

Review

Biomarkers for Screening, Diagnosis, and Monitoring of Ovarian Cancer

Eiji Kobayashi, Yutaka Ueda, Shinya Matsuzaki, Takuhei Yokoyama, Toshihiro Kimura, Kiyoshi Yoshino, Masami Fujita, Tadashi Kimura, and Takayuki Enomoto

Abstract

Serum tumor markers have a major role in the screening, diagnosis, and monitoring of most of the gynecologic cancers. Ovarian cancer is one of the deadliest of the group because it is so frequently asymptomatic until it has advanced to an untreatable stage. Even serum cancer antigen-125 (CA-125), clinically one of the most reliable serum markers for ovarian cancer, is elevated in only half of early-stage still-treatable tumors. Because of the very low prevalence of ovarian cancer in the general population, at present, there is no cost-effective imaging or simple microscopic screening test for ovarian cancer as there is for breast and cervical cancers. However, recent proteomics and nucleic acid-based analyses have shown great promise for the discovery of new and more useful serum biomarkers, which cumulatively might provide such a screening tool. In this review, we will discuss both the currently used serum tumor markers for screening, diagnosis, monitoring of ovarian cancer, and the novel biomarkers that are now under investigation and validation. *Cancer Epidemiol Biomarkers Prev*; 21(11); 1902–12. ©2012 AACR.

Introduction

Endometrial, cervical, and ovarian cancers are 3 of the most common malignancies of the female reproductive tract. Of the 3, ovarian cancer, although rare in occurrence, is the deadliest; in 2008 alone, 224,747 women were diagnosed with ovarian cancer worldwide, and a heart-breaking 62% of these women died from the disease (1). This is primarily because roughly three-quarters of ovarian cancer cases present at an advanced stage, with the disease spread well beyond the ovaries (2). The cancer is insidious, patients usually have their first symptoms only in the advanced-stage of the disease, and these are often related to the presence of a grossly enlarging tumor and extensive ascites fluid; in the early- and midstage disease, most patients are largely asymptomatic (3). Serum cancer antigen-125 (CA-125) levels and transvaginal ultrasonography (TV-USG) screening have contributed to an earlier detection of ovarian cancer; however, the value of tumor markers and USG to screen for epithelial ovarian cancer (EOC) is yet to be clearly established by prospective studies (3, 4).

For any hope of curing ovarian, endometrial, and cervical cancers, it is critical to detect these diseases at the earliest possible stage. These tumors are phenotypically

and genetically heterogeneous, so no single tumor marker will detect all variations; therefore, the discovery of additional useful serum biomarkers for the early detection of gynecologic cancers has thus been highly sought after. Such tumor markers will be molecules arising from the presence of a tumor, which can appear in the surrounding tissues, blood, and excretions because they are secreted or shed by the tumor in excess of the normal tissue or cell phenotype. Sometimes, the marker will be uniquely specific to a tumor subtype, for example, as embryonic, fetal, undifferentiated, or stem-cell phenotypes. Tumor markers can occur as reexpression of genes silenced during differentiation or as anomalous alternative mRNA splicing products of a currently expressed gene. Glycoproteins produced by cancer cells can have detectably altered glycan structures, although the core proteins themselves are ubiquitous (5). Tumor markers might be unique extracellular matrix or cell adhesion molecules, or they can be receptors, growth factors, cytokines, or products of abnormal metabolism. Rarely, the marker molecules can be released by other tissues and organs in response to signals from the tumor. Even the body's autoantibodies against tumor antigens can be markers.

Tumor markers can be associated with patient diagnosis, prognosis, clinical management, and follow-up. In an ideal world, tumor markers would be highly tumor-specific, would always be produced in sufficient amounts to allow fast, easy, cheap, and noninvasive detection of minimal disease, and would quantitatively reflect tumor burden. These idealistic tumor markers would enable their use in screening, diagnosis, monitoring response to therapy, and detecting earlier recurrence during follow-up.

Authors' Affiliation: Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Yamadaoka, Suita, Osaka, Japan

Corresponding Author: Yutaka Ueda, Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-66879-3351; Fax: 81-66879-3359; E-mail: ZVF03563@nifty.ne.jp

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Recent advances in clinical proteomics and serum miRNA analysis have propelled us into an exciting period of discovery of new cancer biomarkers, although the available technologies still have their limitations. The principles of serum marker technology require stringent guidelines for the collection of clinical material, the application of analytic techniques, and for interpretation of the data.

In this review, we will present an overview of the currently used serum tumor markers for the screening of ovarian cancer. Also, we will discuss novel biomarkers that have given us great hope for the future of better detection and management of ovarian cancers.

Serum Markers for Ovarian Cancer

Roughly, three-quarters of all cases of ovarian cancers are diagnosed only after the disease has progressed to stage III or IV, and have involved the peritoneal cavity or other organs. The ultrapoor prognosis for this cancer results directly from the lack of reliable, sensitive screening tests and our limited understanding of the mechanisms of its chemoresistance and relapse. Thus, establishment of an appropriate earlier stage screening test for ovarian cancer has long been sought.

The symptoms that are commonly associated with early to midstage ovarian cancer are typically nonspecific, and the association is often not clinically recognized until the disease is irretrievably advanced (6). Previous studies have shown that USG can provide some degree of high sensitivity; however, its specificity and positive predictive values (PPV) were found to be unsatisfactory (7, 8).

Given the low prevalence of ovarian cancer in the general population, an effective and acceptable screening strategy must have not only a high sensitivity for early-stage disease (>75%), but must also have a very high specificity (99.6%) so as to prompt no more than 10 exploratory operations for each actual case of ovarian cancer diagnosed; that is, it must have a PPV of 10%, even in postmenopausal women more than 50 years of age, who are at a significantly higher risk than younger women (9). At present there is no highly effective screening test for ovarian cancer (such as for breast and cervical cancer). However, the serum markers for ovarian cancers that are currently being used, and those novel biomarkers under investigation, will be discussed later.

Usefulness of CA-125 for screening and surveillance of ovarian cancer

Early detection of ovarian cancer. To date, CA-125 is the serum marker that has received the most use and is the most trusted as an identifying method for ovarian cancer early detection (Table 1). CA-125 was originally developed to monitor patients previously diagnosed with an ovarian cancer but not for its screening. When used as an individual marker on a single occasion, CA-125 is not sufficiently sensitive to detect most cases of early-stage ovarian cancer. Serum CA-125 levels do become more frequently elevated in patients as the disease progresses;

Table 1. Clinical significance of CA125 level for ovarian cancer

Screening of ovarian cancer
Differential diagnosis between primary ovarian cancer and metastatic ovarian cancer ^a
Prediction of prognosis
Surveillance of recurrence

^aIn combination with CEA.

elevations are detected in 50% and 92% of ovarian cancers in early and late stages, respectively (10). Nossov and colleagues (11) found that PPV of CA-125 assay for early detection of ovarian cancer was 57%. Unfortunately, for identifying the source of this tumor marker, elevated CA-125 occurs in other cancers as well, such as endometrial, breast, pancreatic, gastrointestinal, and lung cancers. Elevated CA-125 levels can also be found in patients with benign gynecologic conditions, such as during menstruation, pregnancy, endometriosis, and pelvic inflammatory disease, and even in nongynecologic conditions, such as hepatitis and pancreatitis (12). The physician, therefore, has to always consider the possibility that this tumor marker is creating a false positive case due to another pathologic condition. A one-time determination of CA-125 is thus neither sufficiently sensitive nor specific enough to be used as a biomarker for screening the general population.

To augment its usefulness for screening, CA-125 has been combined with TV-USG. Various combinations of CA-125 and imaging screening, both concurrent testing as well as sequentially, are being tested. There are currently 4 major ovarian cancer screening trials, 2 of which are still ongoing and 2 that have been completed (Table 2). The prostate, lung, colorectal, and ovarian (PLCO) trial in the United States was a randomized control trial of 78,216 women, ages 55 to 74 years, assigned either to annual screening ($N = 39,105$) or usual care ($N = 39,111$; ref. 13). The "intervention group" received annual screening with CA-125 for 6 years and TV-USG for 4 years at 10 medical centers throughout the country. The control "usual care" group was not offered this advanced screening for 6 years but did receive their usual medical care. Twenty-two percent of patients with screening-detected cancers had stage I or II disease, versus 22% in the control group, and there was no evidence of a shift to early-stage disease associated with screening. There was equivalent ovarian cancer mortality in both groups.

The second completed study, a multicenter screening trial in Japan, was a prospective randomized trial conducted between 1985 and 1999, in which asymptomatic postmenopausal women were assigned either to a screening group ($N = 41,688$) or a control group ($N = 40,799$; ref. 14). Women in the screening arm received an annual pelvic examination, a serum CA-125 test, and an ultrasound examination. Ovarian cancers were detected by

Table 2. Results from major ovarian cancer screening trials

Screening trial	Years	Study design	Screening test	Nonscreened	Cancers detected	Stage I and II	Stage III and IV	Survival benefit
PLCO (USA)	1993–2001	Randomized control	Ultrasound C125 vs. usual care	34,253	212	22%	77%	(–)
UKCTOCS (UK)	2001–2005	Randomized control	Ultrasound C125 or ultrasound alone vs. usual care	101,279	58	48%	52%	Analysis pending ^a
SCSOCS (Japan)	1985–1999	Randomized control	Ultrasound C125 vs. usual care	41,688	27	67%	33%	Analysis pending ^a
University of Kentucky (USA)	1987–2011	Population control	Ultrasound	37,293	47	70%	30%	(+)

^aNot reported until present. PLCO, The prostate, lung, colorectal, and ovarian trial; UKCTOCS, The United Kingdom Collaborative Trial of Ovarian Cancer Screening; SCSOCS, The Shizuoka Cohort Study of Ovarian Cancer Screening.

screening in 27 women, of which 67% had a stage I or stage II disease. Thirty-two women in the control group developed ovarian cancer, 44% of whom had stage I or II disease. Analysis of site-specific ovarian cancer mortality in the screening and control groups has not yet been reported.

The largest ongoing screening trial is the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS; ref. 15). From 2001 to 2005, 202,638 postmenopausal women, ages 50 to 74 years, were randomly assigned to annual TV-USG screening ($N = 50,639$), multimodal screening with sequential serum CA-125 testing and ultrasound ($N = 50,640$), or no treatment ($N = 101,359$). Fifty-eight invasive ovarian cancers were detected by screening, 28 patients (48%) had stage I or II disease, versus 26% in the control population, and 22% in the prevalence screen of the PLCO trial. This trial is ongoing, therefore, the effect of the screening program on ovarian cancer mortality awaits further analysis.

The University of Kentucky Ovarian Cancer Screening Trial has been in progress from 1987 to the present time, and 37,293 women have been screened (16). To date, 47 EOCs have been detected, with 70% of patients having stage I or II disease. Twelve women developed detectable ovarian cancers within 12 months of a negative screen. The stage at detection and the site-specific ovarian cancer mortality in women with screen-detected cancers have been compared with women from the same geographic area whose cancers were detected clinically during the same time period. Screening produced a stage shift, in which 70% of women with screening-detected ovarian cancers had stage I or II disease versus 27% in the unscreened control group ($P < 0.01$). The 5-year survival of all women whose EOCs were detected by this screening study, including the interval cancers, was $74.8\% \pm 6.6\%$, as compared with $53.7\% \pm 2.3\%$ for women with routine clinically detected ovarian cancers treated at the same institution with the same surgical and chemotherapy protocols ($P < 0.01$).

Although in several of the trials described earlier, screening seems to have allowed for detection of the tumor at an average of an earlier stage, the effects of screening on ovarian cancer mortality has varied significantly, and disappointingly, in the different trials, and that itself is the subject of further investigations. In addition, these tests (combined TV-USG and CA-125) are not cost-effective as currently conducted and are thus still not used routinely to screen for ovarian cancer.

Differentiation from other malignancies. The differentiation of a primary ovarian cancer from a tumor metastatic to the ovary is still tremendously challenging. In a previous study, Yedema and colleagues (17) described the preoperative discrimination of ovarian cancer from colorectal cancer. They reported that the specificity increased significantly when using a combination of a CA-125 positive score (>35 U/mL) and a simultaneous negative tumor marker CEA; carcinoembryonic antigen score (5 ng/mL; specificity 100%, sensitivity 81%). A CA-125/CEA serum ratio of more than 25 resulted in the highest discriminative power, with a specificity of 100% and a sensitivity of 91%, resulting in an overall test accuracy of 94%. They concluded that a combination of CA-125 and CEA are helpful in the preoperative differential diagnosis between a primary ovarian cancer and a colorectal origin.

Sørensen and Mosgaard (18) also reported the ability of CEA in combination with CA-125 to differentiate between malignant ovarian and malignant nonovarian disease. They reported that, among the patients with CEA levels of more than 5 ng/mL, 68% had nonovarian malignancies. In patients with a CA-125/CEA ratio of more than 25, ovarian cancer was found in 82%. The specificity increased to around 85% when the cut-off value of the CA-125/CEA ratio was increased from 25 to 100 (18). From these results, a combination of CA-125 and CEA may be helpful in the preoperative differential diagnosis between ovarian cancer and another originated cancer.

Prediction of prognosis and surveillance of recurrence. The predictive value of pretreatment CA-125

levels for prognosis is controversial. While some studies did not find preoperative CA-125 levels to be an independent prognostic factor (19–21), others reported that it could identify poor prognostic subgroups, independent of stage (22, 23). However, changes in CA-125 levels can also correlate with regression, stability, and progression of the disease in 87% to 94% of instances (12).

Elevation levels in CA-125 can be used to document progressive disease in patients who achieve a normal CA-125 after primary treatment. Rustin and colleagues (24) found that a doubling of CA-125 level from the upper limit of normal had a sensitivity of 86% and a specificity of 91% for detecting progression. A second confirmatory value reduces the false-negative rate to less than 2%. Similarly, a doubling of CA-125 from baseline in patients with persistently elevated CA-125 following primary treatment accurately predicts progression (25). Increase in CA-125 levels tend to precede symptomatic relapse by a median of 4.5 months (range 0.5–29.5 months), and there is considerable debate about whether additional treatment should be commenced on the basis of increasing CA-125 alone. In the recent Medical Research Council/European Organisation for Research and Treatment of Cancer (MRC/EORTC) trial, Rustin and colleagues showed no difference in overall survival (HR, 1.00) between patients who received chemotherapy based on increasing CA-125 and those who did not receive chemotherapy until they were symptomatic (26). Thus, whether or not early reintroduction of treatment produces a survival advantage remains unclear.

Although a high probability exists that some tumor response can be achieved with chemotherapy, a complete cure of these patients is rarely possible. Potential advantages of early treatment of relapse include delaying cancer-related symptoms, providing psychologic reassurance, and possibly improved survival. Potential

disadvantages include loss of time without treatment and associated toxic effects. Patients should be counseled on these advantages and disadvantages before deciding whether to have their CA-125 concentrations routinely measured during follow-up.

Other tumor markers. Serum levels of CA 19-9 (a monosialoganglioside antigen widely used in gastrointestinal adenocarcinoma diagnostics) are elevated in 68% to 83% of mucinous ovarian cancers but in only 28% to 29% of nonmucinous types, whereas CA-125 is elevated in 80% of nonmucinous ovarian tumors (27–30) providing a differential diagnostic tool for nonmucinous versus mucinous subtypes. Other markers, alone or in combination, have also been used; serum CA 15-3, CA 72-4, and CEA levels are elevated, respectively, in 50% to 56%, 63% to 71%, and 25% to 50% of patients with ovarian cancer (27, 31–38; Table 3). According to Gadducci and colleagues, the levels of the markers CA 19-9, CA 15-3, and CA 72-4 were poorly correlated with the clinical course of the disease, when compared with CA-125, and thus these markers did not offer additional clinical benefit for monitoring ovarian cancer. However, the serial measurement of these markers may still play an important role in the management of the relatively large group of patients with a CA-125 negative tumor (12). This would be similar to monitoring Her-2-negative/estrogen receptor-negative breast tumors with other breast tumor markers.

There are additional serum markers for ovarian cancer that are under active investigation (Table 3). For example, HE4 has recently been accepted by the U.S. Food and Drug Administration (FDA) as a monitoring method for patient management with EOC. In a review by Li and colleagues, they found that HE4 displayed the highest sensitivity (72.9%) among all single markers, including CA-125, in the detection of ovarian cancer, in both the early (62%–83%) and late (75%–93%) stages

Table 3. Diagnostic serum markers for ovarian cancer

	Cut-off	Ref. No.	SE (%)	SP (%)	PPV	NPV
CA125	>35 U/mL	26	82.2	67.3	47.1	91.4
	>65 U/mL	26	75.6	86.6	66.7	90.9
CA19-9	>40 U/mL	26	35.6	81.1	40	78
CA15-3	>32 U/mL	26	57.1	93.9	75.9	86.7
CA72-4	>3.8 U/mL	26	70.7	91.8	75.7	89.6
CEA	>3 ng/mL nonsmoker, >5 ng/mL smoker	37	16	93	37	83
HE4	>70 pmol/L	41	72.9	95	NA	NA
LPA	1.3 μ mol/L	41	98	90	NA	NA
IAP	482 μ g/mL	34	93.3	91	NA	NA
HP- α	65 μ g/mL	44	64	90	NA	NA
OVX-1	7.2 μ mL	49	70	95	NA	NA
Methothelin	–	43	60	98	NA	NA

Abbreviations: IAP, immunosuppressive acidic protein; NA, not assessed; NPV, negative predictive value; Ref. No., reference number; SE, sensitivity; Spec, specificity; –, not shown.

Table 4. Level of circulating exosome in patients with ovarian cancer by tumor stage

Stage	Number of patients	Level of circulating tumor-derived exosomes
I	10	0.320 ± 0.056 mg/mL
II	10	0.640 ± 0.053 mg/mL
III	20	0.995 ± 0.084 mg/mL
IV	10	1.42 ± 0.228 mg/mL

NOTE: Table modified from ref. (71).

(39). In addition, serum levels of HE4 are elevated in at least a third of the patients with EOC who do not have tumors that overexpress CA-125, suggesting a complementary application of the 2 tests would be useful (40, 41).

Elevated serum lysophosphatidic acid (LPA) levels, another potentially useful marker, were found in 90% and 98% of ovarian cancer in early and late stages, respectively; however, serum levels of LPA do not correlate well with the stage of the disease, and nonspecific elevation of LPA was detected in healthy and benign gynecologic conditions (11, 42, 43).

Significantly, elevated sFas levels are detected in some patients with ovarian cancer as compared with healthy women, and serum sFas level was shown to be a statistically significant indication factor for survival, as well as histologic grade, in ovarian carcinomas (44). Another antigen marker, mesothelin (41), is a protein of unknown biologic function, which is present in normal mesothelium and has been detected at elevated levels in the serum of patients with mesothelioma, ovarian cancer, and some squamous cell carcinomas. Through transcriptional profiling, Mesothelin was found to be elevated in the serum of 76% of patients with ovarian cancer and was also found to be informatively complementary to CA-125 in early detection of ovarian cancer (45).

Haptoglobin- α (HP- α) is a liver glycoprotein (with α -electrophoretic mobility on a gel) that binds to free hemoglobin released from red cells. Using surface enhanced laser desorption and ionization (SELDI) and mass spectrometric (MS) protein profiling, HP- α has been identified as being a potential tumor marker having a 64% sensitivity and a 90% specificity (46).

Bikunin is a glycosylated protease (glycoprotein) that inhibits tumor cell invasion and metastasis. Preoperative plasma bikunin levels have been reported to be a strong prognostic marker for ovarian cancer. A large study showed that low plasma level of bikunin were associated with late-stage disease, probable suboptimal debulking with a large residual tumor (>2 cm) outcome, low response to chemotherapy, and reduced survival time (47).

OVX1 is an epitope of a high molecular weight mucin-like glycoproteins, which can be detected by radioimmu-

noassay. OVX was found to be elevated in 67% of patients with ovarian cancer who were CA-125 negative (48, 49).

Other novel biomarker panels have also been investigated for early detection of ovarian cancers. Zhang and colleagues identified a panel of markers that consisted of 3 proteins, including apolipoprotein A-I (apoA-I), a truncated form of transthyretin (TTR), and a cleavage fragment of H4 (inter- α -trypsin inhibitor heavy chain) to detect early-stage ovarian cancer with a sensitivity of 83% and a specificity of 94% (50). Su and colleagues used a multiple logistic regression model (MLRM), with values for CA-125, ApoA-I, transferrin (TF), and TTR for early detection of ovarian cancer (51). This model provided a sensitivity of 89% and a specificity of 97% for detection of early-stage ovarian cancer. The sensitivity and the specificity in distinguishing normal and mucinous ovarian cancer samples were 95% and 92%, respectively. Nosov and colleagues applied this same MLRM model and marker panel to analyze serous and endometrioid histologic types of ovarian carcinomas; they showed a sensitivity of 94% and a specificity of 94% for serous ovarian carcinoma in its early stage, and a sensitivity of 98% and a specificity of 98% for endometrioid ovarian carcinoma in its early stage (52).

Visintin and colleagues proposed a panel of serum biomarkers that consisted of leptin, prolactin, osteopontin, insulin-like growth factor II (IGF-II), macrophage inhibitory factor (MIF), and CA-125 to discriminate between patients with ovarian cancer and healthy women. The panel had a sensitivity of 95% and a specificity of 99% (53). Not surprisingly, this panel provided a significant improvement over CA 125 alone. However, these studies had similar methodologic limitations of excessive numbers of tumor cases versus small numbers of matched population controls.

Still, with all this said, novel proteomics-based investigations and bioinformatics analysis provide great promise for finding ever more accurate and useable biomarkers for these gynecologic cancers.

miRNAs

miRNAs (or miR) are a class of small (18–25 nt) non-protein-coding gene-regulatory RNA molecules that are emerging as immensely important diagnostic and potentially therapeutic tools. miRNAs play important roles in a variety of human biologic processes, including development, organogenesis, metabolism, and homeostasis. miRNAs negatively regulate mRNA translation into protein of a large number of important target genes, either by translational repression or by degradation of the messenger RNA transcript, after targeting, by sequence complementarity, the 3'-untranslated region of the mRNA.

Similar to other cancers, the initiation and development of ovarian cancer is characterized by disruption of oncogenes and tumor suppressor genes by both genetic and epigenetic mechanisms (54). It is now well known that altered or deregulated miRNA expression can also be a determinant of disease development and/or progression

in a host of pathologic conditions. Importantly, for the purposes of this review, miRNAs are functionally involved in the pathogenesis of many tumors (including our subject, ovarian cancer), in which miRNAs can have important roles as regulatory molecules, acting as oncogenes (oncomirs) or tumor suppressors. A variety of miRNA candidates are differentially or aberrantly expressed in ovarian carcinomas, or by adjoining stromal tissues, and even by other tissue in the host body in response to the tumor.

Changes in tumor miRNA expression patterns occur through a variety of mechanisms, such as genetic alterations, epigenetic regulation, or altered expression of transcription factors, which target the miRNA genes. For example, in cancer cells, transcriptional gene silencing has frequently been associated with epigenetic defects. miR-125b1 has been suggested to be an miRNA with tumor suppressor activity, and it has been shown to be deregulated in various human cancers. DNA methylation at its regulatory-region-associated CpG island can reduce miR-125b1 expression, and these effects have been observed in several gynecologic cancers, including ovarian and cervical tumors (55).

RNases are abundant in the bloodstream. Therefore, to be stable, some secretory miRNAs are contained in apoptotic bodies, microvesicles, or bound to the RNA-binding proteins (56). However, the vast bulk of the miRNA in serum and saliva is found in tiny membrane vesicles known as exosomes (57), which are cell-derived extracellular vesicles of endosomal origin. In addition to miRNAs, exosomes can contain proteins and mRNAs, and thus exosomes have been shown to constitute a mode of intercellular communication selectively transmitting several types of information between cells. These "bioactive shuttle vesicles" are known to transfer these various molecules, including the miRNAs, to recipient cells, and to promote cell-cell communication and immunoregulatory functions (58, 59).

Cancer cells can secrete excessive amounts of exosomes as compared with normal cells (60). A new aspect of cancer research is being revealed by the emergence of these "secretory miRNA." The molecular composition and functional role of tumor cell-derived exosomes in tumorigenesis, metastasis, and response to therapy are slowly being decrypted (60). Inappropriate release of miRNAs via exosomes may cause significant alterations in biologic pathways that affect disease development. Their active secretion has functional implications, albeit, it is often still unknown whether they are tumor promoting or suppressing. Notably, the interplay via the exchange of exosomes between cancer cells and between cancer cells and the tumor stroma may promote the transfer or expression of oncogenes (e.g., β -catenin, CEA, HER2, Melan-A/Mart-1, and LMP-1) and onco-miRNAs (e.g., let7, miR1, miR15, miR16, and miR375) from one cell to another, leading to the reprogramming of the recipient cells (60).

Some miRs exert negative control over the expression of numerous oncoproteins in normal cells, and consequent-

ly, their deregulation is believed to be an important mechanism underlying cancer development and progression (61). miRNAs have distinct patterns of expression associated with specific cancer types, and once secreted by the cancer cells, they have remarkable stability in blood and other body fluids (61).

Because of the amount of signal amplification possible with nucleic acid serum markers, the identification of "miR signatures" associating cancer cell phenotypes with disease outcome and specific risk factor exposures will open new avenues for early diagnosis of cancer, as well as for the development of novel strategies for cancer prevention and therapy (61). Because these miRNA signatures can appear in the body fluids in exosomes, they can serve as relatively stable circulating diagnostic biomarkers, and have been shown to do so for ovarian cancer (62). Isolation of an exosome fraction also improves the sensitivity of miRNA amplification from human biologic fluids and reduces the probability of false negative results involving low abundance miRNAs that may be missed by using unfractionated serum or saliva (57).

Moving from merely being biomarkers for ovarian cancer to being targets for therapy, the development of strategies that might block the expression or mimic the functions of miRNAs could represent new therapeutic strategies for any of the aforementioned gynecologic disorders. Exosome vesicles can also be used as gene therapy vehicles for delivery of miRNAs and siRNA with therapeutic effects. The ability to do so has already been shown in mice (59). It thus seems that exosomal RNA has the potential to play important roles in the diagnosis, prognosis, and treatment of such diseases in the future.

Using well-characterized examples from other tumors, clinicians can begin to understand some of the functions of tumor miRs. Some miRNAs, such as let-7 in lung cancer and miRs-15/16 in leukemia, normally act as tumor suppressor genes, in these cases suppressing the expression of the oncogenes *Ras* and *BCL2*, respectively (63, 64). When they are underexpressed, tumor growth is permitted. Tumor overexpressed miRNAs, such as miR-21, and the cluster miR-17-92, can act as oncogenes (oncomirs), targeting tumor suppressors PTEN and E2F1 in solid and hematologic malignancies, respectively (65, 66).

miRNA research in the gynecologic malignancies is now progressing quite rapidly, as the miRNA signature profiles of ovarian cancer were first published in 2007 and 2008 (67-69). The use of miRNA signatures of tumor-derived serum exosomes as a diagnostic biomarker for ovarian cancer was first convincingly showed by Taylor and Gercel-Taylor (70; Table 4). The authors showed that the level of tumor-derived miRNA-containing exosomes in serum is strongly increased in women with invasive ovarian cancer as compared with women with benign ovarian tumors or healthy controls. In addition, the levels of circulating, tumor-derived exosomes increased in parallel to the stage of disease. Furthermore, they showed, by miRNA microarray profiling, that the 218 miRNAs that were identified in tumor samples were also identified in

Table 5. Differently expressed miRNAs in the serum

	<i>P</i> -value
Overexpressed	
miRNAs-21	0.0002
miRNAs-29a	0.0003
miRNAs-92	0.0001
miRNAs-93	0.0003
miRNAs-126	0.007
Underexpressed	
miRNAs-127	0.0001
miRNAs-155	0.0003
miRNAs-99b	0.0001

NOTE: Table modified from ref. (72).

circulating exosomes and that some miRNAs are even more overexpressed in the circulating exosomes than in the original tumor samples.

Differences in serum miRNAs between healthy controls and patients with ovarian cancer were also reported by Resnick and colleagues (71; Table 5). They were seeking an alternative or complementary diagnostic approach to TV-USG and serum CA-125 levels for women at high risk for ovarian cancer, knowing that this would be of great importance because CA-125 remains such a poor marker for early-stage disease, with a documented sensitivity of only 40%. Thus, it was hoped that miRNAs might serve as early detection biomarkers in patients with normal CA-125 levels. They identified 21 miRNAs that were differentially expressed between normal and patient sera with ovarian cancer. Analyzing these miRNAs in more detail, 5 miRNAs were found to be overexpressed and 3 miRNAs were decreased in the serum of patients with ovarian cancer, as compared with controls, establishing a possible set of miRNAs as biomarkers for ovarian cancer.

The Cancer Genome Atlas (TCGA) Network has recently catalogued the most extensive set to date of molecular aberrations in ovarian cancers. Patterns of miRNA expression in 487 high-grade serous tumors revealed multiple tumor subtypes and a set of 34 miRNAs predictive of overall patient survival (72). The miR-29 family and predicted target genes were among the most strongly anticorrelated miR: mRNA pairs, meaning the mRNA targets were suppressed when the miRs were active. In the standard test for miR functionality, overexpression of miR-29a *in vitro* repressed several anticorrelated genes (including *DNMT3A* and *DNMT3B*) and substantially decreased ovarian cancer cell viability. Mining the TCGA microarray database has also shown that the expression level of RAD51AP1 was found to be strongly anticorrelated with the expression of hsa-miR-140-3p, which was significantly downregulated in the tumor samples (73). Other pairs of potentially biologic relevance included: hsa-miR-145/E2F3, hsa-miR-139-5p/TOP2A, and hsa-miR-133a/GCLC (73).

The interplay between various families of miRs is quite complex, resulting in researchers finding "signatures" of expression in which no single component is essential, but overall patterns are consistent. For example, Bentink and colleagues (74) identified a previously undescribed patient stratification based on an "angiogenesis signature" of miRNA expression profiles. These pathways are probably determined early in tumorigenesis. Recent recognition of (HG-SOC) high grade serous ovarian cancer precursor lesions, defined as serous tubal intraepithelial carcinoma (STIC) in fimbria, provides a new venue for the study of early genetic changes in HG-SOC. Using miRNA profiling analysis, Liu and colleagues (75) found that miR-182 expression was significantly higher in STIC than in matched normal Fallopian tube. Further study revealed that miR-182 was significantly overexpressed in most HG-SOC cases. miR-182 overexpression resulted in increased tumor transformation *in vitro*, and enhanced tumor invasiveness *in vitro* and metastasis *in vivo*. Mechanistically, they showed that the oncogenic properties of miR-182 in ovarian cancer were mediated in part by its impaired repair of DNA double-strand breaks and negative regulation of breast cancer 1 (BRCA1) and metastasis suppressor 1 (MTSS1) expression, as well as its positive regulation of the oncogene high-mobility group AT-hook 2 (HMGA2).

Chang and colleagues (76) have suggested that miR-148b may be one of the dysregulated miRs involved in the early stage of ovarian carcinogenesis. They found that miR-148b was overexpressed in 92.21% (71/77) of the ovarian cancer samples they examined, and the overexpression was not associated with any of the clinicopathologic features of patients with ovarian cancer (meaning it correlated with the causation and not the symptoms of the disease).

The human kallikreins are a cluster of 15 kallikrein-related peptidases (KLK). Evidence shows the involvement of KLKs in a wide range of pathologic processes and their potential contribution to cancer. Recently, epigenetic changes (including methylation and miRNA regulation) were shown to control KLK expression. Target prediction showed that KLK mRNAs are potential targets of miRNAs that are dysregulated in tumors, including ovarian cancers, with downstream effect on tumor proliferation (77).

Malignant ovarian disease is characterized by high rates of mortality arising from high rates of recurrent chemoresistant disease due to the chemoresistant properties of cancer stem cells (CSC). Microarray analysis showed a 90% difference between gene expression events involved in early regulation of differentiation in murine EC (mEC) and embryonic stem cells (41). Gene list comparisons have identified a signature set of genes for 'cancer stemness' in data from primary versus recurrent tumors, a subset of which are known to be p53-p21 regulators. Gallagher et al. (78) have proposed that this tumor signature of miRNA expression may, at least partially, differentially regulate the p53-p21 mechanism in

ovarian disease. Targeting CSCs within ovarian cancer via miR expression targeting represents another potential therapeutic avenue.

In ovarian cancer, unique CD44⁺/CD117⁺ stem cells, also known as cancer-initiating cells (CIC), are highly proliferative, have a low degree of differentiation, and are resistant to chemotherapeutics. Therefore, the CD44⁺/CD117⁺ subpopulation is thought to be an important target for novel therapeutic strategies. CD44⁺/CD117⁺ ovarian CICs were enriched from human primary ovarian tumor tissues and studied for miRNA expression and responses to miRs. When miR-199a was cloned and transfected into ovarian CICs, it significantly increased the chemosensitivity of the ovarian CICs to cisplatin, paclitaxel, and Adriamycin, and reduced mRNA expression of the multidrug resistance gene *ABCG2* as compared with miR-199a mutant-transfected and -untransfected cells (79). The expression of "stemness markers" was also significantly reduced. Furthermore, xenograft experiments confirmed that miR-199a suppressed the growth of xenograft tumors formed by ovarian CICs *in vivo*. Thus, expression of an endogenous mature miR-199a may prevent tumorigenesis in human ovarian cancer, via regulating expression of its target gene, *CD44*.

Mesothelin, the aforementioned differentiation antigen present in a series of malignancies, such as ovarian, mesothelioma, lung, and pancreatic cancer, has been studied as a marker for diagnosis and a target for immunotherapy. Wang and colleagues (80) have been evaluating the effects of direct targeting of mesothelin on the viability of cancer cells as the first step toward developing a novel therapeutic strategy. They have shown that the gene-specific silencing for mesothelin by distinct methods (siRNA and miRNA) decreased viability of ovarian cancer Skov3 and Ovar-5 cell lines. In addition, the invasiveness of these cancer cells *in vivo* was also significantly decreased upon such treatment. Mesothelin-silencing revealed a significant decrease in phospho-ERK1 and PI3K/AKT activity. The molecular mechanism of reduced invasiveness was connected to the reduced expression of β -catenin, an important marker of epithelial-mesenchymal transition (EMT). Ero1, a protein involved in clearing unfolded proteins and a member of the ER stress (endoplasmic reticulum-stress) pathway, was also markedly reduced (80).

Tiam1 has been implicated in the aggressive invasive phenotype of ovarian cancer, as Tiam1 expression was remarkably increased in both primary and metastatic ovarian cancer tissues. Li and colleagues (81) showed that miR-22, miR-183, and miR-31 expression had negative regulatory effects on Tiam1 expression and that down-regulation of Tiam1 in SKOV-3ip and HO-8910PM ovarian cancer cells lead to reduced cell migration and invasion and to growth inhibition, without significantly affecting cell apoptosis, suggesting that the differential expression profiles of these miRs may contribute to the dysregulation of Tiam1 abundance, which contributes to

the invasive, migratory, and viability properties of ovarian cancer cells.

miRNA in Prognosis

Recently, miR-100 was reported to be significantly downregulated in human ovarian carcinoma; however, the clinical significance and functional roles of miR-100 expression in human EOC were unclear. Peng and colleagues (82) now report that low miR-100 expression was found to be closely correlated with advanced Federation Internationale des Gynaecologues et Obstetristes (FIGO) stage, higher serum CA-125 expression levels, and lymph node involvement. Also, low miR-100 expression is correlated with shorter overall survival of patients with EOC, and multivariate analysis showed that the status of miR-100 expression was an independent predictor of overall survival in EOC. In addition, they show that miR-100 could affect the growth of EOC cells by posttranscriptionally regulating polo-like kinase 1 (PLK1) expression. Together, these results suggest that low miR-100 expression may be an independent poor prognostic factor and miR-100 can function as a tumor suppressor by targeting PLK1 in human EOCs.

Bagnoli and colleagues (83) delineated a miRNA signature associated with early relapse in advanced-stage patients with EOC. Thirty-two differentially expressed miRNAs in early versus late relapsing patients were identified; 8 of these, belonging to a cluster located on chrXq27.3, were downmodulated in early relapsing patients. Forced expression of the chrXq27.3-cluster selected miRNAs in human EOC cellular models was associated to reduction of cell proliferation and increased sensitivity to cisplatin.

Drug Resistance

miR-93 is significantly upregulated in cisplatin-resistant ovarian cancer cells and inversely correlates with PTEN expression in cisplatin-resistant and -sensitive human ovarian cancer tissues (84). They used *in vitro* assays to show that overexpression and knockdown of miR-93 regulates apoptotic activity, and thereby cisplatin chemosensitivity, in ovarian cells. Furthermore, they found that miR-93 could directly target PTEN, and participated in the regulation of the AKT signaling pathway.

The miR-34 family has a strong role in regulating the genotoxic-response p53 pathway in ovarian cancer. Zhang and colleagues (85) have shown that the miR-449a, miR-449b, and miR-192 family of miRNAs may play the same role. They have shown that the expressions of miR-449a/b, miR-34b, and miR-34c were 19- to 21-fold elevated after p53 activation by genotoxic agent. Ectopic expression of miR-449b, as well as miR-34c, resulted in cell-cycle arrest in SKOV3.ipl cells. Thus, as tumor-suppressor miRNAs, miR-449a/b, miR-34b, and miR-34c cooperate and play important roles in p53 pathway. Their inactivation may contribute to the carcinogenesis and progression of serous ovarian carcinomas.

Conclusions and Future Directions

For gynecologic cancers, only a small handful of tumor-associated antigens, such as SCC and CA-125, have been routinely used as tumor markers. Some markers are useful not only as a diagnostic tool but also as a predictive marker for the prognosis and clinical course after treatment. Some newer serum markers being recently investigated seem to be clinically useful, such as HE4 for endometrial and ovarian cancers. The future of tumor marker research is being rapidly expanded because of the recent technologic advances in genomics and proteomics. While a large amount of information has been gained about the roles and possible therapeutic use of miRNAs in ovarian carcinoma, much remains to be done. In particular, more thorough miR expression profiling will be necessary to understand the intricacies of their expression in ovarian carcinoma of various grades, stages, or drug resistance status. The next step, the identification of relevant therapeutic miRNA targets, will likely be a tedious task, complicated by the fact that miRs can have multiple functional targets and that these targets may be dependent on several factors, including the expression of other miRs. Once relevant miRs and their functional targets are

identified, the investigation of possible clinical use for these molecules will represent the next frontier in cancer research, and may, ultimately, lead to novel strategies for ovarian cancer detection and therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: E. Kobayashi, Y. Ueda, T. Kimura, M. Fujita, T. Enomoto

Development of methodology: E. Kobayashi, Y. Ueda, M. Fujita

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Kobayashi, S. Matsuzaki, T. Yokoyama, M. Fujita

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Kobayashi, Y. Ueda, S. Matsuzaki, T. Yokoyama, M. Fujita

Writing, review, and/or revision of the manuscript: E. Kobayashi, S. Matsuzaki, T. Yokoyama, T. Kimura, K. Yoshino, M. Fujita, T. Enomoto

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Kobayashi, S. Matsuzaki, T. Kimura, M. Fujita

Study supervision: E. Kobayashi, Y. Ueda, K. Yoshino, M. Fujita, T. Kimura, T. Enomoto

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Biomarkers for Screening, Diagnosis, and Monitoring of Ovarian Cancer

Eiji Kobayashi, Yutaka Ueda, Shinya Matsuzaki, et al.

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