD2281 (Olaparib) [54]. Moreover, several clinical trials are studying the efficacy of PARP inhibitors in combination with cytotoxic compounds

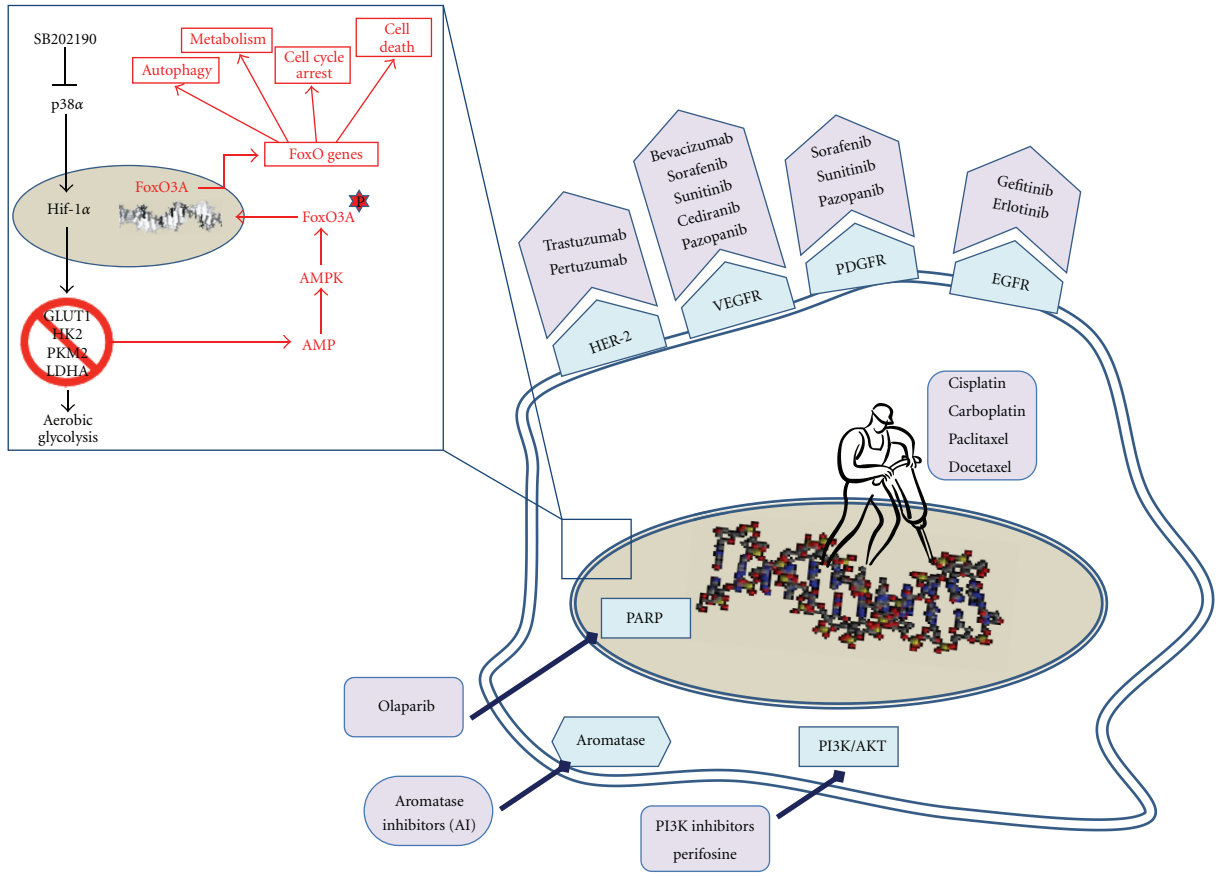

Figure 1

including monofunctional alkylating agents, topoisomerase-I poisons and DNA-crosslinking agents [55].

4. p38α and Ovarian Cancer Cell Survival

The high rate of drug resistance acquisition observed in ovarian cancer patients has led to a recent shift in the design of therapeutic strategies: pathways involved in drug resistance are being investigated in depth in order to identify new putative targets, and the potential to manipulate cancer-specific features is being evaluated with the aim of specifically targeting tumor cells in order to reduce adverse effects (Figure 1). As for this second aspect, major attention has been focused on the metabolic reprogramming occurring in cancer cells, which display increased levels of glycolysis compared with their normal counterparts. Indeed, conventional therapies, such as chemotherapy and radiation, produce heavy adverse effects because they are mainly designed to affect survival of highly proliferating cells and thus also damage healthy tissues characterized by a high cellular turnover. In recent years, the observation made in the 1920s by Nobel Prize winner Otto Warburg that tumor cells produce 50% of their adenosine triphosphate through the glycolytic flux versus the 10% observed in normal cells—the so-called Warburg effect—is being revalorized and is now considered a promising target for new therapeutic approaches [56]. This phenomenon is already successfully exploited for the detection of metastasis of most epithelial tumors by positron emission tomography combined with computed tomography (CT; PET/CT) [57, 58]. The Warburg effect seems to be achieved through stable genetic or epigenetic alterations that promote the constitutive activation of the glycolytic pathway and induce a decrease in mitochondrial oxidative phosphorylation, a phenomenon known as aerobic glycolysis. The transcription factor HIF1α is one of the central players of cancer-specific aerobic glycolysis. Indeed, its stabilization leads to overexpression of target genes involved in key regulation steps of glucose transport, glycolysis, lactate production, and lactate/proton extrusion [59]. Concomitantly, deregulated HIF1α also induces suppression of mitochondrial metabolic pathways, such as oxidative phosphorylation, lipid synthesis, and β-oxidation [60].

The role of HIF1α has been well documented in cancers originating in the ovary. Tumor xenografts obtained from stable HIF1α-silenced ovarian cancer cells show increased cell death and necrosis [61], and the expression levels of HIF1α have been proposed as an independent prognostic factor in patients with epithelial ovarian tumors [62]. HIF1α activity is regulated by several pathways, including the mitogen-activated protein kinase cascade, and p38α has been demonstrated to be involved in the stabilization of HIF1α in various normal and cancer cell types [63, 64]. The p38α pathway regulates proliferation, differentiation, metabolism,

and cell death in a cell type-specific and signal-dependent manner [65]. Starting from our promising results obtained on colorectal cancer, showing that p38α blockade promotes autophagy, cell cycle arrest, and non-apoptotic programmed cell death both *in vitro* and *in vivo* [66–69], we recently demonstrated that ovarian cancer cells are highly sensitive to p38α inhibition [70]. Inhibition of p38α activity by the specific inhibitor SB202190 impairs the expression of genes sustaining the altered metabolism of ovarian cancer cell lines and induces a shift from HIF1α- to FoxO3A-dependent transcription (Figure 1) [70]. SB202190 promotes a time-dependent reduction of HIF1α protein levels, ultimately leading to an acute energy need that triggers the activation of AMPK and the consequent induction of the FoxO3A transcriptional program. In turn, FoxO3A promotes upregulation of crucial mediators of autophagy, cell cycle control, and cell death. Upon p38α inhibition, autophagy is first accompanied by G1 arrest, but prolonged inactivation of p38α leads to autophagic cell death [70]. Autophagy represents a promising target for the design of new therapeutic strategies relying on pharmacological manipulation in tumors displaying resistance to apoptosis. Besides, as aerobic glycolysis represents a differentiating factor between normal and cancer cells, inhibition of genes involved in cancer cell metabolic reprogramming may provide both specificity and efficacy in countering the energetic demand of transformed cells, thus hampering growth and inducing energy failure-dependent death processes. Thus, therapies based on p38α-specific inhibitors could represent a valuable tool against cancer.

The rationale to manipulate the p38 pathway in ovarian cancer is further corroborated by recent findings indicating that p38α is a major mediator of drug resistance in response to chemotherapy with 5-fluorouracil and irinotecan [71, 72]. Moreover, as p38α inhibitory compounds are currently being investigated in clinical trials for inflammatory diseases and cancer [73], these findings might be taken advantage of in the prospect of clinical translation and support the idea that p38α could be one of the special agents engaged by clinicians to hunt down the silent killer.

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Review Article

Regulatory T Cells in Human Ovarian Cancer

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Multiple layers of suppressive components including regulatory T (T_{Reg}) cells, suppressive antigen-presenting cells, and inhibitory cytokines form suppressive networks in the ovarian cancer microenvironment. It has been demonstrated that as a major suppressive element, T_{Reg} cells infiltrate tumor, interact with several types of immune cells, and mediate immune suppression through different molecular and cellular mechanisms. In this paper, we focus on human ovarian cancer and will discuss the nature of T_{Reg} cells including their subsets, trafficking, expansion, and function. We will briefly review the development of manipulation of T_{Reg} cells in preclinical and clinical settings.

1. Introduction

Ovarian cancer is one of the most common and deadliest gynecologic cancers. In 2010, 21880 new cases were diagnosed, and such cancer caused nearly 13850 deaths in the United States alone [1]. Ovarian cancer usually has poor prognosis, and most patients were diagnosed at advanced stages. The five-year survival rate for all stages of ovarian cancer is 46% in 2010 [1]. It has been well documented that patients' clinical outcome and five-year survival rate are positively associated with the number of tumor-infiltrating lymphocytes (TILs) [2], and the ratio of intraepithelial $CD8^+$ TILs to T_{Reg} cells [3], or negatively associated with tumor-infiltrating T_{Reg} cells [4].

T_{Reg} cells are also known as suppressor T cells which consist of a specific subpopulation of cells that functionally suppress the activation of immune system and maintain immune tolerance to self-antigens. T_{Reg} cells contain two major subsets known as natural T_{Reg} cells (nT_{Reg}) and adaptive or induced T_{Reg} cells (iT_{Reg}). nT_{Reg} cells derived from thymus are considered as classic T_{Reg} cells, by contrast, iT_{Reg} cells develop in the periphery in response to self- or tumor antigens by converting naive $CD4^+$ T cells into T_{Reg} cells [5]. Because most tumors express self-antigens, T_{Reg} cells-mediated immunosuppression is believed to be one of the major contributors to immune evasion by tumors and becomes the main obstacle toward successful tumor immunotherapy [6]. In this paper, we will focus on human ovarian cancer and discuss the nature of T_{Reg} cells including their subsets, trafficking, differentiation, and proliferation and the clinical application of manipulation of T_{Reg} cells.

2. Regulatory T-Cell Subsets

In early 1970s, Gershon and Kondo first described the existence of thymus-derived suppressive T cells (later termed as T_{Reg} cell) *in vivo* [7, 8]. After more than a decade, Sakaguchi et al. demonstrated that $CD4^+$ T cells expressing interleukin-2 (IL-2) receptor alpha-chain (CD25) can be defined as the population of T_{Reg} cells with immune-suppressive activities and maintaining immune tolerance to self-antigen [9]. Later in 2003, Hori et al. found that the transcription factor forkhead box P3 (Foxp3) controls the development of T_{Reg} cells and is crucial for maintaining the immune-suppressive function of T_{Reg} cells [10].

Natural T_{Reg} cells differentiate in the thymus and migrate to periphery, which constitute 5–10% of $CD4^+$ T cells [11–13]. In addition, there are several subsets of T_{Reg} cells other than $CD4^+CD25^+Foxp3^+$ T_{Reg} cells. Groux et al. identified another subset of T_{Reg} cells, $CD4^+$ T_R 1 cells, that suppress antigen-specific immune responses by producing high levels of IL-10 [14]. In addition to $CD4^+$ T_{Reg}, $CD8^+$ suppressive T cells have been found playing an important role in the regulation of autoimmune disease [7, 15]. $CD8^+$ suppressive T cells now refered to as $CD8^+$ T_{Reg} cells are characterized as $CD8^+CD25^+$, $CD8^+CD122^+$, or $CD8^+CD45RC^{low}$ T_{Reg} cells, which comprise less than 1% of peripheral $CD8^+$ T cells [15]. Th3 T_{Reg} cells have similar immune-suppressive function; however, in contrast to natural T_{Reg} cells, Th3 exerts its suppressive capacity independent of cell membrane contact but mainly bases on the action of self-produced cytokine TGFβ [16].

3. Regulatory T-Cell Trafficking

T_{Reg} cells consist of ~10% of peripheral $CD4^+$ T cells characterized as $CD4^+CD25^+FOXP3^+$ T cells, which is important for the control of autoimmune reaction [9, 11]. Dysregulation of T_{Reg} can cause autoimmune diseases [17] and may contribute to tumor-initiated immune evasion [18]. As demonstrated by *in vivo* mouse model, the deletion of T_{Reg} cells results in tumor rejection [19]. However, the suppressive capacity of T_{Reg} cells is also determined by the ratio of T_{Reg} cells to effector T cells [3]. A high $CD8^+/T_{Reg}$ ratio is associated with favorable prognosis and improved survival [3, 20]. It has been reported that many human cancers are associated with high frequency of T_{Reg} cells in the circulation or in the tumor tissues, including ovarian cancer [4], lung cancer [21], breast cancer [22], liver cancer [23], head and neck cancer [24], and lymphoma [25]. These increased levels of T_{Reg} cells are linked to high death hazard and poor survival, while the depletion of tumor-infiltrated T_{Reg} cells and the blockade of T_{Reg} trafficking to tumors enhance antitumor immune response [4, 26].

CCR4 and its binding partners CCL22 and CCL17 are believed to be the most predominant axis in chemokine-mediated selective T_{Reg} trafficking to the tumors. Iellem et al. have profiled chemotactic responses and chemokine receptors expression of human T_{Reg} cells and found that T_{Reg} cells specifically express chemokine receptors CCR4 and CCR8 [27]. Chemokine CCL22, the ligand for CCR4, preferentially attracts activated-antigen-specific T cells to dendritic cells [28, 29]. It has also been shown that human ovarian cancer cells and tumor-associated microphages produce chemokine CCL22, which mediates T_{Reg} cells trafficking to tumor [4]. Blockade of CCL22 *in vivo* significantly reduces human T_{Reg} cells trafficking to tumors in ovarian carcinoma [4]. This chemokine-mediated T_{Reg} trafficking has been also observed in other types of cancer, such as gastric cancer [30], Hodgkin's lymphoma [31], and breast cancer [32]. Interestingly, in gastric cancer, CCL22 and CCL17 seem both important to recruit T_{Reg} cells to the tumors as demonstrated by *in vivo* study as well as *in vitro* migration assay, and the levels of CCL22 and CCL17 within tumors are correlated to the increased levels of T_{Reg} cells in early gastric cancer [33].

Besides CCR4 chemokine axis, CCR5/CCL5 axis may also selectively recruit T_{Reg} cells to the tumors. Using human pancreatic adenocarcinoma and murine pancreatic tumor model, it has been found that CCR5 is highly expressed in T_{Reg} cells, while tumor cells produce elevated amount of CCL5, and disruption of CCR5/CCL5 chemokine axis blocks T_{Reg} cells migration and reduces tumor growth [34]. In addition, CCL20 chemokine shows high affinity to CCR6 and can also mediate selective $CCR6^+$ T_{Reg} cells trafficking [35].

4. Regulatory T-Cell Differentiation and Proliferation

$CD4^+CD25^+$ T_{Reg} cells are generated in the thymus. Papiernik et al. found that peripheral T_{Reg} migrates from the thymus and appears in the periphery as early as 10th day of life [36]. They also found that $CD4^+CD25^+$ T_{Reg} cells differentiation is totally dependent on IL-2, because IL-2 knockout mice do not develop $CD4^+CD25^+$ T_{Reg} *in vivo* [36]. Further evidences have been provided from the studies on irradiated rat model [37]. In this study, autoimmune diseases were induced in rats by thymectomy and irradiation; however the xenograft transfer of $CD4^+$ T cells from normal rats can abrogate the autoimmune responses. These observations suggest that normal thymus-derived T cells have immune suppressive functions and thus prevent autoimmunity [37]. In another model system, adoptive transfer of thymocytes or peripheral T cells depleted of $CD4^+CD25^+$ T_{Reg} cells causes autoimmune diseases in mice, which provides further evidences of thymic origin of T_{Reg} cells and their peripheral existence [38].

However, there is little known about the comprehensive requirements for thymic T_{Reg} development. Although there are several arguments about how and what stromal components are involved in thymic T_{Reg} cell differentiation, thymic stromal cells, including cortical and medullary thymic epithelial cells and dendritic cells (DCs), contribute to T_{Reg} cells differentiation and selection [38]. Jordan et al. used TCR-transgenic mice which express the receptor recognizing specific self-antigen and found that thymocytes bearing a TCR with high affinity to a specific self-antigen undergo selection and become $CD4^+CD25^+$ T_{Reg} cells when interacting with a single self-antigen, but thymocytes bearing TCR with low affinity do not undergo selection [39].

In addition to thymus, T_{Reg} can also be generated in the periphery. For instance, tumor microenvironment favors the induction and differentiation of T_{Reg} cells, and that has been extensively studied for several years [40]. In the tumor microenvironment, DC differentiation and function were suppressed by tumor-associated factors IL-10, VEGF, and TGFβ, resulting in immature/dysfunctional DC [6]. Dysfunctional DC directly contributed to the induction of IL-10-producing T_{Reg} cells *in vivo* in human and *in vitro* [41, 42]. Tumor-associated plasmacytoid DC also induced $IL\text{-}10^+$ T_{Reg} generation [43, 44]. Tumor can convert DC into TGFβ-producing

immature DC, which selectively promotes T_{Reg} proliferation in TGFβ-dependent manner [45].

$CD4^+CD25^+$ T_{Reg} cells can also be converted from peripheral naïve $CD4^+CD25^-$ T cells by the action of TGFβ. Tumor microenvironment contains high levels of TGFβ which might mediate tumor-associated T_{Reg} cells conversion [46].

5. Targeting Regulatory T Cells

5.1. T_{Reg} Cell Depletion. In the mouse model, depletion of $CD4^+CD25^+$ T_{Reg} cells using anti-CD25 antibody causes tumor regression, which correlated to the reduced number of T_{Reg} cells [18, 47]. Using the recombinant IL-2 diphtheria toxin conjugate DAB(389)IL-2 (also known as denileukin diftitox and ONTAK), Dannull et al. demonstrated that DAB(389)IL-2 was capable of selectively eliminating $CD25^+$ T_{Reg} cells from the PBMCs of cancer patients without inducing toxicity on other cellular subsets, and DAB(389)IL-2-mediated T_{Reg} depletion enhanced anti-tumor immune responses and significantly reduced the number of T_{Reg} cells present in the blood of cancer patients [48]. Daclizumab (also known as Zenapex) and Basiliximab (also called Simulect) are monoclonal antibodies against CD25 [49, 50], and the administration of Daclizumab in patient with metastatic breast cancer enhanced anti-tumor immunity [51].

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) is constitutively expressed and restricted to $CD4^+CD25^+$ T_{Reg} cells among all $CD4^+$ cells, and the immune-suppressive function of T_{Reg} is mediated by CTLA4 signaling [52, 53]. CTLA4 binds to inhibitory B7 members on APC and transmits an inhibitory signal to T cells. *In vivo* administration of anti-CTLA4 antibody resulted in tumor rejection including preestablished tumors [54]. Periodic infusions of anti-CTLA4 antibody in previously vaccinated patients with cancer created clinically effective antitumor immune response [55]. Patients with metastatic melanoma showed improved antitumor immunity and tumor regression by blockade of CTLA-4 together with peptide vaccination [56].

Glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related protein (GITR or DTA-1) is predominantly expressed on the surface of T_{Reg} cells. An agonistic anti-GITR antibody administration in mice can abrogate T_{Reg}-mediated immune suppression and enhance effective anti-tumor immunity *in vivo* [57, 58]. In addition, treatment with anti-GITR antibody in B16 mice elicited immune response and rejected tumor [59]. However GITR is not exclusively expressed on T_{Reg} cell; it is also expressed by various $CD4^+$ T cells and others. Therefore, the clinical therapeutic relevance of GITR blockade and its side effects on potential deficits of other effective immune cells remain to be determined.

OX40 (CD134) also belongs to TNF receptor family and expressed on activated T cells. Both naïve and activated T_{Reg} express OX40. Similar to GITR, triggering OX40 by an agonistic antibody against OX40 reduces T_{Reg}-mediated immune suppression and restores effector T-cell function both *in vivo* and *in vitro* [60]. It has been also shown that OX40 is necessary for T_{Reg} development, homeostasis, and immune-suppressive activity. However, stimulation of OX40 signal in naïve T cells can abrogate T_{Reg}-mediated suppression [61].

Clinical relevance of the depletion of T_{Reg} cells has been further confirmed by the treatment of cyclophosphamide (CY) in the patients bearing tumor. Cyclophosphamide is a nitrogen mustard alkylating agent that mediates DNA crosslinking. Low dose of CY administration improved patients' immune responses by reducing the number of T_{Reg} cells and by decreasing the suppressive activity of T_{Reg} cells [62]. Effects of T_{Reg} depletion on anti-tumor immune responses were further investigated by the study on B16 melanomas mouse model [63]. Other immunosuppressants like cyclosporine A (CSA) and azathioprine might also inhibit T_{Reg} cells generation [64, 65]. For instance, high dose of CSA abrogates T_{Reg} cell generation; by contrast, low dose of CSA can promote T_{Reg} cell development [64]. It is therefore important to determine whether lowdose of those agents can improve antitumor immunity in patients.

5.2. Targeting T_{Reg} *Trafficking.* Our group has demonstrated that human ovarian cancer cells and tumor-associated macrophage (TAM) produced chemokine CCL22, the ligand for CCR4 which functionally expressed on tumor T_{Reg} cells, mediating T_{Reg} cells trafficking to the tumor and ascites, and the blockade of CCL22 abrogated T_{Reg} cells migration [4]. It has been demonstrated that chemokine receptor CCR4 is selectively expressed by T_{Reg} cells, and the CCR4 and CCR4-associate chemokines axis is one of the most described tumor T_{Reg} recruitment axes [66]. The administration of anti-CCR4 antibody effectively depletes $CCR4^+$ T cells and inhibits T_{Reg} cells migration in Hodgkin lymphoma [31]. Furthermore, the significant correlation between CCL17 or CCL22 chemokines and the number of tumor-infiltrating T_{Reg} cells was found in patients with neoplastic meningitis and gastric cancer [30, 33]. CCL5 and CCL20 chemokines are also involved in T_{Reg} trafficking, and that blockade of those chemokines reduces T_{Reg} cells trafficking and inhibits tumor growth [34, 35]. We have shown that CXCL12/CXCR4 axis mediated T_{Reg} trafficking to bone marrow [67]. Recently, a study has demonstrated that blockade of CXCR4 by a selective antagonist resulted in the significant reduction of intratumoral T_{Reg} cells, which was associated with greatly increased antitumor immunity and an improved survival in an immunocompetent mouse model of ovarian cancer [68].

5.3. Targeting TGFβ Signaling Pathway. TGFβ is implicated in T_{Reg} differentiation, conversion, and function. It is thought that blockade of TGFβ signaling pathway may alter T_{Reg} phenotype and function and in turn enhances antitumor immunity [6]. In addition to T_{Reg} cells, ovarian carcinoma cells can also produce TGFβ [69]. Notably, TGFβ is not only important for T_{Reg} cell functional integrity, but also inhibits the proliferation and functional differentiation of T lymphocytes, NK cells, and macrophages [46, 70]. This may induce T-cell unresponsiveness to TCR stimulation, failure to produce Th1 cytokines, and production of additional TGFβ [46]. TGFβ signaling may also be crucial for tumor cell

transformation. Therefore, targeting TGFβ signaling may be therapeutically meaningful. TGFβ inhibitor AP 12009 was tested in a Phase I/II clinical trial for advanced pancreatic cancer and other malignancies [71]. LY2109761, an inhibitor of TGFβ I/II receptors, can suppress pancreatic cancer metastases [72]. In a preclinical model, we have shown that anti-TGFβ can reduce T_{Reg} cells in tumors and tumor-draining lymph nodes. This effect is enhanced by B7-H1 blockade [73]. Nonetheless, it is clear that blocking TGFβ signaling may affect T_{Reg} compartment. However, as TGFβ is implicated in multiple layers of biological activities, the ultimate clinical therapeutic efficiency and side effects of TGFβ signaling blockade remain to be investigated.

5.4. Targeting Inhibitory B7 Family Members. The expression, regulation, functional, and clinical relevance of inhibitory B7 family members have been reviewed elsewhere [74]. Human ovarian cancer and cancer-associated myeloid antigen-presenting cells express high levels of B7-H1 (PD-L1), which are negatively associated with patient survival [74, 75]. Patients with high expression of B7-H1 had a significantly poor prognosis compared to the patients with low expression of B7H1 [76]. B7-H1 expression was also found inversely correlated to the intraepithelial $CD8^+$ T lymphocyte count, indicating that B7-H1 on tumor cells may suppress antitumor $CD8^+$ T cells [76]. The receptor, programmed death 1 (PD-1), is expressed on activated T-cell subsets, antigen-specific $CD8^+$ T cells [77], and T_{Reg} [78]. Interestingly, B7-H1/PD-1 has been reported to be involved in the development of induced T_{Reg} cells [79]. Therefore, targeting B7-H1/PD-1 signaling pathway may reduce T_{Reg} development and function. As anti-PD-1 is in clinical application to treat patients with melanoma, renal cell carcinoma, and other cancers, further mechanistic studies on these patients will determine if the effects of anti-PD-1 on T_{Reg} cells are mechanistically and clinically relevant.

In addition to B7-H1, human ovarian cancer and cancer-associated myeloid antigen-presenting cells also express high levels of B7-H4 (B7x, B7s1), which are negatively associated with patient survival [74, 80, 81]. Interestingly, T_{Reg} cells can induce IL-10 expression by APCs and indirectly stimulate B7-H4 expression on APCs and convey suppressive activity to APCs [74, 80, 81]. Thus, it is tempting to speculate that blocking B7-H4 signaling pathway may disable the suppressive effects of T_{Reg} cells on APCs. Notably, as the receptor for B7-H4 has not been identified, B7-H4 signaling is much less understood in both mouse and human system. Nonetheless, studies on ovarian cancer patients and preclinical cancer models suggest that interruption of B7-H4 signaling may lead to improved antitumor T-cell response and decreased T_{Reg} suppressive function.

6. Conclusions

T_{Reg} cells infiltrate tumor including ovarian cancer. Their phenotype, trafficking mechanism, suppressive activity, and clinical relevance have been defined in human cancer. However, recent evidence indicates that T_{Reg} cells may not be stable and are subject to environmental regulation. In this regard, it remains poorly understood how T_{Reg} cells evolve in human tumor microenvironment. Although their action mode of mechanisms has been investigated in many different physiological and pathological scenarios, the key suppressive mechanisms may be differed in different tumors or/and in different stages. Therefore, further patient-oriented studies are essential for dissecting T_{Reg} cell biology. Nonetheless, targeting T_{Reg} cells or/and reprogramming T_{Reg} cells is an important strategy to treat patients with cancer. It is suggested that combinatorial therapy by incorporating T_{Reg} manipulation may be ideal direction to develop novel therapeutic regimen to efficiently treat patients with cancer.

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Review Article

Optimizing Molecular-Targeted Therapies in Ovarian Cancer: The Renewed Surge of Interest in Ovarian Cancer Biomarkers and Cell Signaling Pathways

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The hallmarks of ovarian cancer encompass the development of resistance, disease recurrence and poor prognosis. Ovarian cancer cells express gene signatures which pose significant challenges for cancer drug development, therapeutics, prevention and management. Despite enhancements in contemporary tumor debulking surgery, tentative combination regimens and abdominal radiation which can achieve beneficial response rates, the majority of ovarian cancer patients not only experience adverse effects, but also eventually relapse. Therefore, additional therapeutic possibilities need to be explored to minimize adverse events and prolong progression-free and overall response rates in ovarian cancer patients. Currently, a revival in cancer drug discovery is devoted to identifying diagnostic and prognostic ovarian cancer biomarkers. However, the sensitivity and reliability of such biomarkers may be complicated by mutations in the *BRCA1* or *BRCA2* genes, diverse genetic risk factors, unidentified initiation and progression elements, molecular tumor heterogeneity and disease staging. There is thus a dire need to expand existing ovarian cancer therapies with broad-spectrum and individualized molecular targeted approaches. The aim of this review is to profile recent developments in our understanding of the interrelationships among selected ovarian tumor biomarkers, heterogeneous expression signatures and related molecular signal transduction pathways, and their translation into more efficacious targeted treatment rationales.

1. Introduction

Ovarian cancer is the major cause of gynecological cancer deaths worldwide [1–6]. It is widely accepted that the distinctive genotypic and phenotypic characteristics of ovarian cancer not only promote its metastatic potential but are also responsible for the development of resistance to conventional modes of cancer therapy, disease recurrence, and poor prognosis [2, 4, 7–19]. In particular, epithelial ovarian cancer (EOC) presents a considerable impediment to successful treatment outcome because of its propensity to embark on a program of epithelial-to-mesenchymal transition (EMT), a transdifferentiation process that is almost invariably associated with tumor progression and invasiveness [2, 15, 19–24].

Furthermore, self-renewing ovarian cancer stem cells (OCSCs) or ovarian cancer-initiating cells (OCICs), as well as mesenchymal stem cells (MSCs), have been implicated in ovarian tumorigenesis, intra- and extraperitoneal metastases, and chemoresistance [2, 19, 25–27]. Since cancer stem cells (CSCs) are predominantly quiescent, have upregulated DNA repair capacity, are noncommittal to apoptosis, and overexpress ATP-binding cassette (ABC) drug efflux transporters, for example, ABCG1 (MDR1/P-glycoprotein/Pgp), ABCG2, and breast cancer resistance protein (BCRP), and a profusion of cancer gene signatures, they sustain the succession of clonal tumor cell proliferation and repopulation in the tumor microenvironment [2, 22, 25, 26, 28–38]. Many CSC-derived or EMT-induced tumors, including ovarian cancer, also express this aggressive, malignant, and multidrug resistance (MDR) phenotype and other tumor prosurvival repertoires which pose significant challenges for cancer drug development, therapeutics, prevention, and management [2, 19–22, 28, 33, 34, 39].

The optimal management modality for ovarian cancer includes histopathological diagnosis and staging, debulking (surgical resection) of the tumor, and several cycles of intravenous (IV) or intraperitoneal (IP) chemotherapy with carboplatin and paclitaxel at maximum tolerated doses (MTDs), followed by maintenance or salvage treatments, in cases of disease recurrence [3, 12, 15, 40, 41]. Although refinements in tumor ablation procedures and IP combination chemotherapy with carboplatin and paclitaxel can achieve beneficial response rates, for example, median progression-free survival (PFS) range of 16 to 21 months and median overall survival (OS) range of 24 to 60 months, most patients with advanced disease ultimately relapse [15, 23, 40, 42–46]. Likewise, the majority of contemporary or tentative regimens of more than two cytotoxic drugs as well as low-dose chemosensitizing abdominal radiation have not yielded radically improved efficacy or significantly reduced adverse effects over the dual combination of carboplatin and paclitaxel, suggesting that other therapeutic avenues need to be explored to prolong PFS and OS rates in ovarian cancer patients [23, 39, 41, 47–55].

Recently, there has been a resurgence of efforts to identify ovarian cancer biomarkers for use in initial detection, staging, disease prognosis, molecular therapeutic targeting, and individualized clinical management of patients [14, 56–73]. Nonetheless, the sensitivity and reliability of ovarian cancer biomarkers may be confounded by several characteristics of the disease such as mutations in the *BRCA1* or *BRCA2* genes and their arcane absence in sporadic ovarian cancer, diverse genetic risk factors, unidentified initiation and progression elements, molecular tumor heterogeneity, and transition time between different stages of the disease. Correspondingly, the lack of a one-fit-all (i.e., highly sensitive and specific) biomarker for different histotypes of ovarian cancer—for example, EOC can be classified into four distinct histotypes: fallopian tube (serous), endometrium (endometrioid), endocervix (mucinous), or nests within the vagina (clear cell), coupled with differential overexpression of homeobox (Hox) genes—suggests that combination panels of biomarkers may offer greater diagnostic and prognostic probability [2, 12, 71, 73–75]. There is a critical need to develop broad-spectrum as well as individualized molecular-targeted therapies for ovarian cancers. Ingenious approaches are currently being applied to precisely map signal transduction pathways and target key molecular role players that direct ovarian tumor sensitivity and resistance to therapy and OS rates in patients. These include improved ultrasound and imaging technologies, molecular genetic analysis, as well as genomic, transcriptomic, and proteomic profiling of novel ovarian tumor biomarkers [2, 7, 14, 16, 56, 61, 72, 76–94]. In view of the complexities and variable response rates experienced with ovarian cancer patients clinically, the aim of this review is to outline recent developments in our understanding of the interrelationships among selected ovarian tumor biomarkers, heterogeneous expression signatures and related molecular signal transduction pathways, and their translation into futuristic as well as more efficacious targeted treatment rationales.

2. The Molecular Therapeutic Targeting Paradigm

The recurrence of ovarian tumors implies resistance to therapy regardless of encouraging response rates to cytoreductive surgery and combination chemotherapy, and most patients who relapse will eventually succumb to the disease [3, 15, 43, 44, 65, 95–98]. The poor prognosis in ovarian cancer patients may be broadly ascribed to distinct tumor histotypes or heterogeneity, disparate genomic expression profiles, and strikingly different molecular abnormalities [2, 12, 16–18, 39, 56, 69, 99–104]. Thus, the likelihood of ovarian cancer recurrence and resistance to therapy warrants serious alternative or complementary strategies to conventional oncologic modalities [1–4, 23, 42, 96, 105–107]. The potential for molecular-targeted therapy of ovarian cancers is increasingly being recognized and empirically validated [61, 108, 109]. Molecular therapeutic targeting is an approach that exploits specific hallmarks of cancers and the tumor microenvironment and their rationalization into clinically relevant and potent anticancer drugs with fewer side effects [1, 2, 23, 37, 39, 110–118]. Moreover, the application and exploitation of the dynamics of molecular-targeted system networks hold great promise for the design of personalized cancer therapies [119, 120]. This review provides a concise insight into recent advances in the molecular mechanisms of signal transduction pathways, the development MDR, DNA repair mechanisms, and tumor biomarkers of prognostic indicators and their therapeutic potential as translational targets in ovarian cancer.

3. Ovarian Cancer Biomarkers and Cell Signaling Pathways

A number of reliable, complementary, or potential diagnostic and prognostic biomarkers have been reported to be overexpressed or deregulated in different types of ovarian cancer. These will be considered in Sections 3 and 4.

3.1. Breast Cancer 1 and 2 (BRCA1/2) Oncogenes. Ovarian cancers are associated with breast cancer 1 (*BRCA1*) and *BRCA2* oncogenes, variously inherited as germline mutations [121–124]. Wild-type *BRCA1/2* genes are critical for DNA repair by the homologous recombination (HR) pathway—hence their deletion causes genomic instability and predisposes affected females to familial breast and ovarian cancers [103, 104, 125–127]. Ovarian cancers with mutated *BRCA1/2* genes are particularly sensitive to agents that cause DNA double strand breaks (DSBs) and DNA interstrand cross-links, like the platinum compounds (e.g., cisplatin and carboplatin) and poly(ADP-ribose) polymerase (PARP) inhibitors (e.g., olaparib, iniparib, veliparib) [128–132]. It is conceivable, therefore, that secondary or reversion mutations of the *BRCA1/2* genes, through multiple complex mechanisms, may favor DNA repair by HR and increase tumor cell survival and so trigger resistance to these compounds [133–138].

In addition, upregulation of *ABCB1* genes encoding the P-glycoprotein drug efflux pump has been found to be responsible for acquired resistance in a genetically engineered mouse model (GEMM) for BRCA1-associated breast cancer, following prolonged exposure to olaparib [139]. Such resistance mechanisms need to be demarcated in order to realize the full potential of molecular targeting of *BRCA1/2* mutations in ovarian cancer [140, 141]. Nonetheless, a recent phase II clinical trial with orally active olaparib in women with confirmed genetic *BRCA1/2* mutations and recurrent measurable ovarian cancer has provided tangible proof of concept of the efficacy and tolerability of molecularly targeted treatment with PARP inhibitors, and validated *BRCA1/2* mutations as biomarkers for predicting responses of ovarian cancer patients to PARP inhibition [142]. Several other reports have, in the context of $BRCA1^{-/-}$ ovarian cancers and their sensitivity to small molecule PARP inhibitors, presented preclinical and clinical evidence that the concept of synthetic lethality which defines a condition whereby two mutations, each with viable phenotypes, produce a lethal phenotype when they are combined can thus be exploited as a molecular-targeted strategy [133, 135, 143–147].

3.2. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR). Tumor neovascularization or angiogenesis, a process dictated by complex cellular pathways that fine-tune proangiogenic and antiangiogenic factors (i.e., an angiogenic switch) in the tumor microenvironment, allows cancers to develop new blood vessels for nutrient and oxygen supply, elimination of metabolic waste products, growth, acquisition of an invasive phenotype, and metastastic spread [148–153]. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) occupy a position of prominence in angiogenesis signaling in normal ovarian physiology and in ovarian cancer progression [1, 2, 4, 15, 23, 65, 114, 154–156]. Therefore, inhibition of the angiogenesis signal transduction pathway via its ligands and receptors in ovarian cancers represents a perfectly cogent molecular targeting strategy [1, 4, 95, 98, 113, 153, 154, 157]. VEGF has long been recognized as a biomarker for predicting ovarian cancer patient responses to VEGF and other therapies and may as well have applications in formulating individualized therapies [4, 71, 72, 158–161]. Inhibitors of the VEGF pathway include bevacizumab (a humanized antibody that targets the ligand VEGF) and VEGF-trap (aflibercept, a high-affinity VEGFR decoy fusion protein that binds and inactivates VEGF and other ligands) [1, 3, 51, 95, 98, 114, 162, 163].

Besides blocking the VEGF pathway with VEGF antibodies, the angiogenic pathway can be targeted with small molecule VEGFR tyrosine kinase inhibitors (TKIs)—those currently used in ovarian cancer include, sorafenib, sunitinib cediranib, vandetanib, and intedanib (BIBF 1120) [7, 15, 65, 153, 154, 162, 164–166]. Since multiple ligands and their receptors are involved in neovascularization, including platelet-derived growth factor (PDGF/R), epidermal growth factor (EGFR/R), placenta growth factor (PlGF/R), KIT, fibroblast growth factor (FGF/R), and hepatocyte growth factor (HGF/R), resistance to single antiangiogenic drugs may occur in ovarian cancer patients, blocking such alternative pathways with rational drug combinations that have cross-specificity would be an appropriate molecular targeting strategy [1, 4, 15, 23, 114, 148, 150, 153, 156, 167–170].

3.3. The EGFR/ErbB Family of Receptor Tyrosine Kinases. In humans, the epidermal growth factor receptor EGFR/ErbB family of receptor tyrosine kinases (RTKs) comprises four members: EGFR/ErbB1/HER-1, ErbB2/Neu/HER-2, ErbB3/HER-3, and ErbB4/HER-4 [171, 172]. ErbB2 lacks ligand-binding capacity because its ectodomain is fixed and in an unfolded conformation, but it is the preferred ally for heterodimerization with EGFR to increase the duration and intensity of the signal triggered by high-affinity ligand binding to EGFR. Thus, ErbB2 is an amplifier of the ErbB signaling network [171]. Aberrant coexpression and collaboration of EGFR and ErbB2 is widespread in cancers and has been associated with poor prognosis [172–175]. Therefore, EGFR is deemed to be a useful biomarker for ovarian cancers [1, 2, 4, 61, 176, 177]. In ovarian cancers, mutant or isoforms of EGFR RTKs transactivate signaling transduction cascades such as PI3K/AKT and Ras/Raf/MEK/MAPK/ERK that result in diverse effects, including cell proliferation, dedifferentiation, adhesion, migration, invasion, angiogenesis, and apoptosis evasion [177–183]. Accepted tenets of molecular targeting of EGFR signaling in ovarian and non-ovarian cancers encompass small molecule TKIs (e.g., erlotinib, gefitinib), ATP-binding site inhibitors (e.g., CI-1033), anti-EGFR/ErbB2monoclonal antibodies (e.g., matuzumab, pertuzumab, cetuximab, trastuzumab), and multikinase inhibitors (e.g., vandetanib, sorafenib) [164, 166, 174, 184–194].

A recent phase II trial in women with predominantly platinum-resistant recurrent ovarian cancer concluded that vandetanib, a multikinase inhibitor designed to perturb both angiogenesis (i.e., VEGFR) and tumor cell growth (i.e., EGFR), did not produce translational clinical benefit since the drug inhibited EGFR and AKT levels in tumor biopsies, but had no effect on VEGFR [164]. Likewise, EGFR gene mutations and EGFR protein expression do not necessarily correlate with clinical outcome [182, 195–197]. Previous phase II clinical studies with imatinib and gefitinib in patients with refractory or recurrent EOC suggested that although these agents have marginal benefits as monotherapies in EOC, their ability to modulate molecular targets (e.g., EGFR, c-Kit, PDGFR, ERK, AKT) and demonstrate proof of concept corroborates their applicability in combinatorial molecular therapeutics [198, 199]. A number of reports have reinforced the notion that inhibition of a single transduction pathway may be insufficient since activation of alternative signaling cascades may conceal efficacy, and that it would be more advantageous to target integrated cancer signals, for example, VEGFR- and EGFR-interdependent pathways [170] and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [200, 201]. Remarkably also, the mammalian target of rapamycin (mTOR) is a central intracellular kinase that not only orchestrates proliferation, survival, and angiogenic pathways, but has also been linked to resistance

to EGFR antagonists, and thus mTOR inhibition could be explored to interfere with tumor growth and expansion at multiple levels [4, 83, 84, 92, 159, 170, 202–205]. Another multiple molecular targeting platform is provided by EGFR-induced EMT in EOC, possibly via mechanisms that incorporate estrogen signaling, E-cadherin downregulation and expression of matrix metalloproteinase-9 (MMP-9), and Snail transcription family members (SNAIL and SLUG) [79, 206, 207]. Additionally, oncolytic viruses engineered to deliver anti-EGFR antibodies to intraperitoneal ovarian cancer cells show great potential as a future gene therapeutic focus [208]. Irrespective of the prospects for molecular targeting of EGFR RTKs in ovarian cancer, resistance to EGFR inhibitors and unwanted adverse events in ovarian and non-ovarian tumors are major clinical concerns that need to be circumvented [16, 166, 174, 191, 209, 210].

3.4. Mucin 16 (MUC16) and Lewis X Mucin Determinant (OVX1). The role of mucins in epithelial cancer, including ovarian cancer, pathogenesis is well established [211–213]. Mucin 16 (MUC16)—also called carcinoma antigen 125 (CA125)—is arguably the most consistently used biomarker for ovarian cancer [58, 59, 61, 64, 72, 73, 211, 214–221]. MUC16 is overexpressed in EOC and correlates with decreased E-cadherin, elevated N-cadherin and vimentin levels, and heightened invasivesness, tumorigenesis, tumor cell proliferation, and metastases, as confirmed by MUC16 knockdown which completely abolished the development of subcutaneous tumors in nude mice [222]. Interestingly, the C-terminal domain of MUC16 promotes cisplatin resistance and MUC16 selectively modulates the sensitivity of EOC cells to DNA-damaging drugs such as cyclophosphamide, doxorubicin and etoposide, effects validated by downregulation of cell surface MUC16 [223]. The strong interaction between MUC16 and mesothelin, a glycosylphosphatidylinositol- (GPI-) anchored glycoprotein, promotes cell adhesion and peritoneal metastasis of ovarian cancer cells [224, 225]. Furthermore, MUC16 suppresses natural killer (NK) cell-induced cytolysis in EOC patients, indicating that it compromises immune-mediated tumor surveillance and destruction [226]. In preclinical and clinical studies, antibodies and vaccines directed against mucins, evaluated for their potential to delay or limit the spread of tumor cells, produced significant survival benefits [211, 227–229]. The usefulness of MUC16 as a target antigen in ovarian carcinomas is hampered by cleavage and secretion of its extracellular domain. However, a recent study has shown that the introduction of a gene encoding a chimeric antigen receptor (CAR) targeted to the retained extracellular fraction (MUC-CD) and its retroviral transduction into human T cells specifically targets and lyses MUC-CD$^+$ tumor cells and may thus signify an innovative design to adoptive immunotherapy of cancer [230–232]. In view of the previous assertions, MUC16 needs to be probed for its plausibility as a molecular target in the immunotherapy of ovarian cancers [233, 234].

MUC16 is used along with multiple serum biomarkers for the early detection and screening of ovarian cancer [235]. One such biomarker is the Lewis X mucin determinant (OVX1) which is increased in the majority of patients with EOC [59, 71, 72, 125, 218, 221, 236–238]. Monoclonal antibodies to OVX1 are internalized by ovarian cancer cell lines in vitro and may prove useful in the molecular targeting of this neoplasm with conjugated antibodies and immunotoxins [232, 238–241]. Curiously, alterations of the sugar moieties of the glycosylated Lewis X and Lewis Y antigens are frequent in epithelial ovarian cancers and, besides having obvious prognostic implications, may be prime arbiters along with extracellular matrix component interactions (e.g., β-integrin/fibronectin, CA125/mesothelin, CD44/hyaluronan) in CD44-mediated adhesion and peritoneal spreading (metastasis) of ovarian cancer cells [242]. These mechanisms should be explored as a molecular targeting principle in ovarian cancers.

3.5. The IL-6R-JAK-STAT3 Axis and Nuclear Factor Kappa-B (NF-κB). The upregulation of several proinflammatory cytokines in ovarian cancers confirms a link between inflammation and immunogenic-tumor microenvironment interactions in the increased risk of ovarian tumor initiation and progression [243–251]. IL-6 is a proinflammatory cytokine that modulates pleiotropic cellular and immune responses. Binding of the ligand, IL-6, to the α-subunit of its receptor (IL-6R) results in the formation of a heterodimeric complex (IL-6R/gp130) which activates Janus kinase (JAK) and various downstream effectors such as signal transducer and activator of transcription 3 (STAT3), SHP-2/Ras, mitogen-activated protein kinase (MAPK), and phosphatidylinositol-triphosphate kinase PI3K/Akt, critical for cell proliferation, apoptosis evasion and survival, drug resistance, and inactivation of tumor suppressors [252–258]. STAT3 is also activated by growth factor receptor signaling, including EGFR, HER2, VEGFR, PDGFR, IGFR, and FGFR [252]. Indeed, raised levels of IL-6 in ascites and serum from ovarian cancer patients correlate with cisplatin and paclitaxel resistance and poor disease prognosis [259], whereas blockade of STAT3 expression in ovarian cancer cells increases their sensitivity to paclitaxel [254]. The expression of IL-6 and its downstream signaling proteins is upregulated in ovarian clear cell adenocarcinoma (OCCA) and EOC [7, 260].

A recent study has shown unequivocally that siltuximab (a monoclonal anti-IL-6 antibody) significantly reduced ovarian cancer expression of STAT3 downstream proteins such as Mcl-1, Bcl-X(L), and survivin, implying proapoptotic effects. In the same study, metastatic and drug-resistant recurrent ovarian tumors expressed significantly higher IL-6 levels than primary ovarian cancer tissue [261]. By the same token, administration of sunitinib, a potent multikinase (VEGFR, PDGFR, and KIT) inhibitor, to two OCCA patients with progressive disease and refractory to conventional chemotherapy resulted in markedly lower levels of CA125 and notable reduction in tumor mass [7]. The possible mechanistic correlation for the favorable responses seen in these patients had been advanced as inhibition by sunitinib of IL-6, STAT3, and hypoxia-induced factor (HIF). Thus, the upregulation of the IL6-STAT3-HIF pathway in OCCA may be exploited as a biomarker to clinically differentiate

OCCA from other ovarian tumor types [7], and inhibition of the IL-6-STAT3 signaling autocrine pathway may offer yet another molecular targeting strategy in the management of cisplatin- and paclitaxel-resistant ovarian cancers [259, 262]. The observation that crosstalk between the EGFR and IL-6R signaling through JAK/STAT3 mediates EMT in ovarian cancers further adds to the number of exploitable opportunities that are emerging to target the molecular intricacies that underscore the aggressive phenotype of ovarian cancer and its recurrence in patients [258, 263, 264]. Generic strategies to target the IL-6R-JAK-STAT3 signaling axis include receptor-ligand antagonists or antibodies, tyrosine or serine kinase inhibitors, transcription factor decoy (siRNA), physiological protein modulators of STAT3 activation, disrupters of STAT dimerization, inhibitors of STAT3 nuclear translocation, and target gene transcription [257].

Nuclear factor kappaB (NF-κB) is a highly inducible transcription factor which regulates several inflammatory response and cancer signaling pathways [252, 265, 266]. NF-κB is constitutively expressed in the majority of tumors, including ovarian cancer [80, 256, 257, 266]. Many cytokine-induced signaling pathways that control inflammation and cancer converge on NF-κB and STAT3 [252]. The mammalian NF-κB family comprises five members, namely, RelA (p65), RelB, cRel (Rel), NF-κB1 (p50 and its precursor p105), and NF-κB 2 (p52 and its precursor p100) which form homo- and heterodimers whose activities are regulated by two key NF-κB activation pathways. In the first (classical or canonical) pathway, RelA:p50 dimers are sequestered in an inactive conformation in the cytoplasm through interactions with inhibitory proteins, I-κB. Upon binding of ligands such as TNF-α or IL-1, viruses, genotoxic agents, and exposure to ionizing radiation, the I-κB molecules become phosphorylated at specific serine residues by the I-κB kinase complex (IKK, made up of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO/IKKγ) which results in their ubiquitylation and proteasomal degradation. The liberated RelA:p50 dimers translocate to the nucleus to activate transcription of several target genes that regulate innate immunity and inflammation. In the second (alternative or non-canonical) pathway which is stimulated almost exclusively by members of the TNF superfamily, an upstream NF-κB-inducing kinase (NIK) activates IKKα, causing phosphorylation and proteasomal processing of p100, the principal RelB inhibitor, followed by RelB:p52 and RelB:p50 nuclear translocation and binding to genes responsible for regulating development, organization, and function of secondary lymphoid organs, B-cell maturation, and survival. Even though many genes are regulated by STAT3 and NF-κB, these two master regulators both favor the transcriptional activation of protumorigenic and antiapoptotic genes such as *Bcl-xL*, *Bcl-2,* and *c-IAP2*, while *A1* and *c-FLIP* genes are predominantly NF-κB-dependent and *Mcl-1* and *survivin* genes are STAT3-dependent [252, 256, 265, 266]. NF-κB (RelA/p65) is overexpressed in advanced-stage metastatic serous ovarian carcinoma, and its localization to the nucleus is associated with poor PFS [267]. Using specimens from patients with IKKβ-positive ovarian tumors and ovarian cancer cell lines, a recent study showed that activation of the NF-κB pathway by downregulating IKKβ activity with highly specific kinase inhibitors or through short hairpin RNA (shRNA), depletion of IKKβ correlated not only with a number of cellular expressions associated with the invasive phenotype of this cancer, but also with poor OS [80]. These findings are in agreement with the notion that constituent expression of NF-κB in OCSCs, which may be the trigger of chemoresistance and disease recurrence, can be targeted by inactivation of NF-κB signaling [25, 247].

Although IL-6 signaling has been studied extensively in ovarian cancers, several reports have indicated the involvement of many other interleukins in the development of this neoplasm [248, 252]. These will not be considered further in this review, except to mention that IL-8 has previously been identified to have autocrine growth factor, tumorigenic and angiogenic effects in human ovarian cancer [268–273], but conflicting reports have also appeared [274]. Particularly noteworthy is the fact that activation of G-protein-coupled receptor protease-activated receptor-1 (PAR1) by matrix metalloproteinase (MMP1) is a principal promoter of angiogenesis and metastasis in peritoneal mouse models of ovarian cancer. In ovarian carcinoma cells, activated MMP1-PAR1 induces the release of angiogenic factors such as interleukin-8 (IL-8) and growth-regulated oncogene-alpha (GRO-α) which, through paracrine signaling, act on endothelial CXCR1/2 to effect endothelial cell proliferation, tube formation, and migration [110]. This pathway may be targeted to identify novel ovarian cancer therapies.

3.6. PI3K/AKT/mTOR Cell Signaling Pathway. The mammalian target of rapamycin (mTOR) is a central intracellular kinase that coordinates mitogenic, angiogenic, antiapoptotic, and survival pathways in cancers through crosstalk with VEGF, HIF-1, and the EGFR/ErbB family of RTKs [202]. PI3K/Akt/mTOR signaling thus confers a selective survival advantage on tumor cells [397]. Activators of this pathway include defective tumor suppressor PTEN, upregulation or mutation of PI3K and AKT, and ligand binding to growth factor receptors. Mutation or amplification of PI3K or Akt triggers mTOR phosphorylation and increased ovarian tumor cell survival [398]. A recent study has shown that PI3K/AKT/mTOR signaling is involved in EOC development and resistance to cisplatin, since downregulation of AKT with triciribine or shRNA transfection of ovarian cancer cells decreased their resistance to cisplatin via mTOR/survivin signaling [92]. In advanced-stage ovarian cancer, the mTOR pathway is upregulated, and hence its blockade will enhance ovarian cancer cell sensitivity to antitumor drugs [204]. In patients with serous ovarian carcinoma undergoing cisplatin-taxane-based therapy, activation of VEGFR2/AKT/mTOR pathway was significantly correlated with raised ascites levels and decreased OS [205]. mTOR has been implicated in the resistance of various cancers to EGFR inhibitors [202] and mTOR pathway activation is a poor prognosticator of EOC [84]. Furthermore, treatment of highly metastatic ovarian tumor cells with

Table 1: Candidate biomarker profiles and the molecular basis for their targeting in ovarian cancers.

Biomarker†	Molecular basis for biomarker targeting in ovarian cancer	References
M-CSF	Hematopoietic cytokine that stimulates differentiation, activation, and proliferation of monocyte and macrophages; can also act as an autocrine or paracrine growth factor for some epithelial cancers; promotes vasculogenesis; modulates CSCs, and can thus be targeted in OCSCs to induce immune-mediated tumor cell lysis; a phase II trial with GM-CSF and recombinant interferon gamma 1b (rIFN-γ1b) in women with recurrent, platinum-sensitive ovarian, fallopian tube, and primary peritoneal cancer produced reasonable OS.	[14, 16, 33, 34, 59, 275]
HNF-1β	Overexpressed in ovarian clear cell adenocarcinoma (OCCC); reduction of HNF-1β expression by RNA interference induces apoptotic cell death in ovarian OCCC cells; HNF-1β is hypomethylated in OCCC and can thus be targeted in ovarian cancers.	[276–280]
HE4	A glycoprotein highly expressed in ovarian cancers that might have a role in ovarian carcinogenesis; HE4 expression is highest in endometrioid and serous ovarian cancer	[214, 281, 282]
OPN	A glycophosphoprotein cytokine secreted by activated T-lymphocytes, macrophages, and leukocytes at the inflammation site; higher levels occur in patients with ovarian cancer versus normal control; correlates significantly with tumor response to surgery, chemotherapy, and disease recurrence; implicated in tumorigenesis, tumor invasion, metastasis, and poor prognosis; binding of OPN as an ECM component to integrin and CD44 receptors in the tumor microenvironment regulates signaling cascades associated with adhesion, migration, invasion, chemotaxis, and cell survival; alternative splicing of OPN leads to 3 isoforms, OPNa, OPNb, and OPNc; the latter possess ovarian protumorigenic properties mediated by PI3K/Akt signaling pathway which serves as a critical cancer molecular target.	[14, 111, 283–285]
MES	Binding of MUC16 to MES, a GPI-anchored glycoprotein, is thought to facilitate cell adhesion and peritoneal metastasis of ovarian tumors; this function can be exploited as a molecular targeting strategy, for example, anti-MES antibodies, to limit the metastatic spread of the tumor; MES is an attractive candidate for adenoviruses-mediated gene therapy of ovarian cancers; diffuse mesothelin expression is associated with prolonged survival in patients with high-grade ovarian serous carcinoma.	[224, 225, 286, 287]
HP-α	Glycoprotein synthesized in the liver, but also present in ascites and serum of ovarian cancer patients; proteomic profiling identified HP-α as a potential biomarker with high specificity for ovarian cancer; high levels of this acute phase protein correlate with poor prognosis, but attenuate with chemotherapy—this mechanism should be explored further.	[14, 71, 288–291]
BIK	This glycosylated protease suppresses ovarian tumor cell invasion and metastasis by downregulating PI3K and Ca^{2+}-dependent TGF-β signaling pathways; plasma BIK is a strong prognostic indicator of ovarian cancer; a combination of BIK and paclitaxel significantly reduced tumor burden and ascites in a mouse model of ovarian cancer; BIK overexpression has been shown to suppress TNF-induced apoptosis in ovarian cancer cells; BIK also downregulates uPA/R and HBP gene expression in ovarian cancer cells; other target genes of BIK include transcriptional regulators, oncogenes/tumor suppressor genes, signaling molecules, growth/cell cycle, invasion/metastasis, cytokines, apoptosis, ion channels, and ECM proteins; the evidence cited here underlines the applicability of BIK in therapeutic strategies targeting the inhibition of peritoneal invasion and dissemination of ovarian cancer.	[14, 292–299]
FRα	This protein is an alternative folate transporter which may confer an increased DNA synthesis and growth advantage on tumor cells; ovarian cancer patients have elevated blood levels of this protein, identified as a diagnostic marker and molecular target in high-grade, high-stage serous tumors; the status of FRα apparently does not change in response to chemotherapy and has no effect on overall patient survival; however, farletuzumab, a humanized monoclonal antibody against FRα, demonstrated anticancer efficacy in patients with platinum-refractory/resistant EOC; FRα expression is preserved on metastatic foci and recurrent tumors, suggesting that novel folate-targeted therapies may have therapeutic potential for the majority of women with newly diagnosed or recurrent ovarian cancer.	[300–304]
TTR	This is a highly sensitive biomarker used in the screening of prostate, lung, colorectal, and ovarian (PLCO) cancers; was found to be downregulated in grade 3 ovarian tumors; and has been validated for its high specificity and sensitivity in early-stage ovarian cancer; further research on TTR is needed to explore its molecular targeting possibilities.	[58, 71, 305–307]
IαI	The expression of this protein is reportedly upregulated in ovarian cancer patients and it is used mainly to complement MUC16/CA125 in the screening for EOC; however, proteomic analysis showed its levels to be significantly reduced in the urine of patients with ovarian carcinoma.	[14, 125, 308]
CRP	Is one of a panel of plasma biomarkers used for the identification of women with ovarian cancer and to significantly increase diagnostic performance compared to MUC16/CA125 used singly; raised serum levels of CRP is associated with high levels of Il-6 and haptoglobin, considered as adverse prognostic factors in ovarian cancer; CRP are also a marker of high-grade inflammation in advanced-stage ovarian cancer and anemia in EOC (i.e., CRP correlates negatively with hemoglobin levels); high levels of prediagnostic CRP may indicate an inflammation stage that precedes ovarian cancer development and might denote increased risk.	[235, 291, 309–313]

Table 1: Continued.

Biomarker†	Molecular basis for biomarker targeting in ovarian cancer	References
PRSS	This channel-activating serine protease is overexpressed in EOC; it is localized to the apical surface of normal epithelial cells and suppresses cancer cell invasion in vitro; in various cancer cell lines, PRSS downregulates EGFR signaling by cleaving its extracellular domain and hence interferes with cell proliferation and tumor expansion; this property should be investigated as a molecular target.	[14, 71, 72, 314–316]
CLDNs	Large family of integral membrane proteins essential for tight junction formation and function; CLDN3 and CLDN4 expression levels are upregulated in EOCs of all subtypes and correlate with MMP-2 activity; CLDNs may promote ovarian cancer invasion and metastasis; CLDN upregulation in ovarian carcinoma effusions is associated with poor survival; cells that overexpress CLDN4 exhibit low DNA methylation and high histone H3 acetylation of the critical CLDN4 promoter region, while the converse is true for cells that do not overexpress it; CLDN4-expressing EOC cells secrete proangiogenic factors (e.g., IL-8) and downregulate genes of the angiostatic IFN pathway; CLDN5 overexpression is associated with aggressive behavior in serous ovarian adenocarcinoma; CLDNs are, therefore, suitable biomarkers for different types of ovarian cancer and promising molecular targets for ovarian cancer therapy.	[317–326]
APOA1	Is the protein component of HDL; the *APOA1* gene is upregulated in chemoresistant EOC and has an established role in tumorigenesis; algorithmic proteomic profiling of postdiagnostic/pretreatment sera of women with ovarian cancer revealed that the ApoA1 and TTR combination yield high specifity, but low sensitivity as tumor markers; further investigations into the mechanistic roles of APOA1 in ovarian tumorigenesis are crucial for its consideration as a molecular target in ovarian cancer.	[306, 327]
LPA	Generated by the action of the enzyme, lysophospholipase; LPA is the ligand for GPCRs (LPAR2 and LPAR3) which are upregulated during ovarian tumorigenesis; LPA is a bioactive lipid central to the initiation and progression of ovarian cancer; LPA is preferable to MUC16/CA125 as a biomarker for the diagnosis, but not the prognosis of EOC; in human EOC tissues obtained from patients, LPA-induced POSTN (an ECM constituent, see the following) expression in cancer-associated stromal fibroblasts correlates with poor survival and recurrence; remarkably, LPA also regulates IL-6 expression and STAT3 phosphorylation via the Gi/PI3K-Akt/NF-κB pathway in ovarian cancer cells; LPA enhances growth and invasion of ovarian cancer cells and tumor angiogenesis; active RTK and EGFR signaling is required for LPA-mediated Gi-dependent cellular responses in ovarian cancer cells; LPA antibodies, LPA antagonists, and LPAR gene silencing may thus be useful molecular targeting strategies in ovarian cancer.	[2, 268, 328–339]
POSTN	POSTN is an ECM protein which normally functions as a homophilic adhesion molecule in bone formation; 5 isoforms have so far been identified; targeted comparative glycotranscriptome analyses of ovarian cancer and normal ovarian tissues have shown that POSTN and thrombospondin may be useful biomarkers for specific tumor-specific glycan changes in benign ovarian adenomas, borderline ovarian adenocarcinomas, as well as malignant ovarian adenocarcinomas; POSTN binds to numerous cell-surface receptors, predominantly integrins, and signals effectively via the PI3K/Akt and other pathways to promote cancer cell survival, EMT, invasion, metastasis, and angiogenesis; ovarian cancer cells actively secrete the protein; interaction of the ligand, POSTN, with integrins facilitates ovarian cancer cell motility; antibodies directed against POSTN have been shown to inhibit growth and metastasis of subcutaneous and ovarian tumors derived from a POSTN-expressing ovarian cancer cell line; thus, POSTN represents a novel molecular-targeted therapy for ovarian cancer.	[330, 340–345]
KLK	Largest family of flanking proteases in the human genome, comprising at least 15 members; KLKs are secreted serine proteases that stimulate or inhibit tumor progression; KLK5-11 levels are typically elevated in sera of ovarian cancer patients and regarded as predictors of poor disease prognosis; aberrant *KLK* gene expressions in different types of ovarian cancers may complicate generalizations; for example, high tumor KLK6 protein expression correlates with inferior patient outcome in ovarian cancer, while raised KLK8 is an independent marker of favorable prognosis in ovarian cancer, whereas KLK5 levels are low in serum of patients with benign ovarian tumors; elevated KLK5 antigen in serum and ascitic fluid of ovarian cancer patients is a prognostic factor for PFS; KLK5-specific antibodies have been detected in patients with benign masses, borderline tumors, and ovarian carcinomas compared with healthy controls; the presence of KLK5 antibodies suggests that KLK5 might represent a possible target for immune-based therapies; KLK6 exemplifies the altered glycosylation hallmark of ovarian cancer; KLK7 is associated with negative characteristics of ovarian cancer, but is not considered an independent prognosticator for the disease; a combined panel of KLK6, KLK13, and MUC16/CA125 affords improved sensitivity in the detection of early stage ovarian cancer than MUC16/CA125 alone; KLKs have recently been shown to be subject to posttranscriptional control by multiple miRNAs which can be exploited in the differential diagnosis of ovarian cancer and as a molecular targeting opportunity.	[60, 346–365]
AGR2	This is a mucinous metastasis-inducing protein detectable in the plasma of ovarian cancer patients; elevated AGR2 levels in ovarian cancer patients are associated with disease stages II and III in both serous and nonserous tumors; AGR2 is thought to promote cell proliferation and migration; it is currently being validated for its diagnostic and prognostic significance in ovarian cancers.	[67, 366–368]

Table 1: Continued.

Biomarker[†]	Molecular basis for biomarker targeting in ovarian cancer	References
HDACs	Posttranslational modification of histones by HATs results in acetylation of the histone structure which exposes chromatin of transcriptionally active genes; the acetylation status of histones governs access of transcription factors to DNA and determines levels of gene expression; HDACs catalyze the removal of acetyl groups from histone tails and thus suppress transcription; accordingly, homeostatic control of HATs and HDACs activities is essential for maintaining nuclear and genomic stability; HDACs also act on various other transcription factors such as p53, Rb, and E2F1; HDACs are often activated or mutated in human cancers; in ovarian tumors, type-specific overexpression and roles for these enzymes have been delineated; for example, HDAC1 promotes cell proliferation whereas HDAC3 induces cell migration by downregulating E-cadherin; HDACs have become critical drug targets for cancer therapy and HDACi shows tremendous promise in preclinical and clinical trials (www.clinicaltrials.gov); SAHA (vorinostat, Zolinza) has been approved by the FDA for treatment of cutaneous T-cell lymphoma; HDACi promotes cell cycle arrest by inducing CDK inhibitor p21 (WAF1/CIP1); moreover, HDACi has pleiotropic actions, including the upregulation of proapoptotic proteins of Bcl-2 family (Bim, Bmf, Bax, Bak, and Bik) and downregulation of antiapoptotic proteins of Bcl-2 family (Bcl-2, Bcl-XL, Bcl-w, Mcl-1) and XIAP and survivin which may be significant in apoptosis targeting approaches [369, 370]; HDACi, such as NaB, SAHA, and TSA, enhanced in vitro ovarian cancer cell killing with concomitant increased mRNA expression of MDR1 but decreased mRNA expression of MRP1 and MRP2; the novel hydroxamic acid-derived HDACi, MHY218, has been shown to be more potent than SAHA in suppressing ovarian tumor cell viability and transplanted tumor growth in an in vivo tumor carcinomatosis model; MHY218 also raised expression levels of the cell cycle inhibitor, p21WAF1/CIP1, induced apoptosis via caspase-3 activation, and increased release of cytochrome c and Bax/Bcl-2 ratio; previously, similar results have been reported for another novel HDACi, apicidin; in view of the above, it is clear that HDACi is an emerging molecular-targeted approach to the management of ovarian cancer, but prudent forethought should be given to specific targeting of different HDAC family members, for example, HDAC1 and HDAC2 coregulator complexes, and more especially since acetylated HDAC1 can transregulate HDAC2 through heterodimerization.	[327, 371–386]
miRNAs[‡]	MicroRNAs belong to a family of endogenous, small RNAs (~22 nucleotides); these noncoding, yet functional RNAs are key regulators of coding genes in the human genome; microarray analysis of altered expression of miRNAs provides useful information on the ontogeny and differentiation status of various cancers; genomic and epigenetic modifications are known to deregulate miRNA expression in human EOC; a recent study showed that several miRNAs (let-7e, miR-30c, miR-125b, miR-130a, and miR-335) were differentially expressed and upregulated in paclitaxel- and cisplatin-resistant ovarian cancer cell lines and concluded that the development of drug resistance in ovarian cancer may be linked to distinct miRNA fingerprints that could be used as biomarkers to monitor disease prognosis; deregulation of miRNA-27a may correlate with the development of drug resistance by regulating the expression of MDR1/P-glycoprotein targeting HIPK2 in ovarian cancer cells; deregulation of miR-214, miR-199a, miR-200a, and miR-100 has also been demonstrated to occur in ovarian cancers; miR-214 promotes cell survival and cisplatin resistance by targeting the PTEN/Akt pathway; lack of miRNA-31 expression has been linked to a defective p53 pathway in serous ovarian cancer patients, raising hopes that treatment with miRNA-31 may offer an efficacious strategy in the management of such patients; miRNA-125a is a negative regulator of EMT since it induces reversion of highly invasive ovarian cancer cells from a mesenchymal to an epithelial histotype; this finding represents a landmark in ovarian cancer therapeutics since overexpression of EGFR is coupled to EMT in ovarian cancer cells which correlates with poor prognosis; the expression of miRNA-200 family members in ovarian tumors obtained from patients correlated with raised levels of β-tubulin and poor PFS to paclitaxel-based treatment; some miRNAs have been identified as putative tumor suppressor genes in ovarian tumors; thus specific miRNA signatures may be exploited as biomarkers for progression and recurrence of advanced stage ovarian carcinoma patients, and as molecular targets in ovarian cancer.	[68, 387–396]

[†] Granulocyte/macrophage-colony stimulating factor (G/M-CSF); hepatocyte nuclear factor-1β (HNF-1β); human epididymis protein 4 (HE4); osteopontin (OPN); mesothelin (MES); haptoglobin-α (HP-α); Bikunin (BIK); phosphoinositide-3-kinase (PI3K); transforming growth factor-beta (TGF-β); tumor necrosis factor (TNF); urokinase plasminogen activator and its receptor (uPA/R); hyaluronan-binding protein (HBP); extracellular matrix (ECM); folate receptor alpha (FRα); transthyretin (TTR); inter-α-trypsin inhibitor (IαI); C-reactive protein (CRP); prostasin (PRSS); claudin/s (CLDN/s); matrix metalloproteinase-2 (MMP-2); interferon (IFN); apoliprotein A1 (APOA1); high-density lipoprotein (HDL); lysophosphatidic acid (LPA); G-protein coupled receptors (GPCRs); receptor tyrosine kinase (RTK); periostin (POSTN, also called osteoblast specific factor 2, OSF2); kallikrein/s (KLKs); human anterior gradient 2 (AGR2); histone acetyltransferase/s (HAT/s); histone deacetylase/s (HDAC/s); histone deacetylase inhibitors (HDACi); suberoylanilide hydroxamic acid (SAHA); sodium butyrate (NaB); trichostatin A (TSA); multidrug-resistant protein (MDR1, P-glycoprotein); multidrug resistance-associated proteins 1 and 2 (MRP1/2); microRNAs (miRNAs); extracellular matrix (ECM); homeodomain-interacting protein kinase-2 (HIPK2); glycosylphosphatidylinositol (GPI). All these biomarkers are used in various multimodal combinations in the screening/detection of ovarian cancer in high risk women. [‡] For more information, see (http://www.sanger.ac.uk/Software/Rfam/mirna/).

bikunin (BIK) or upregulating *BIK* gene expression in these cells significantly attenuated PI3K/p85 gene expression, and decreased their urokinase-type plasminogen activator- (uPA-) dependent invasive potential in nude mice [292]. Therefore, the molecular targeting of multiple signaling pathways such as EGFR, VEGFR, HIF-1, and PI3K/PTEN/AKT/mTOR may improve responses in recurrent and resistant ovarian cancers [4, 83, 92, 203, 205, 399–403].

3.7. ATP-Binding Cassette (ABC) Drug Transporters. Despite the encouraging response rates of ovarian cancer patients to a combination regimen of carboplatin and paclitaxel, most will experience recurrence and/or relapse. Disease recurrence is mostly associated with the development of multidrug resistance (MDR) which is mediated by the overexpression of tumor ATP-binding cassette (ABC) drug transporters. In ovarian cancer cells, the *ABCB1 (MDR1)* gene encodes P-glycoprotein, which targets to the luminal surface and actively effluxes a wide array of anticancer drugs, including carboplatin and paclitaxel [404–406]. P-glycoprotein expression has been shown to be a predictor of unfavorable response (recurrence) and poor survival in uniformly treated and followed cohorts of advanced ovarian cancer patients [407–409]. Reversal of MDR in ovarian cancer cell lines is possible with siRNA knockout of *ABCB1 (MDR1)* and *ABCB4 (MDR3)* genes [410, 411], combination drug treatments [412, 413], chitosan/pshRNA plasmid nanoparticle targeting of *MDR1* genes [414], and perturbation of P-glycoprotein N-glycosylation [415]. The prognostic value of *ABCB1* gene polymorphisms in ovarian cancer patients is conflicting, for example, whereas a recent study found that ABCB1 G2677T/A and ABCB1 C3435T gene polymorphisms did not correlate with survival and prognosis in Caucasian women with ovarian cancer [416, 417], another study found such a relationship [418]. Analogous earlier reports concluded that although *MDR1* expression profiles may be closely related to histologic subtype of ovarian cancer, they were not accurate predictors of survival [419, 420]. Remarkably, elevated expression of MDR-1 in tumor tissue sampled after first cytoreductive surgery was associated with a higher risk of brain metastases in women with epithelial ovarian, fallopian tube, or peritoneal cancer [421]. Noteworthy also is the observation that chemoresistance induced by IL-6R signaling correlated with enhanced expression of MDR genes (*MDR1 and GSTpi*), antiapoptotic proteins (Bcl-2, Bcl-xL, and XIAP), and upregulation of Ras/MEK/ERK and PI3K/Akt signaling [259]. Undoubtedly, more research is required to unravel the complex expression of the MDR phenotype in ovarian cancers.

4. Candidate Ovarian Cancer Biomarkers as Molecular Targets

Candidate biomarker profiles and the molecular basis for their targeting in ovarian cancers are summarized in Table 1.

5. Conclusion

This aim of this review was to present a broad overview of how improved diagnostic and prognostic specificity and sensitivity of tumor biomarkers and signaling molecules can be translated into more efficacious molecularly targeted therapies that will prevent resistance, recurrence, and relapse in ovarian cancer patients. The different types of ovarian cancers variously express the major hallmarks of cancer such as genomic instability, gain of oncogenes, loss of tumor suppressors, immeasurable self-renewal potential, epithelial-to-mesenchymal transition, and reversed mutational capacities, autocrine signaling and self-sufficiency in growth factor requirements, host immune co-option, escape from immune surveillance and natural killer cell mediated oncolysis, apoptosis evasion, increased DNA repair mechanisms, sustained angiogenesis, invasion, and metastatic spread. The rapid increase in our understanding of the molecular processes that regulate cancer signatures in general has raised an equally strong desire to eradicate ovarian cancer before resistance, recurrence, and relapse can set in and claim more lives. It is becoming increasingly evident that traditional approaches to ovarian cancer management such as surgical debulking and carboplatin-paclitaxel chemotherapy will have to be complemented with molecularly targeted and personalized treatment approaches to impact positively on PFS and OS rates. The molecular therapeutic targeting paradigm and the concept of synthetic lethality as exemplified by BRCA1/2 mutations and PARP inhibition offer profound opportunities for ovarian cancer drug development and discovery. The targeting of multiple signaling pathways such as VEGFR, EGFR, IL-6R-JAK-STAT3/NF-κB, PI3K/AKT/mTOR, and ABC drug transporters in ovarian cancer may be an auspicious start to favourable PFS and OS outcomes. The Wnt/β-catenin signaling pathway should not be overlooked since it has recently been implicated in regulating the immunoreactivity and chemosensitivity to anticancer drugs in ovarian cancer cells, which may be a useful prognostic indicator in patients with ovarian cancer [422]. The interaction between MUC16 and MES should be seen as an opportunity to block intra- and extraperitoneal metastasis of highly aggressive ovarian cancers and to develop effective antibodies and vaccines against this type of cancer which is a major contributor to the high mortality rate among women worldwide. Finally, candidate or emerging biomarkers, especially HDACi and miRNAs, and their molecular interactions with cancer signaling pathways should be translated into cross-spectrum and individualized therapies for the different histological subtypes of ovarian cancer.

Conflict of Interests

The author declared that he has no conflict of interest.

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Review Article

Integrin Inhibitors as a Therapeutic Agent for Ovarian Cancer

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Ovarian cancer is a deadly disease, with a cure rate of only 30%. Despite aggressive treatments, relapse remains almost inevitable in patients with advanced-stage disease. In recent years, great progress has been made towards targeting integrins in cancer treatment, and clinical studies with various integrin inhibitors have demonstrated their effectiveness in blocking cancer progression. Given that the initial critical step of ovarian cancer metastasis is the attachment of cancer cells onto the peritoneum or omentum, in addition to the proven positive clinical results of anti-angiogenic therapy, targeting integrins is likely to be one of the most feasible approaches. This paper summarizes the current understanding of the integrin biology in ovarian cancer metastasis and the various therapeutic approaches attempted with integrin inhibitors. Although no integrin inhibitors have shown favorable results so far, integrin-targeted therapies continue to be a promising approach to be explored for further clinical investigation.

1. Introduction

Ovarian cancer is a highly metastatic disease characterized by widespread peritoneal dissemination and ascites and is the leading cause of death from gynecologic malignancies. It is often diagnosed at a late stage after tumor cells are disseminated within the peritoneal cavity. Despite aggressive treatments which consist of surgical cytoreduction and chemotherapy, more than two-thirds of all patients succumb to the disease within 5 years [1]. The initial step of ovarian cancer metastasis is that cancer cells, detached from the ovarian surface epithelium, attach to the layer of mesothelial cells that line the inner surface of the peritoneum. Several integrins have been identified as important mediators of ovarian carcinoma metastasis to the mesothelium, suggesting that integrin inhibitors could be a new therapeutic strategy to prevent cancer cells from attaching onto the peritoneal cavity. During the last 10 years, novel insights into the mechanisms that regulate cell survival as well as cell migration and invasion have led to the development of novel integrin inhibitors for cancer treatments [2]. In this short review, we describe the critical roles of integrins during the metastatic process of ovarian carcinoma and discuss the potential of integrin inhibitors as a new therapeutic agent for the treatment of ovarian cancer.

2. Biology of Integrin

The role of integrins in cell migration and invasion is one of their most studied functions in tumor biology [3, 4]. Integrins are cellular surface glycoprotein receptors consisting of a heterodimer of α- and β-subunits that are mutually noncovalently associated. In mammals, integrins have extensive distributions throughout the whole body, and there are 18 α- and 8 β-subunits assembling 24 functionally different heterodimers [5, 6]. Each individual integrin subunit has a large extracellular domain, a single membrane-spanning domain and a short noncatalytic cytoplasmic tail. The assembled integrin heterodimer can bind to a unique set of ligands. Natural integrin ligands include the components of the extracellular matrix (ECM) such as collagen, laminin, fibronectin, and vitronectin. Many integrins bind their ligands by recognizing the short amino acid sequences on exposed loops, such as Arg-Gly-Asp (RGD) (integrin $\alpha 5\beta 1$) or Arg-Glu-Asp-Val (REDV) (integrin $\alpha 4\beta 1$). On ligation to the ECM, integrins recruit complex signaling events, alone or in combination with growth factor receptors. Integrin signaling regulates diverse functions in tumor cells, including migration, invasion, proliferation, and survival through the activation of various pathways, such as integrin-linked kinase (ILK), mitogen-activated protein kinase (MAPK),

protein kinase B (PKB/Akt), or nuclear factor kappa B (NF-κB) [7]. In recent years, great progress has been made towards targeting integrins in cancer treatment. Preclinical as well as clinical studies with various integrin antagonists have demonstrated their effectiveness in blocking tumor progression [3]. Almost all such Phase 1 clinical trials showed that the integrin inhibitors are nontoxic and well tolerated by patients, suggesting that they can be used concurrently with the conventional cytotoxic chemotherapy or radiotherapy. Some reports showed that radiotherapy results in up-regulation of integrin expression in several types of cancer, leading to cellular resistance to radiotherapy-induced cancer cell death [8, 9]. Nam et al. demonstrated in their preclinical works that targeting β1-integrin enhances the efficacy of radiation therapy in several cancers including breast cancer [9]. Integrins are also involved in innate multidrug resistance, allowing tumor cells to survive chemotherapy (cell-adhesion-mediated drug resistance: CAM-DR) [8]. It has been proposed that CAM-DR is caused by the activation of β1-integrin-stimulated tyrosine kinase that suppresses apoptosis from chemotherapy [10, 11]. Integrin-targeted therapies in addition to conventional cytotoxic treatments, thus, have great potential to enhance the efficacy of overall treatments with minimal side effects.

3. Ovarian Cancer Metastasis and Current Treatment Options

In 2010, the American Cancer Society estimated that there were 21,880 cases of epithelial ovarian carcinoma and 13,850 disease-related deaths, identifying that ovarian cancer has the highest mortality rate of all gynecologic tumors. Sixty-three percent of all patients with ovarian carcinoma will succumb to their disease, making it the fifth leading cause of cancer death among USA women [12]. The high mortality of this tumor is largely explained by the fact that the majority of patients present at an advanced stage, with widespread metastatic disease within the peritoneal cavity. Only 20% of ovarian cancers are diagnosed while they are still limited to the ovaries, and patients at this early stage have an 85–90 percent 5-year survival [13].

In spite of several efforts made for early screening of ovarian cancer, no effective screening methods have been established to reduce ovarian cancer incidence and mortality [14]. Current treatment strategies for advanced ovarian carcinoma consist of aggressive "cytoreductive" or "tumor-debulking" surgery, followed by a combination of platinum- and taxane-based chemotherapy. The surgical treatment goal is "optimal" surgical cytoreduction, which is generally defined as residual disease of 1 cm or less. No gross residual tumors should be left throughout the abdominal cavity, because several studies have convincingly shown that cytoreduction results in improved patient survival [15, 16]. This effect of cytoreduction is indicative of a dramatic difference in the biological behavior of ovarian cancer as compared with other malignancies, because in most other cancers the removal of metastatic tumors does not necessarily lead to improved survival [13]. One of the main reasons for this difference is that, unlike other malignancies, ovarian cancer directly disseminates within the abdominal cavity and rarely disseminates through the vasculature unlike other malignancies, although metastasis in pelvic and/or para-aortic lymph nodes can be found occasionally [17]. Once the cancer cells have detached as single cells or clusters from the primary ovarian tumor, it is thought that they metastasize through a passive mechanism, carried by the physiological movement of peritoneal fluid to the peritoneum and omentum. However, in spite of the execution of primary aggressive cytoreductive surgery as well as meticulously-designed chemotherapy regimens, the overall cure rate of ovarian cancer patients remains approximately 30% [13]. Even though no apparent tumors remain throughout the peritoneal cavity after the initial surgery, invisible cancer cells are left and endure through the postoperative chemotherapy. Small numbers of drug-resistant cells can persist for many months and remain dormant in the peritoneal cavity, only to grow progressively, leading to death of the patient despite aggressive treatment of the recurrent disease. There is, thus, a critical need for novel targeted therapies to overcome this situation. In particular, efficacious consolidation or maintenance therapy after the cytoreduction surgery needs to be explored.

Novel molecularly directed therapies which aim to target tumor cells and the tumor microenvironment in ovarian tumorigenesis are rapidly emerging. Antiangiogenic agents have led the field so far. Preclinical and clinical studies have demonstrated the efficacy of antiangiogenic approaches against ovarian cancer both alone and in combination with cytotoxic chemotherapy [18]. Bevacizumab, a humanized monoclonal antibody directed against VEGF, has been tested in several epithelial malignancies, including ovarian cancer. Several prospective Phase II trials have shown that bevacizumab in combination with chemotherapy (carboplatin-paclitaxel, cyclophosphamide, or topotecan) is efficacious in advanced ovarian cancer [19], and Phase III evaluation is currently ongoing. Although these results are promising and it appears to be clear that bevacizumab is efficacious in a subset of ovarian cancer patients, resistance to bevacizumab is a major obstacle even for patients in whom bevacizumab was initially efficacious [18]. One potential alternative treatment option is targeting integrins, which regulate diverse functions in tumor cells including adhesion, migration, invasion, proliferation, and survival. In addition to tumor cells, integrins are also found on tumor-associated host cells, such as the vascular endothelium, fibroblasts, or bone marrow-derived cells. Targeting integrin signaling has the potential to inhibit the contribution of these cell types to cancer progression [3]. Several integrin-targeted therapeutic agents are emerging and currently in clinical trials for cancer therapy including ovarian carcinoma.

4. Integrin Biology in Ovarian Cancer

Most ovarian cancer cells are derived from the epithelial cells that cover the surface of the ovary [1]. Before the ovarian carcinoma cells detach from the basement membrane, they often undergo an epithelial-mesenchymal transition (EMT),

which loosens the intercellular adhesions between the cancer cells. EMT often starts from the loss of E-cadherin, one of the molecules crucial for the adhesion between neighboring epithelial cells. During the process of EMT, cancer cells acquire a more invasive phenotype and proliferate and spread throughout the abdominal cavity, carried by the physiological movement of massive ascites. Indeed, the knockdown of E-cadherin was reported to induce the up-regulation of the fibronectin receptor, $\alpha 5\beta 1$-integrin, which promotes the adhesion of ovarian cancer cells to secondary metastasis sites, such as omentum and peritoneum [17, 20]. According to an immunohistochemical analysis using clinical samples conducted at the University of Chicago (Chicago, IL), about 40% (42 of 107) advanced (Stages II–IV) ovarian cancer patients showed $\alpha 5\beta 1$-integrin positive staining. Among these positive cases, 10 cases (9%) were considered to show overexpression and the median survival of the patients with $\alpha 5\beta 1$-integrin overexpression was significantly worse (26 months) than that of those with low or negative integrin expression (35 months) [20]. Once the cancer cells have detached from the primary tumor, they float in the ascites as single cells or as multicellular spheroids. Casey et al. reported that the $\beta 1$-integrin stimulating antibody or exogenous treatment with fibronectin promoted the spheroid formation of ovarian cancer cells, while blocking antibodies against $\alpha 5$- or $\beta 1$-integrin inhibited the formation, indicating that interactions between $\alpha 5\beta 1$-integrin and fibronectin mediate the formation of ovarian carcinoma spheroids and their adhesion to ECMs at the secondary tumor growth sites [21]. The initial key step of ovarian cancer metastasis is the attachment of ovarian cancer cells onto the layer of mesothelial cells which cover the peritoneal cavity. Integrins have also been identified as important mediators between ovarian carcinoma and the mesothelium. Strobel and Cannistra reported that blocking antibodies against $\alpha 5$- and $\beta 1$-Integrin as well as RGD peptide inhibited the binding of ovarian cancer cells to mesothelial cells, suggesting that $\alpha 5\beta 1$-integrin was the major receptor responsible for fibronectin-mediated ovarian cancer binding to the mesothelium [22]. These accumulating results strongly suggest that inhibition of $\alpha 5\beta 1$-integrin is a potential new therapeutic target, at least for a subset of ovarian cancer patients [21]. Not only fibronectin but also collagen and laminin are the most abundant extracellular proteins in the mesothelium covering the peritoneum and the omentum. Primary ovarian carcinoma cells adhere preferentially to type I collagen, which can be blocked with an $\alpha 2\beta 1$-integrin antibody [23]. The other important adhesion molecules which interact with cancer cells and the mesothelial cells are $\alpha 4\beta 1$-integrin and its adhesion receptor, cell adhesion molecule-1 (VCAM-1) [24]. $\alpha 4\beta 1$-integrin expressed on ovarian carcinoma cells binds to VCAM-1, which is present on the mesothelial cells and function-blocking antibodies directed against VCAM-1 and $\alpha 4\beta 1$-integrin block migration and metastasis in a xenograft model [24]. The expression of $\alpha v\beta 6$ integrin in ovarian cancer cell lines correlates with the invasive potential of cells by inducing the secretion of proteinases such as urokinase plasmin activator (uPA) and matrix metalloproteinases (MMPs) [25]. Inconsistent results have been reported regarding the role of the vitronectin receptor, $\alpha v\beta 3$-integrin, in ovarian cancer metastasis. Although it was initially thought to be expressed on aggressive ovarian cancer cells and to be correlated with ovarian cancer cell adhesive, migratory, and proliferative properties, recent data question this assertion and indicate that it is expressed on well-differentiated tumors and acts as a tumor suppressor in ovarian cancer [17]. Kaur et al. reported that $\alpha v\beta 3$-integrin-expressing ovarian cancer cells showed impaired invasion, protease expression, and colony formation and that patients with tumors expressing high levels of $\beta 3$-integrin had significantly better prognoses [26]. Given that Reynolds et al. recently showed that nanomolar concentrations of RGD-mimetic $\alpha v\beta 3$-/$\alpha v\beta 5$-integrin inhibitors enhance tumor growth and tumor angiogenesis in preclinical xenograft models [27], therapies aimed at blocking $\alpha v\beta 3$-integrin may have detrimental effects.

5. Clinical Trials Targeting Ovarian Cancer

Preclinical studies have shown that integrin antagonists inhibit tumor growth by affecting both tumor cells and tumor-associated host cells, especially the angiogenic endothelium. Integrin antagonists currently in clinical trials include monoclonal antibodies and Arg-Gly-Asp (RGD) peptide mimetics [3, 31]. The candidate integrin inhibitors which could be applied for ovarian cancer treatment are summarized in Table 1 [20, 26, 29, 30]. Volociximab, a chimeric monoclonal antibody directed against $\alpha 5\beta 1$-integrin, inhibits angiogenesis and impedes tumor growth. Bell-McGuinn et al. reported on their Phase II data of platinum-resistant ovarian cancer patients treated with volociximab as a monotherapy [32]. Of 14 patients who were evaluable for efficacy, only one patient had stable disease at 8 weeks, and the remaining 13 progressed on treatment, although weekly volociximab was well tolerated. Beside the antibodies, synthetic peptides that mimic the structure of natural integrin binding ligands are alternative candidates for integrin inhibitors [6]. ATN-161 is a non-RGD-based pentapeptide binding to $\alpha 5\beta 1$- and $\alpha V\beta 3$-integrins, derived from fibronectin by replacing an arginine residue of the primary sequence with cysteine moiety [6]. It has been shown to inhibit tumor growth, angiogenesis, and metastasis in multiple animal models [28, 33]. In Phase I safety trials, ATN161 was well tolerated, and several patients exhibited stable disease, including one ovarian carcinoma [34]. Since the 1990s, $\alpha v\beta 3$-integrin has been identified as a target for antiangiogenic therapy, as it expresses in proliferating vascular endothelial cells and regulates endothelial cell migration in sprouting vessels [24]. LM609, a mouse anti-human monoclonal antibody raised against $\alpha V\beta 3$-integrin, showed considerable antiangiogenic activity in preclinical models [35]. As a result of these studies, etaracizumab (MEDI-522), a humanized version of LM609, was developed as one of the first integrin antagonists introduced into clinical trials. However, clinical trials found it to have limited effectiveness as a metastatic cancer treatment, probably owing to the single integrin ($\alpha V\beta 3$-) targeting [6, 36]. The human αv-integrin specific monoclonal antibody, intetumumab (CNTO-95),

Table 1: Candidate integrin inhibitors for ovarian cancer treatment.

Drug name	Type	Target	Preclinical data in gynecologic cancer	Manufacturer	Ref.
Volociximab (M200)	Chimeric antibody	$\alpha5\beta1$	*i.p.* treatment reduced tumor burden and ascites in SKOV-3ip1 ovarian cancer mouse xenografts by 83% and 97%, respectively.	Protein Design Labs	[20]
ATN-161	Peptide	$\alpha5\beta1$	*i.v.* (1 mg/kg) injection inhibited the outgrowth of metastases at lung, liver, or spleen in a metastasis model mouse of MDA-MB-231 breast cancer cell lines.	Attenuon LLC	[28]
Etaracizumab (MEDI-522)	Humanized antibody	$\alpha v\beta3$	*i.p.* treatment decreased tumor burden in the SKOV3ip1 and the HeyA8 mouse models by 36 and 49%, respectively and reduced the number of proliferating cells but not microvessel density.	Medimmune	[29]
Intetumumab (CNTO95)	Human antibody	$\alpha v\beta3$ $\alpha v\beta5$	Low doses (0.15–1.25 μg/mL) of intetumumab were effective in inhibiting adhesion and migration of 6 uterine serous papillary carcinoma cell lines i*n vitro*.	Centocor	[30]
Cilengitide (EMD-121974)	Peptide	$\alpha v\beta3$ $\alpha v\beta5$	$\alpha v\beta3$-integrin overexpression on SKOV3ip1 cells impaired invasion, protease expression, and colony formation *in vitro*. Cilengitide may have detrimental effects against ovarian cancer.	Merck KGaA	[26]

which targets both $\alpha v\beta3$- and $\alpha v\beta5$-integrins, also showed antitumor and antiangiogenic effects in xenograft tumor models [37, 38]. In a Phase I clinical trial, intetumumab was nontoxic, localized to tumors, and showed signs of antitumor activity [39]. A complete response imaged by FDG-PET was observed in one patient with ovarian carcinosarcoma whose disease remained stable for 6 months while receiving intetumumab [40]. This antibody should be further evaluated in additional clinical trials. Among various available RGD mimic peptides, cilengitide (c-[RGDf(NMe)V-]) has emerged as a promising agent. It can bind to both $\alpha V\beta3$- and $\alpha V\beta5$-integrins with high affinity and inhibit their function strongly [6]. Cilengitide has shown significant promise in patients with late-stage glioblastoma by extending patient survival with minimal side effects [41, 42]. It is currently being tested in Phase II trials in patients with lung and prostate cancer, and Phase II and Phase III trials are currently underway for glioblastoma [3]. However, in moving toward ovarian cancer clinical trials with cilengitide, a serious concern needs to be addressed. As noted above, Kaur et al. suggested that increased $\alpha v\beta3$-integrin expression on ovarian cancer cells correlates with a favorable outcome and that inhibiting its activity could increase the severity of the disease [26]. Therefore, it is critical to further investigate and clarify the effects of anti-$\alpha v\beta3$-integrin therapy on ovarian cancer tumors and the surrounding endothelial cells, before embarking on clinical therapeutic trials [18].

6. Conclusion

Recognition of the need for cytoreduction along with the evolution of surgical techniques and the establishment of chemotherapy regimens through multiple clinical trials allows a majority of ovarian cancer patients to achieve "disease-free" status after the initial treatment. One of the major disappointments with the current ovarian cancer treatments is failure to achieve a complete cure, even in optimally debulked or chemosensitive patients. The establishment of efficacious consolidation or maintenance therapies would be a powerful tool for improving the miserable outcomes of patients with advanced-stage disease.

The biological behavior of ovarian carcinoma is unique, differing from the classic and well-studied pattern of hematogenous metastasis found in most other cancers. Once ovarian cancer cells have detached as single cells or clusters from the primary ovarian tumor, they are carried by the physiological movement of peritoneal fluid and finally metastasize to the peritoneum and omentum, suggesting that the attachment of cancer cells onto the mesothelial cells covering the basement membrane is the initial key step in metastasis. Bevacizumab has already shown significant utility in ovarian cancer treatment not only in combination with current chemotherapy but also as a single agent, indicating that antiangiogenic therapy has considerable promise. Given that targeting integrins can affect not only the diverse functions of tumor cells, including adhesion, migration, invasion, proliferation, and survival, but also tumor microenvironments, especially the angiogenic endothelial cells, integrin inhibitors obviously have the potential for clinical use in the near future. Unfortunately, although several clinical trials have been attempted against ovarian cancer, no integrin inhibitor has shown sufficiently promising efficacy to progress to further clinical investigation; the agents targeting only a single integrin, such as $\alpha v\beta3$ and $\alpha5\beta1$, failed to show evident clinical benefits in metastatic cancer treatment. In cancer progression, more than one integrin pathway is involved. For

example, even if inhibition of the function of $\alpha 5\beta 1$-integrin as a fibronectin receptor could be adequately achieved, the other integrins, such as $\alpha v\beta 3$ or $\alpha 3\beta 1$, would eventually compensate for its function. Therefore, a combination of different integrin receptor pathways is likely to be more effective in the clinical setting and should be explored for the future clinical application.

Collectively, although there remain many questions and challenges, integrin-targeted therapies continue to be a promising approach to improve the outcomes of women with ovarian cancer.

Conflict of Interests

The authors state no conflict of interests and have received no payment in preparation of this paper.

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Review Article

Ovarian Cancer: Opportunity for Targeted Therapy

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Ovarian cancer is a common cause of cancer mortality in women with limited treatment effectiveness in advanced stages. The limitation to treatment is largely the result of high rates of cancer recurrence despite chemotherapy and eventual resistance to existing chemotherapeutic agents. The objective of this paper is to review current concepts of ovarian carcinogenesis. We will review existing hypotheses of tumor origin from ovarian epithelial cells, Fallopian tube, and endometrium. We will also review the molecular pathogenesis of ovarian cancer which results in two specific pathways of carcinogenesis: (1) type I low-grade tumor and (2) type II high-grade tumor. Improved understanding of the molecular basis of ovarian carcinogenesis has opened new opportunities for targeted therapy. This paper will also review these potential therapeutic targets and will explore new agents that are currently being investigated.

1. Introduction

Ovarian cancer is the most common cause of gynecologic neoplasm and is the fifth cause of cancer mortality in women. The high mortality rate in women with epithelial ovarian cancer (EOC) is due to its detection at advanced stages. Even though there have been improvements in surgical techniques and treatment options, five-year survival for stage III and IV ovarian cancer still remains at approximately 45% [1].

Known risk factors of EOC include nulliparity, early menarche, late menopause, and age. A particularly significant risk factor is a strong family history of breast and ovarian cancer. 10%–15% of women with ovarian cancer have genetic predispositions of BRCA1 and BRCA 2 mutations [2]. BRCA1 is associated with a 40% lifetime risk of ovarian cancer, and BRCA 2 has an approximately 15% lifetime risk of ovarian cancer. Epidemiological studies show a reduction in the incidence of EOC in developed countries [2].

Part of the complexity of EOC lies in its heterogeneity. EOC can be classified into diverse group of tumors on the basis of morphology and molecular genetic features. This paper will review the current understanding of the molecular and morphologic heterogeneity of EOC as well as possible explanations of pathogenesis that contribute to the heterogeneity.

2. Tumor Origin and Pathogenesis

EOC origins are difficult to ascertain, because the majority of cases are diagnosed at late stages. Thus, there are limited records regarding early-stage disease. Historically, EOC is thought to originate from the ovarian epithelial surface and undergoes progressive dedifferentiation and spreads to the pelvic and abdominal cavities prior to metastasizing to distant organs [2, 3]. However, EOC which predominantly consists of serous, endometrioid, and mucinous cell types is morphologically columnar and ciliated, similar to Mullerian epithelial cell lining of the endometrium, endocervix, fallopian tube, and gastrointestinal tract [3]. The ovarian epithelial surface, where these cells are purported to have originated from, consists of a single mesothelial layer of cells that are flattened and squamous-like. To explain this discrepancy, the traditional theories suggest that the mesothelial lining of the ovary invaginates to form paraovarian cysts that acquire

Mullerian cell lining features and undergo malignant transformation [4]. The enlarging tumor envelops the ovary and is diagnosed as an adnexal mass of ovarian origin [5, 6].

Increasing evidence now suggests that the Fallopian tube may be an alternative site of tumor origin in many diagnosed as primary EOC [5]. In older studies, the origin of EOCs were presumed to be the ovaries, and Fallopian tubes were typically not examined. However, more recently, observational studies have shown that in situ and early invasive tubal carcinomas occur in women with a genetic predisposition for ovarian cancer [5, 7, 8]. Furthermore, over 70% of nonhereditary ovarian cancer and peritoneal high-grade serous carcinomas revealed serous epithelial carcinoma in the Fallopian tube and mucosal tubal involvement [9].

The fimbria of the Fallopian tube are abundant with angiolymphatic vasculature and are in direct contact with the basement membrane of the Fallopian tube. Through this vasculature, the serous tubal intraepithelial carcinoma may conceivably disseminate to the surface of the ovary and peritoneum without invasive growth from the Fallopian tube [10, 11]. Therefore, rather than the tumor originating from a cyst that developed from the mesothelial lining of the ovary, tubal epithelium may directly implant into the surface of the ovary to form an inclusion cyst which subsequently develops into tubal epithelial carcinoma [3, 10]. An alternative possibility is that normal tubal intraepithelial cells implant into the ovary at the time of ovulation and develop into inclusion cysts that transform into carcinoma over time [4, 10].

Similarly, the endometrioid and clear cell carcinomas are thought to originate from endometriosis. According to this theory, endometrial cells "escape" the uterus via retrograde menstrual flow and implant the ovary or pelvic cavity secondarily. This mechanism has been supported by multiple morphologic and molecular studies [12, 13].

3. Morphologic and Molecular Characteristics

EOC was initially categorized into invasive serous carcinoma and serous borderline tumor (SBT) which was defined as a low malignant potential carcinoma lacking invasive growth. More recently, SBT was further subdivided into (1) atypical proliferative serous tumor (APST) and (2) micropapillary serous carcinoma (MPSC), a possible precursor to low-grade serous carcinoma (LGSC) [10]. Previously, serous carcinoma was thought be a spectrum of disease, where LGSC progressed to high-grade serous carcinoma (HGSC) over time. However, with the understanding that LGSC arises from SBT, high-grade invasive carcinoma is thought to be a disparate entity. This resulted in a model of ovarian carcinogenesis that consists of two distinct pathways and subtypes [3, 4, 14].

The two subtypes are low-grade (type I) and high-grade (type II). Type I tumors are typically indolent, slow growing tumors that are often detected at early stages. Type I tumors include low-grade serous, low-grade endometrioid, clear cell, and mucinous carcinomas [15, 16]. Type II includes high-grade serous, high-grade endometrioid, mixed mesodermal (carcinosarcoma), and undifferentiated carcinomas. Type II tumors are typically diagnosed in advanced stages. The majority of EOC, approximately 75%, are type II aggressive tumors [17, 18]. Type I tumors have a median survival of 81 months compared to 57 to 65 months in type II tumors.

Molecular and genetic differences are now being recognized to further understand the distinction between these two subtypes. The main genetic difference between the two subtypes that explains duality in their malignant potentials is (1) type I tumors have genetically stable and isolated mutations and (2) type II tumors have significant genetic instability and involve p53 mutations that result in a more aggressive and invasive phenotypic expression [4].

Mutational analysis and genetic expression studies have shown that APST, MPSC, and LGSC share molecular mutations that are significantly different from molecular alterations in HGSC [10]. Type I tumors typically involve mutations in a number of genes such as KRAS, BRAF, PTEN, PIK3-CA, and CTNNB1 which encodes beta-catenin [10, 14]. Mutations in these upstream regulators result in constitutive activation of the MAPK signaling pathway. Up to 70% of MPSC and LGSC have been shown to express active MAPK signaling [4, 15]. Her2/neu mutations have been detected in approximately 10% of Type I tumors and appear to be mutually exclusive with having KRAS and BRAF mutations [10, 15]. Type I tumors rarely express p53 mutations. Furthermore, to further explain the phenotypic association between LGSC and MPSC that are not shared with APST, MPSC is more molecularly similar to LGSC than APST. On the basis of mutational analysis of clear cell carcinomas, the PI3K/PTEN pathway appears to be the most commonly deregulated [19]. While PTEN mutations are present in low-grade endometrioid histologies, they also have alterations in the Wnt/β-catenin pathways and CTNNB1 mutations [20]. Therefore, among the type I tumor subtypes, variable genetic alterations have been identified that explain their phenotypic differences.

In contrast, type II tumors have TP 53 mutations in up to 95% of the cases. They are also characterized by genetic instability and high frequencies of DNA copy number gains and losses. They rarely contain KRAS and BRAF mutations. A recent publication in Nature identified 9 significantly mutated genes in high-grade serous ovarian cancer. The most common were RB1 mutations (67%), TP 53 mutations (96%), PI3/Ras pathways (47%), and BRCA 1 or 2 mutations (22%) [21]. De novo, nonfamilial cases of BRCA1 and BRCA2 inactivation are often associated with hypermethylation. In a genomic analysis of high-grade ovarian cancer, 11% of BRCA1 silencing was the result of hypermethylation and epigenetic modification rather than mutations. Studies have shown that mutated or hypermethylated BRCA carriers respond to PARP inhibitor therapy [22, 23].

4. MicroRNA

MicroRNAs are short nucleotide sequences that are noncoding RNAs that are involved in the regulation of posttranscriptional genes. MicroRNAs are critical in cell development but may also contribute to tumor origin [24–27]. MicroRNAs have been found to be differentially expressed between ovarian carcinoma compared to normal ovarian epithelial cells [28]. Upregulation or downregulation of microRNA

that regulate oncogenes and tumor suppressor genes, respectively, can induce malignant transformation. Dysregulation of microRNA expression has been associated with high-grade serous ovarian carcinoma. The relationship between such microRNA dysregulation and BRCA 1 or 2 expression is being explored [28]. Furthermore, microRNA have previously been found to regulate cellular differentiation [29, 30] and may play a role in epithelial to mesenchymal cell transformation during carcinogenesis of ovarian epithelial cells. Exploration of microRNA involvement in ovarian carcinogenesis has recently focused attention on Let-7 microRNA and HMGA2 gene regulation.

5. HMGA2 and Let-7

HMGA2, a high-mobility-group AT-hook protein, is a nonhistone DNA-binding factor that attaches to AT-rich sequences in the minor groove of the DNA helix. It is an important regulator of cell growth, differentiation, apoptosis, and malignant transformation. HMGA2 expression is regulated by the microRNA Let-7. Preclinical studies have evaluated the role of let-7 as a tumor suppressor gene to the oncogenic mechanism of HMGA2 [31]. When the let-7 activity is downregulated, HMGA2 activity is no longer repressed and contributes to malignant transformation [31]. HMGA2 overexpression has been identified in 65% of ovarian carcinoma but are rarely expressed in normal ovarian epithelial cells [32–34]. Furthermore, HMGA2 overexpression has been associated with high-grade serous ovarian carcinoma [32, 33]. The Let-7 HMGA2 dysregulation may be a key factor in ovarian carcinogenesis distinguishing low-grade or type I from high-grade or type II EOC [35].

In our institution, we compared the HMGA2 expression in serous OC and endometrioid OC surgical specimens. In this study, we attempted to distinguish expression patterns on the basis of the phenotypic grade of the OC. Fourteen consecutive endometrioid OC were analyzed and twelve consecutive high-grade serous carcinomas were analyzed for HMGA2 expression by immunohistochemical staining. High-grade serous carcinoma was associated with greater HMGA2 expression compared to endometrioid OC. Longitudinal evaluation is pending to evaluate the prognostic significance of HMGA2 expression and clinical outcome [41].

Other studies have further evaluated the correlation of HMGA2 overexpression and p-53 mutations [32] further demonstrating the tumorigenesis of type II high-grade EOC that is distinct from type I low-grade EOC. Furthermore, animal models have shown that HMGA2 silencing is associated with reduction in tumor growth and increased apoptosis of tumor cells [34]. This suggests that HMGA2 may be a possible target in ovarian cancer therapy.

6. Opportunities for Targeted Therapy

Despite optimal surgical and cytotoxic treatment of advanced ovarian cancer, only 10% to 15% achieve long-term remission, and the majority will face recurrent disease [42, 43]. While there is a role for chemotherapy in recurrent disease, the effects are often short lived due to development of chemoresistance [44]. Targeted therapies in ovarian cancer are currently being investigated to find novel ways to overcome chemoresistance. These molecular targets of therapy include: VEGF (antiangiogenesis), EGFR tyrosine kinase, HER2 receptor, PARP, and MAPK/BRAF/MEK pathways (See Table 1).

7. Antiangiogenesis

Angiogenesis has been shown to be a key component in ovarian cancer metastasis and ascites development [45]. Vascular endothelial growth factor (VEGF) is a critical regulator of angiogenesis and is involved in various aspects of ovarian carcinogenesis [46]. Antiangiogenic therapy has been shown to have activity in ovarian cancer. Bevacizumab is a monoclonal antibody that targets VEGF-A. Two phase II trials with single-agent bevacizumab have shown 16% to 21% response [47, 48]. Phase III studies involving chemotherapy with or without bevacizumab presented during the 2011 Annual Meeting of the American Society of Clinical Oncology (ASCO) showed a trend toward an overall survival benefit in patients treated with bevacizumab in addition to chemotherapy in the first line setting with HR = 0.64 P = 0.0022 in those with poor prognosis disease (ICON7). Similar data were reported in patients treated for platinum sensitive recurrent ovarian cancer with median overall survival of 35.5 months (+bevacizumab) versus 29.9 months (−bevacizumab) and HR = 0.751 P = 0.094 (OCEANS) [49].

Mammalian target of rapamycin (mTOR) is involved in cell growth and proliferation and induces increases in VEGF and platelet derived growth factor (PDGF) that ultimately activate angiogenesis. mTOR inhibitors have also shown single agent activity in the treatment of clear cell ovarian carcinoma [50]. The antiangiogenic effects are thought to be synergistic with that of bevacizumab. Results of a phase II study combining the mTOR inhibitor, temsirolimus, with bevacizumab suggest possible synergy [49]. Other phase I and II trials involving antiangiogenic therapy are showing activity as single agents or synergistically with chemotherapy. These agents include (1) VEGF trap (Aflibercept) which is a fusion protein that acts as a high-affinity VEGF receptor blocker, (2) Sunitinib (a PDGF and VEGF receptor inhibitor), (3) Vatalanib (a pan-VEGF receptor inhibitor), (4) Motesanib (a multikinase inhibitor of PDGF, VEGF, and cKIT), and (5) Cedarinib (a VEGF tyrosine kinase receptor inhibitor).

8. Anti-HER2 Therapy

Her2 expression in ovarian cancer has been variable. Many studies evaluate HER2 expression in ovarian cancer as a positive or negative expression and do not describe the staining intensity to characterize the overexpression of HER2 [51]. The studies that have evaluated HER2 overexpression demonstrate significant variability in intense HER2 staining from 1.8% to 35% [43, 51–53]. Studies have suggested that gene amplification does not always correlate with HER2

Table 1: Potential targets of therapy in ovarian cancer.

Targets	Inhibitors	Studies	NCI study
VEGF	Bevacizumab	Phase III [49]	GOG 218 ICON 7 GOG 213 OCEANS
VEGF TKI	Sunitinib	Phase II	NCT00979992 NCT00768144 NCT00388037
	Vatalanib	Phase I (+doce)	NCT00268918
	Sorafenib	Phase II (+carb/pacli)	NCT00390611
		Phase II (+topo)	NCT01047891
		Phase II	NCT00522301
		Phase II (+bev)	NCT00436215
		Phase II [36]	NCT00093626
		Phase II (+carb/pacli)	NCT00096200
	Vandetanib	Phae I/II (+bortezomib)	NCT00923247
		Phase II	NCT00445549
		Phase II(+/− doce)	NCT00872989
	Cediranib	Phase I/II(+olaparib)	NCT01116648
		Phase II	NCT00275028
		Phase II (+temsirolimus)	NCT01065662
	Pazopanib	Phase I/II (+cyclophosphamide)	NCT01238770
		Phase II	NCT01262014
		Phase I/II [37]	NCT01035658
		Phase II	NCT00281632
		Phase III	NCT00866697
	Vargatef	Phase I (+everolimus)	Pending
	AMG 706	Phase II	NCT00574951
VEGF TRAP	Aflibercept	Phase II	NCT00327171
		Phase II (+doce)	NCT00436501
PI3K-PTEN-Akt-mTOR pathway	Temsirolimus	Phase I (+lip Doxo)	NCT00982631
		Phase II	NCT00926107
		Phase II (carb/pacli)	NCT01196429
		Phase II	NCT00429793
		Phase I/II(+bev)	NCT01010126
	Everolimus	Phase II (+bev)	NCT01031381
		Phase II (+/− bev)	NCT00886691
		Phase II (+lip doxo)	NCT01281514
	Ridaforolimus	Phase I (+carb/pacli)	NCT01256268

Table 1: Continued.

Targets	Inhibitors	Studies	NCI study
EGFR	Cetuximab	Phase II (carb/pacli)	NCT00063401/
		Phase II (+carb)	NCT00086892
	Erlotinib	Phase II (+bev) [38]	NCT00130520
		Phase II (+topo)	NCT01003938
		Phase I/II (+carb/pacli)	NCT00217529
		Phase II (+carb)	NCT00030446
		Phase II (+bev after carb/pacli/bev)	NCT00520013
	Lapatinib	Phase II (+topo) [39]	NCT00436644
		Phase II	NCT00113373
		Phase I/II (+carb/pacli)	NCT00316407
HER2	Trastuzumab	Phase II [40]	
	Pertuzumab	Phase II	NCT00058552
		Phase II (+gemcitabine)	NCT00096993
	Lapatinib	See above	
PARP	ABT 888	Phase II (+temoz versus lip doxo)	NCT01113957
		Phase II (+cyclophosphamide)	NCT01306032
		Phase I/II (+topo)	NCT01012817
		Phase I (+carb/pacli/bev)	NCT00989651
	Olaparib	Phase II (+carb/pacli)	NCT01081951
		Phase I (+carb)	NCT00647062
		Phase II	NCT00679783
		Phase II	NCT00494442
		Phase II (versus lip doxo)	NCT00628251
		Phase II	NCT00753545
		Phase II	NCT01033123
	Iniparib*	Phase II (carb/gemcitabine)	NCT01033292
		Phase II	NCT00677079
Epigenetic	Decitabine	Phase II (+carb)	NCT00477386
		Phase I (+doxorubicin/vaccine)	NCT00887796
	Belinostat	Phase I/II (+carb or pacli)	NCT00421889
MAPK/RAF/MEK pathway	Cabozantinib	Phase I	NCT00940225
HMGA2	Let-7 microRNA	Preclinical	

* Iniparib as a true PARP inhibitor is currently under investigation.
Doce: docetaxel, Carb: carboplatin, pacli: paclitaxel, topo: topotecan, bev: bevacizumab, temoz: temozolomide, and lip doxo: liposomal doxorubicin.

protein overexpression [54]. Furthermore, some data suggest that HER2 overexpression is mostly found in high-grade serous histology as opposed to low-grade endometrioid [55]. Her2 positive expression has demonstrated association with survival in patients with EOC [53, 56–58]. More recently, HER2 gene status was evaluated from the GINECO study, and no survival differences were detected in patients with or without HER2 overexpression who were treated with carboplatin/paclitaxel [59]. This suggests an association between HER2 status and paclitaxel sensitivity.

As a result of such variability in HER2 expression in ovarian cancer, the role of anti-HER2 therapy is unclear. Anti-HER2 therapy with trastuzumab and pertuzumab have shown modest activity in ovarian cancer [40, 60]. Preclinical studies initially suggested activity in tumors without HER2 overexpression [61]. However, subset analyses of phase II trials indicate that pertuzumab has better activity in those with HER2 overexpression [60]. HER2 expression in EOC has not been studied as extensively as in breast cancer, and there are many inconsistent data. Thus, the limitations to

anti-HER2 therapy hinges on further careful examination of HER2 oncogene as a potential prognostic, predictive, and therapeutic target.

9. EGFR Tyrosine Kinase Inhibitors

EGFR tyrosine kinase inhibitors are also being explored including cetuximab, lapatinib, and erlotinib [62, 63]. A phase II study of erlotinib with carboplatin has shown activity in platinum sensitive recurrent ovarian cancer (57% objective response rate in platinum sensitive and 7% in platinum-resistant patients) [64]. A phase II study with erlotinib, carboplatin, and paclitaxel in the first line setting showed no statistically significant pathologic complete response rates compared to historical controls [65]. A Phase I/II study of lapatinib with carboplatin and paclitaxel in recurrent stage III or IV ovarian and breast cancer proved safe with favorable response rates [66]. Recently, a phase II study was published which explored lapatinib combined with topotecan in platinum refractory or resistant ovarian cancer. This study showed minimal clinical activity with significant hematologic grade 3 and 4 toxicities [39]. A Phase II study with cetuximab and carboplatin in EGFR-positive ovarian cancer showed modest activity where 9 of 28 patients achieved objective response and 8 of 28 patients had stable disease [67]. Toxicities in this study included acneiform rash and hypersensitivity reactions. Another phase II study with cetuximab, carboplatin, and paclitaxel failed to demonstrate progression-free survival benefit compared to historical data [62].

10. PARP Inhibitors

Poly (ADP-ribose) polymerase (PARP) inhibitors block base excision repair. For tumors that lack DNA repair mechanisms due to BRCA1/BRCA2, HNPCC, Fanconi Anemia, and other genetic mutations, inhibiting alternate repair pathways with PARP inhibition may increase antitumor selectivity and improve chemotherapy sensitivity. Three phase II studies with PARP inhibitors, olaparib and iniparib, show activity in recurrent platinum sensitive ovarian cancer in combination with chemotherapy or as single-agent maintenance after chemotherapy [49]. Olaparib as a maintenance therapy has also been shown to improve progression-free survival by 3.6 months compared to placebo in platinum-sensitive relapsed serous ovarian cancer. (ASCO 2011 abstract 5003) There is currently an ongoing NCI sponsored randomized Phase II study that explores ABT 888 (veliparib) with cyclophosphamide in BRCA positive ovarian and triple negative breast cancer [68].

11. Epigenetic Studies

The pathophysiology of cancer is not only the result of inherited or sporadic genetic mutations but is also the result of epigenetic modifications in the genome. Histone hypoacetylation and abnormal DNA methylation also contribute to tumorigenesis and chemotherapy resistance. A phase II study with the DNA hypomethylating agent, decitabine, showed improved response rates in platinum-resistant ovarian cancer when added to carboplatin therapy at low dosages, where 9 out of 17 patients had progression-free survival at 6 months (ASCO 2011 abstract 5011) [49]. Other studies with the histone deacetylase inhibitor, belinostat, and the proteosome inhibitor, carfilzomib, are being explored in ongoing phase II trials.

12. MAPK/BRAF/MEK Pathway

One of the major pathways that regulate cellular growth is extracellular-related kinase (ERK) which triggers a cell surface-receptor mediated signaling cascade involving Ras, Raf, MEK [mitogen-activated protein (MAP)/ERK kinases] and ERK. As described previously, BRAF mutations are commonly associated with low-grade ovarian carcinoma. BRAF mutations result in the constitutive activation of the MAP kinase/MEK pathways. Furthermore, preclinical models have shown that in addition to BRAF mutations in low-grade tumors, Raf-1 isoform predominantly mediates ovarian cancer cell growth compared with Raf-A or B-Raf isoforms [69]. There have also been reports demonstrating reduced survival in ovarian cancer patients with increased expression of Raf-1 [70]. Thus, the MAPK/BRAF/MEK pathway can be a target in both high-grade and low-grade ovarian cancer. MET tyrosine kinase cell surface receptor is involved in RAF and MAP kinase activation pathways, and its inhibition results in downstream suppression of RAF and MAP kinase activity. The potent MET inhibitor, cabozantinib, showed activity in advanced ovarian cancer irrespective of platinum sensitivity [49].

13. Let-7 MicroRNA Therapy

Among the emerging next-generation therapies are the microRNA therapeutics. Preclinical mouse models with exogenous Let-7 microRNA have shown suppression of cell proliferation in breast cancer cells [71, 72]. There are significant limitations to clinical applicability of microRNA technology at this time due to a limited understanding of the Let-7 mechanism and with methods of delivery.

14. Conclusion

Epithelial ovarian carcinoma is a highly heterogeneous disease process that is associated with significant mortality and morbidity. Recent progress in molecular characteristics of ovarian cancer has helped delineate the origins of carcinogenesis, particularly, a model of tumorigenesis which is based on a dichotomous theory of (1) low-grade or type I ovarian cancer associated with gene stability and multiple isolated mutations and (2) high-grade type II ovarian cancer associated with genetic instability and p53 mutations. Continued evaluation of the molecular makeup of ovarian carcinoma is critical in the further identification of treatment targets and improved clinical outcome.

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Research Article

DNA Damage Response is Prominent in Ovarian High-Grade Serous Carcinomas, Especially Those with Rsf-1 (HBXAP) Overexpression

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DNA damage commonly occurs in cancer cells as a result of endogenous and tumor microenvironmental stress. In this study, we applied immunohistochemistry to study the expression of phosphorylated Chk2 (pChk2), a surrogate marker of the DNA damage response, in high grade and low grade of ovarian serous carcinoma. A phospho-specific antibody specific for threonine 68 of Chk2 was used for immunohistochemistry on a total of 292 ovarian carcinoma tissues including 250 high-grade and 42 low-grade serous carcinomas. Immunostaining intensity was correlated with clinicopathological features. We found that there was a significant correlation between pChk2 immunostaining intensity and percentage of pChk2 positive cells in tumors and demonstrated that high-grade serous carcinomas expressed an elevated level of pChk2 as compared to low-grade serous carcinomas. Normal ovarian, fallopian tube, ovarian cyst, and serous borderline tumors did not show detectable pChk2 immunoreactivity. There was no significant difference in pChk2 immunoreactivity between primary and recurrent high-grade serous carcinomas. In high-grade serous carcinomas, a significant correlation ($P < 0.0001$) in expression level (both in intensity and percentage) was found between pChk2 and Rsf-1 (HBXAP), a gene involved in chromatin remodeling that is amplified in high-grade serous carcinoma. Our results suggest that the DNA damage response is common in high-grade ovarian serous carcinomas, especially those with Rsf-1 overexpression, suggesting that Rsf-1 may be associated with DNA damage response in high-grade serous carcinomas.

1. Introduction

Ovarian carcinomas comprise a diverse group of neoplasms that demonstrate distinct clinicopathological features and unique molecular genetic aberrations with respect to different histologic subtypes [1, 2]. Based on clinicopathological and molecular genetic features, we have previously proposed that ovarian carcinoma can be classified into two major types, type I (low-grade serous, low-grade endometrioid, clear cell, and mucinous carcinomas) and type II (mainly high-grade serous carcinomas) [3]. Given the fact that different subtypes of ovarian tumors develop along distinct molecular pathways, we asked if the DNA damage response (DDR) is different among high-grade and low-grade serous carcinomas, the prototypes of type II and type I tumor, respectively.

It has been well established that the DDR pathway is activated by endogenous and environmental cellular stress that is associated with DNA damage and has profound effects on determining cell fate. DDR signaling has been reported to be associated with several types of human cancer including colorectal, pancreatic, and oral squamous cell carcinomas [4–6]. Given its critical role in tumor development, it has been proposed that harnessing the activity of DDR pathways may improve cancer treatment outcome after cytotoxic chemotherapy and irradiation therapy [7]. Molecularly, DDR is initiated by the rapid recruitment of several nuclear proteins involved in the repair process to the site of DNA damage to form a complex, which acts to repair DNA damage and promote cell survival. DDR is mediated by a signal transduction cascade involving the ataxia telangiectasia mutated (ATM-) check point kinase 2 (Chk2)-p53 axis

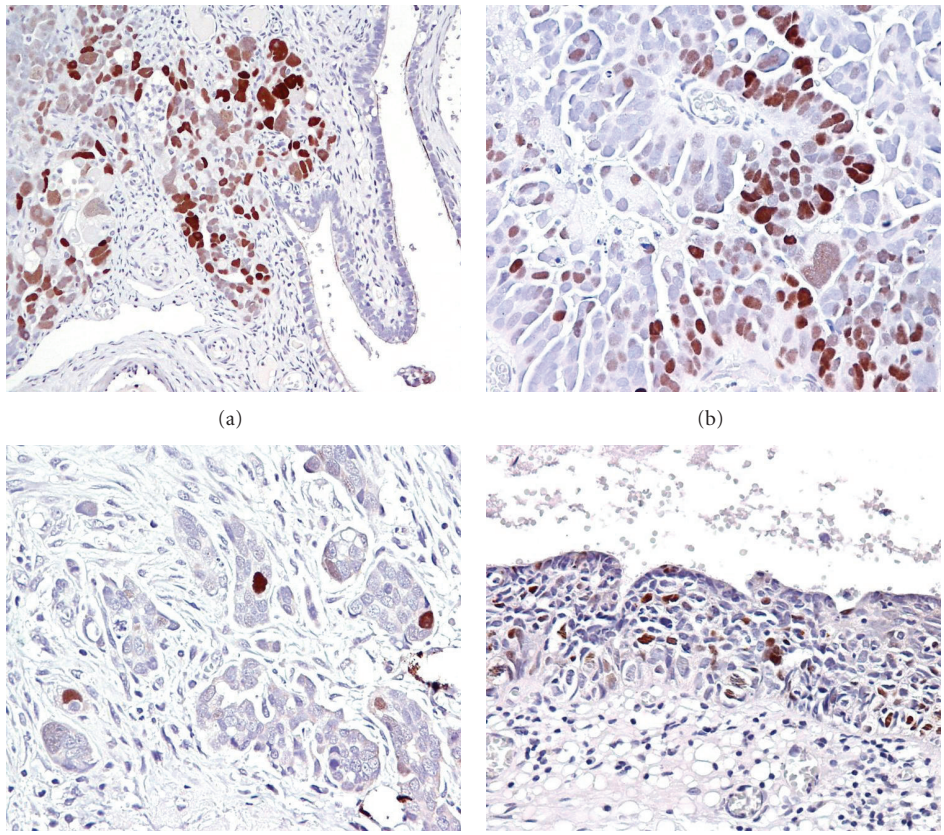

Figure 1: pChk2 immunoreactivity in representative high-grade serous carcinomas. (a) A high-grade serous carcinoma shows relatively diffuse positive staining for pChk2. In contrast, pChk2 immunoreactivity is undetectable in the adjacent fallopian tube epithelium. (b) At a higher magnification, pChk2 immunoreactivity is exclusively present in the nuclei of tumor cells. (c) Another high-grade serous carcinoma shows focal pChk2 staining in cancer cells. (d) A serous tubal intraepithelial carcinoma, a presumable precursor of high-grade serous carcinoma, contains pChk2 positive cells in the lesion.

Table 1: Immunostaining intensity of pChk2 in ovarian serous tumors, normal ovaries, and fallopian tubes.

Staining intensity	Low-grade SC	High-grade SC (primary)	High-grade SC (recurrent)	SBT	Serous cystadenoma	Normal ovaries and FTE
0	31 (73.8%)	47 (29.6%)	28 (30.7%)	16 (100%)	10 (100%)	5 (100%)
1+	5 (11.9%)	37 (23.3%)	15 (16.5%)	0	0	0
2+	3 (7.1%)	39 (24.5%)	18 (19.8%)	0	0	0
3+	3 (7.1%)	36 (22.6%)	30 (33%)	0	0	0

SC: serous carcinoma; SBT: serous borderline tumor; FTE: fallopian tube epithelium.

[8, 9]. In this cascade, Chk2 plays a pivotal role; ATM phosphorylates Chk2 to generate pChk2, the active form of Chk2, which then activates several downstream pathways, leading to cell cycle arrest through p53, BRCA1, Cdc25A, and Cdc25C phosphatase [10, 11]. Because threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks and the specific antibody that binds pChk2 at threonine 68 is available [12], pChk2 immunoreactivity has been used in many studies as a surrogate tissue biomarker for DDR [4–6, 13, 14]. In fact, it has been reported that Chk2 with Thr 68 phosphorylation was detected in more than 50% of primary untreated lung and breast tumor specimens [15].

In this study, we address three main questions: whether there is a difference in the level of DDR between high-grade and low-grade serous carcinoma; whether recurrent high-grade serous carcinomas have an altered DDR as compared to their primaries; whether there is a significant correlation in the expression levels between pChk2 and Rsf-1 (HBXAP), a gene that is frequently upregulated in high-grade serous carcinoma and participates in generating DNA damage. The Rsf-1 gene, located at ch11q13.5, is frequently amplified, and its expression is upregulated in most high-grade serous carcinomas but not in type I tumors [16–18]. Here we applied pChk2 immunohistochemistry to assess

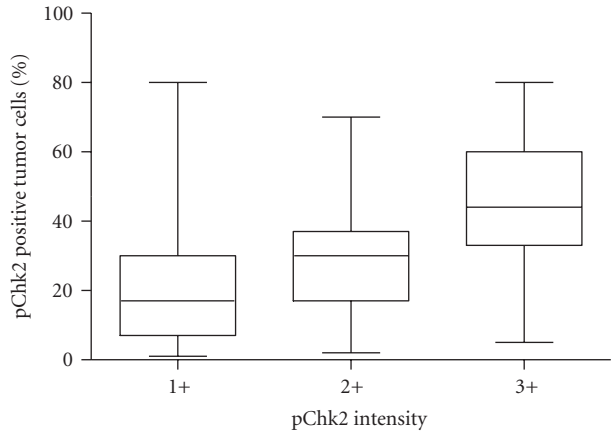

Figure 2: Box plot for the correlation of pChk2 immunostaining intensity and percentage of tumor cells showing pChk2 staining among 206 ovarian tumor tissues. The percentage of pChk2 positive cells correlates with intensity grade: percent pChk2 positive cells in 3+ cases > 2+ cases > 1+ cases ($P < 0.0001$, nonparametric one-way ANOVA test).

the levels of DDR in high-grade serous carcinoma, low-grade serous carcinoma, serous borderline tumor, and serous cystadenoma. Comparing the levels of DDR in cancer cells should help understand the pathogenesis of different subtypes of ovarian serous carcinoma.

2. Methods

An antibody specific for threonine 68 (Thr 68) of Chk2 (Cell Signaling, Danvers, Mass) was used for immunohistochemistry on ovarian carcinoma tissues at a dilution of 1 : 200. Phosphorylation of Chk2 at Thr 68 is a prerequisite for full activation by ATM (3). The specificity of the antibody was reported in a previous study [19]. A total of 292 ovarian carcinomas were analyzed, including 250 high-grade serous carcinomas (159 primary and 91 recurrent tumors) and 42 low-grade serous carcinomas. In addition, normal ovaries and fallopian tubes from 5 patients, 10 serous cystadenomas, and 16 serous (atypical proliferative) borderline tumors were also analyzed. All the ovarian tumors tissues except 14 low-grade serous tumors were arranged in tissue microarrays in triplicate (1.5 mm core) to facilitate immunohistochemistry. pChk2 immunoreactivity was semiquantitatively scored by two pathologists using a four-tiered grading system (0 to 3+) for intensity and percentage for prevalence of positive cells. Correlations of intensity and percentage of pChk2 with clinical data including grade (Chi square), disease-free interval, and overall survival (Kaplan-Meier curves) were determined. A monoclonal anti-Rsf-1 antibody, clone 5H2/E4 (Upstate, Lake Placid, NY), was used for immunostaining in 75 high-grade ovarian serous carcinomas arranged in tissue microarrays. Rsf-1 immunoreactivity was semiquantitatively scored by two pathologists independently using a five-tier grading system (0 to 4+) as previously described [17]. A nonparametric Kruskal-Wallis test was used to determine the statistical significance of correlation between pChk2 expression (both intensity and percentage) and Rsf-1 immunostaining intensity.

3. Results

pChk2 immunoreactivity was exclusively localized in the nuclei of tumor cells (Figure 1). In general, the intensity of pChk2 and the percentage of pChk2 positive cells varied among tumor samples (Figures 1(a)–1(c)) but were highly correlated. The percentage of positive cells in 3+ cases was greater than that in 2+ cases which in turn was higher than that in 1+ cases ($P < 0.0001$, nonparametric one-way ANOVA test) (Figure 2). For example, among high-grade serous carcinomas, using a percentage of 30% as an arbitrary cutoff, 77% of 3+ tumors, 33% of 2+ tumors, and 21% of 1+ tumors contained pChk2 positive cells in more than 30% of cells. The pChk2 immunoreactivity in different types of ovarian serous tumors and normal tissues was summarized in Table 1. Comparing low-grade to high-grade carcinomas, we found that 66 (26.4%) of 250 high-grade serous carcinomas, including primary and recurrent tumors, showed intense pChk2 immunoreactivity (3+) while only 3 (7.1%) of 42 low-grade serous carcinomas had immunostaining scores of 3+. If the cutoff of <2 versus ≥2 was used, 123 of 250 (49.2%) type II carcinomas and 6 (14.2%) of 42 low-grade serous carcinomas had scores ≥2. As compared to low-grade carcinomas, high-grade serous carcinomas demonstrated a statistically higher frequency of intense staining with an intensity score >2 ($P = 0.0002$, Chi-square, two-sided). In high-grade serous carcinomas, no significant difference in staining intensity or percentage was observed between recurrent and primary tumors based on different cutoffs of intensity score and percentage of pChk2 positive cells. In contrast to carcinoma tissues, ovarian surface epithelium, ovarian surface inclusion cysts, serous cystadenomas, serous borderline tumors, and tumor stromal tissues showed undetectable levels of pChk2 immunoreactivity (Figure 3). There was no significant association of pChk2 expression (intensity or percentage) and clinical outcome, including disease-free survival and overall survival, based on Kaplan-Meier survival analysis in high-grade serous carcinomas (data not shown).

We then tested the correlation between the expression levels of pChk2 and Rsf-1 (HBXAP), because we have recently shown that amplification and overexpression of Rsf-1 (HBXAP) contribute to chromosomal instability by inducing DNA strand breaks [20]. Rsf-1 immunostaining results were available in 75 primary high-grade serous carcinomas from our previous study [17], enabling correlation with pChk2 data. Because Rsf-1 immunoreactivity is usually homogenous, we used intensity scores for Rsf-1 expression as previously described [17]. Rsf-1 immunostaining intensity significantly correlated with both percentage ($P < 0.0001$) and intensity ($P < 0.0001$) of pChk2 (Figures 4(a) and 4(b)). Representative immunostained tumor sections of pChk2 and Rsf-1 from the same tissues are shown in Figure 4(c).

4. Discussion

It has been well established that DNA damage in cancer cells and associated DNA damage response pathway activation

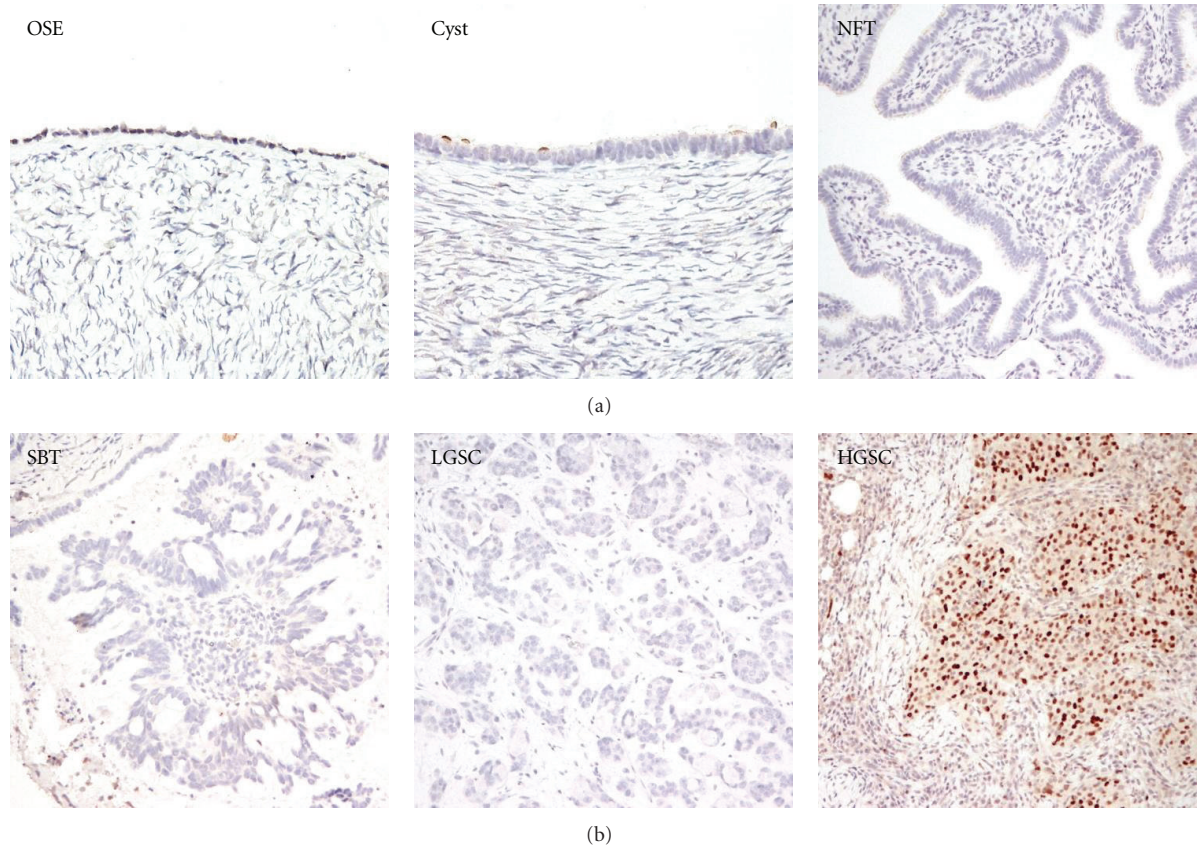

Figure 3: pChk2 immunoreactivity in ovarian tissues and different histologic subtypes of ovarian carcinomas. (a) pChk2 staining is undetectable in ovarian surface epithelium (OSE), cystadenoma (Cyst), normal fallopian tube (NFT). (b) serous borderline tumor (SBT) and low-grade serous carcinoma (LGSC), are negative for pChk2 staining. In contrast, a high-grade serous carcinoma (HGSC) is diffusely positive for pChk2.

play an important role in chromosomal instability and tumor development [21–24]. The results from this study underscore the fundamental molecular differences between high-grade and low-grade serous carcinomas in terms of DDR activation. We have previously proposed that high-grade and low-grade serous carcinomas develop along distinct pathways [3]. Low-grade serous carcinoma develops from the precursor lesion, serous borderline tumor, tends to present at early stages, and is slow growing. In contrast, high-grade serous carcinoma, which has been generally referred to as ovarian cancer, behaves in a highly aggressive fashion, almost always present at advanced stages, and is associated with a dismal clinical outcome. They are typically not associated with borderline tumors, and in fact, a growing body of evidence has supported the view that many high-grade serous carcinomas develop from serous tubal intraepithelial carcinomas and involve the ovary secondarily [25–29]. At the molecular genetic level, low-grade serous carcinomas are characterized by frequent somatic sequence mutations in genes that are involved in signal transduction including *KRAS*, *BRAF*, *ERBB2,* and *PIK3CA* [1, 30–33]. In contrast, mutations in those genes are rarely detected in high-grade serous carcinomas; however, almost all high-grade serous carcinomas harbor *TP53* mutations. In addition to unique sequence mutations, high-grade serous carcinomas and low-grade serous carcinomas (the prototype of type I tumors) have distinct transcriptome and methylation profiles [3, 34, 35].

The higher pChk2 expression levels in high-grade serous carcinomas suggest that DDR is prominent in high-grade serous carcinomas as compared to low-grade serous carcinomas. This is likely due to frequent DNA damage in high-grade serous carcinomas. The pronounced DDR in high-grade serous carcinomas may be related to DNA replication stress due to activation of oncogenes and telomere shortening among several others [36, 37]. Furthermore, high-grade serous carcinomas usually have higher proliferative activity than low-grade serous carcinomas, precipitating the effects of DNA replication stress. On the other hand, tumor microenvironmental changes such as oxidative stress, hypoxia, and the presence of cytotoxic agents may potentiate DNA damage. Furthermore, recent evidence has suggested that excessive remodeling of chromosomal structures can be related to DNA strand breaks followed by DDR. In fact, we have recently shown that overexpression of a chromatin remodeling gene, Rsf-1 (HBXAP), leads to DNA double-strand breaks and DDR, resulting in p53-depdenent cell cycle arrest and apoptosis in *TP53* wild type, nontransformed

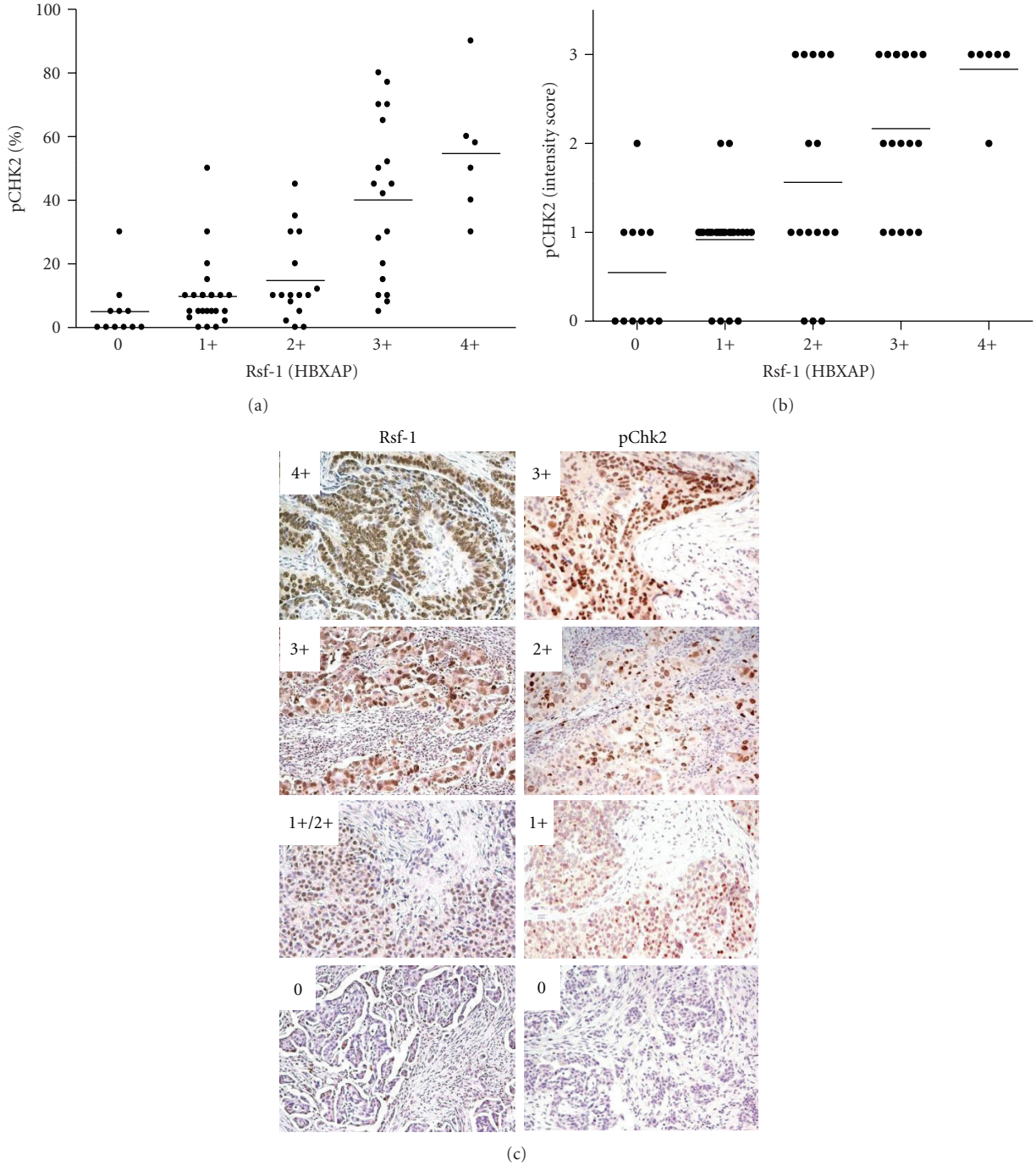

Figure 4: Correlation of the expression levels of pChk2 and Rsf-1 (HBXAP). (a) Scatter plots showing the distribution of pChk2 (percentage) and Rsf-1 (HBXAP) (immunostaining intensity) in 75 high-grade serous carcinomas. The correlation between the percentage of pChk2 positive cells and Rsf-1 immunostaining intensity was significant ($P < 0.0001$). Horizontal bars: means. (b) Scatter plots showing the distribution of pChk2 intensity scores and Rsf-1 (HBXAP) intensity scores in the same set of high-grade serous carcinomas. The correlation between immunostaining intensity of pChk2 and Rsf-1 was significant ($P < 0.0001$). Horizontal bars: means. (c) Four representative cases of high-grade serous carcinomas showing different staining patterns of pChk2 and Rsf-1. The intensity scores are shown in the left upper corner of each panel.

cells [20]. DNA damage goes unchecked, and cells continue proliferating in the presence of DNA strand breaks if *TP53* is mutated, as what occurs in the great majority of high-grade serous carcinomas. Thus, the result from the current immunohistochemistry study further supports the biological link between Rsf-1 upregulation and DDR in ovarian cancer tissues either by aberrant chromatin remodeling or by oncogene/replicative stress. As *TP53* mutation represents one

of the very early event in the development of high-grade serous carcinoma, it is currently not clear if DNA damage occurs before or after *TP53* mutations, although it has been suggested that activation of Chk2 and other DDR members may precede p53 inactivation in human epithelial tumors [22, 38].

It has been demonstrated that high-grade serous carcinoma, as compared to low-grade serous carcinoma, exhibits a higher level of chromosomal instability as reflected by abnormal mitosis, DNA copy number alterations, and karyotypic abnormalities [39, 40]. Thus, the increased pChk2 immunoreactivity in high-grade serous carcinoma raises a possibility that in high-grade serous carcinoma cells, DDR may not be fully capable of repairing DNA damage and maintaining genomic integrity, as the DNA damage may overwhelm the DNA repair capacity. As a result, clonal selection favors tumor cells harboring sustained DNA damage at a level that allows cells to survive but at the same time causes chromosomal instability, serving as the driving force in the evolution of highly aggressive tumor cell species. The finding of pChk2 expression in the majority of high-grade serous carcinomas implies that it may serve as a therapeutic target because inhibiting Chk2 may reduce DNA repair and increase genomic instability to a level that is not compatible with cellular survival and ultimately leads to tumor cell death. Indeed, it has been recently reported that a newly developed Chk2 small compound inhibitor could potentiate the cytotoxicity effect of PARP inhibitors, a finding supporting this view [41].

The majority of low-grade serous carcinomas do not contain prominent pChk2 immunoreactivity. Although our favored view is that less DNA damage is present, and, thus, attenuated DDR occurs in those tumors, other interpretations should also be pointed out. For example, the lack of pChk2 immunoreactivity might be a consequence of inactivation of upstream components in the DDR pathway [36]. As a result, ineffective DDR is present despite DNA damage in tumor cells. However, this scenario is less likely because unchecked and unrepaired DNA damage is generally detrimental to cells and is incompatible with sustained survival and proliferation in tumor cells.

In summary, using pChk2 immunoreactivity as a surrogate marker for DDR, we found that high level DDR was detected more frequently in high-grade serous carcinomas than in low-grade serous carcinoma. This finding provides further support to the view that both tumors are molecularly distinct and develop along different molecular pathways. The significant correlation of expression between pChk2 and Rsf-1 (HBXAP) suggests that excessive chromatin remodeling activity as a result of upregulation of Rsf-1 (HBXAP) is associated with DDR in high-grade serous carcinoma tissues, a result supporting our previous observations that Rsf-1 upregulation contributes to DNA strand breaks and subsequent DDR. Future studies should aim at deciphering the mechanisms responsible for prominent DNA damage in high-grade serous carcinomas as compared to low-grade serous carcinomas and perhaps other type I tumors. Although our current study did not demonstrate an association of pChk2 expression levels with overall survival and disease-free survival, future clinical studies should be conducted to assess the biological significance of pChk2 in ovarian cancer patients.

Abbreviations

Ch: Chromosome
Chk2: Check point kinase 2
DDR: DNA damage response.

Authors' Contribution

Malti Kshirsagar and Wei Jiang contribute equally to this work.

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