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Prognostic Biomarkers in Ovarian Cancer

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Abstract

Epithelial ovarian cancer (EOC) remains the most lethal gynecological malignancy despite several decades of progress in diagnosis and treatment. Taking advantage of the robust development of discovery and utility of prognostic biomarkers, clinicians and researchers are developing personalized and targeted treatment strategies. This review encompasses recently discovered biomarkers of ovarian cancer, the utility of published prognostic biomarkers for EOC (especially biomarkers related to angiogenesis and key signaling pathways), and their integration into clinical practice.

Keywords

biomarkers; ovarian carcinoma; prognosis

1. Introduction

Ovarian cancer remains the most lethal gynecologic cancer due, in part, to the advanced stage at presentation in most patients.¹ For these patients, surgery followed by chemotherapy remains the standard of care.² Given the diversity in responses, there is a need for highly reliable predictive and prognostic biomarkers. Since the introduction of CA125 as a biomarker for epithelial ovarian cancer (EOC) in 1981, the field of tumor biomarkers has grown substantially. Prognostic markers can help to identify patients at different levels of risk for specific outcomes, facilitate treatment choice, and aid patient counseling.³ In oncology, hundreds of prognostic marker studies are published each year. This review provides an overview of current biomarkers in ovarian cancer and their potential utility in clinical practice as prognostic biomarkers (i.e., those that can be used to estimate the chances of disease recurrence) and predictive markers (ie, those associated with response or

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lack of response to a particular therapy). A summary of these biomarkers is provided in Table 1.

2. Classic Biomarkers of Epithelial Ovarian Cancer

2.1. Clinical utility of CA125

CA125, the first ovarian cancer biomarker ever identified, is a high-molecular-weight glycoprotein recognized by the monoclonal antibody OC125. CA125 is expressed by epithelial ovarian tumors and other tissues of Mullerian origin. Serum CA125 levels are initially elevated in >80% of ovarian cancer patients and may be used to reflect the clinical course of disease.⁴ Since Bast and colleagues first produced the OC125 monoclonal antibody in 1981,⁵ serum CA125 measurement has been used extensively in clinical practice to screen women at high risk for ovarian cancer, to plan treatment, and to predict clinical course and response to chemotherapy. Despite having limited sensitivity and specificity, serum CA125 estimation is generally agreed to have clinical value during preoperative work-up and during post-treatment monitoring.^{4, 6, 7} Serum CA125 is also of prognostic significance in predicting patient survival and response to chemotherapy.^{4, 8–10} Therefore, CA125 remains one of the most important prognostic biomarkers for EOC patients in the clinical setting.^{9–11}

It is commonly agreed that patients with early-stage EOC are less likely to have an elevated serum CA125 level preoperatively and more likely to have a better prognosis.^{4, 6, 7, 12} One previous study showed a significantly higher survival rate for patients with preoperative serum CA125 <65 U/ml than for patients with preoperative serum levels >65 U/mL.¹³ Postoperative serum CA125 >65 U/mL was also associated with a significantly worse 5 year survival than was postoperative serum CA125 < 65 U/mL (42% versus 5%). Makar and colleagues found that elevated serum CA125 was an independent prognostic factor for survival, regardless of residual disease status after primary surgery.¹⁴

2.1.1. CA125 as prognostic factor—In patients without residual disease after primary surgery, significant prognostic factors for survival include histology type, postoperative CA125 level with a cutoff value 35 U/mL, and tumor grade ; for those with residual disease, they are histology type, postoperative CA125 level with cutoff value 65 U/mL, postoperative treatment, and size of residual disease. Dynamic change in serum CA125 level before and after treatment is also an independent prognostic factor for survival.^{14, 15} In a 10–year European study, Han and collaborators recruited 296 patients who met the criteria of CA125 progression and doubling during surveillance after first-line therapy. During surveillance, the median CA125 doubling time was 40 d; the median survival was shorter for patients whose CA125 doubling time was < 40 d than for those whose doubling time was > 40 d (10.6 months versus 22.1 months). On multivariate analysis, short CA125 doubling time (< 40 d) and short time to disease progression (6180 d) remained the only 2 independent adverse prognostic factors. Han et al also confirmed these findings in a set of 28 new patients. Together, their findings suggested that the rate of CA125 increase during surveillance has independent prognostic significance.¹³ Therefore, serum CA125 level should be considered when making therapeutic decisions for EOC patients.

2.1.2. CA125 as chemoresponsiveness predictor—Dynamic changes in serum CA125 levels can also be used to predict response to first-line and second-line chemotherapy.^{9, 16–21} This enables clinicians to plan primary treatment and to make the most appropriate second-line treatment decisions for patients with EOC. Some investigators have proposed using serum CA125 as a marker in clinical trials of new therapeutic drugs for ovarian cancer,²² which is now the case in new EOC drug development.²³

Karam et al proposed that withholding treatment in the event of isolated rising CA125 levels would not negatively affect these patients' overall survival,²⁴ thereby highlighting the need for more efficient clinical use of serum CA125 testing and for new EOC biomarkers. Clinical and basic research aimed at overcoming the drawbacks of CA125 by means of (1) dynamic CA125 monitoring, (2) combination with ultrasonography and other clinical factors such as TNM and stage, and (3) combination with other biomarkers has produced encouraging results.^{11, 15, 24–26}

3. Novel biomarker development in EOC

Thanks to the burgeoning development of genomics, proteomics, metabolomics, and other “-omics” and an improved understanding of cancer biology over the past 3 decades, hundreds of prognostically valuable EOC biomarkers have been discovered. Some of the most promising of these biomarkers are VEGF, HE4, mesothelin, M-CSF, osteopontin, kallikrein(s), bikunin, EphA2, and soluble EGF receptor, although none surpass CA125.^{11, 25, 27–35} Those most relevant to the recent clinical focus on targeted and personalized therapy are biomarkers of angiogenesis and related signaling pathways.

3.1. EOC biomarkers with angiogenic features

It is well established that angiogenesis (ie, the formation of new blood vessels) is necessary for the growth and metastasis of solid tumors.³⁶ Tumor angiogenesis is regulated by a complex, and still not fully understood, interplay between endogenous angiogenic stimulators (such as VEGF and FGF family members) and inhibitors (such as endostatin and thrombospondin-1).³⁷ Angiogenic biomarkers have been the subject of recent comprehensive reviews. Alterations in the expression of pro- and anti-angiogenic factors serve not only as markers of solid tumor progression but also as prognostic indicators for EOC.^{28–33, 35, 38–40}

VEGF was the first, and is now the most extensively studied, family of angiogenic factors. VEGF, the most prominent of the angiogenic stimulators, exists in four main forms of 121, 165, 189 and 206 amino acids in length. Each has a variety of functions, including recruitment and mitogenic stimulation of endothelial cells.⁴¹ In a study of VEGF expression in tumor specimens from 68 FIGO stage I and II EOC patients, median disease free survival was better in VEGF negative than VEGF positive patients (18 months vs. 120 months), and elevated VEGF expression was associated with poorer survival.²⁹ Serum VEGF elevation pretreatment, thought by some to be a good predictor of tumor angiogenesis, correlates significantly with poorer disease-free survival and overall survival.³² VEGF-A and other VEGF family members such as VEGF-D are independent prognostic factors in EOC.³⁸ Other researchers have reported similar results.^{28, 30, 31, 33, 35, 39, 42} Because VEGF is one of

the more prominent angiogenic mediators of new tumor vessel growth and also an important endothelial cell survival factor,⁴³ it is both a potential biomarker and a potential therapeutic target. Indeed, VEGF targeted therapy has shown clinical promise,^{44, 45} but its therapeutic effect is largely limited to newly forming tumor vessels and is dependent on complicated interactions with other factors such as EGF, PDGF, IL-8, EphA2, and the matrix metalloproteinases. Meanwhile, there is a lack of effective biomarkers for monitoring response to new antivascular agents.

EphA2, a transmembrane receptor tyrosine kinase overexpressed by many human cancers, is another promising angiogenesis-related prognostic biomarker.^{28, 46, 47} It is often associated with poor prognostic features and tumor angiogenesis.^{46, 47} EphA2 is a member of the largest family of tyrosine kinase receptors. The EphA2 family consists of 16 known receptors and 9 known ligands that are categorized into subtypes A and B by the ephrins with which they interact. EphA2 is involved in many processes crucial to malignant progression, such as migration, invasion, metastasis, proliferation, survival, and angiogenesis. It is overexpressed in cancers of the ovary, breast, prostate, lung, colon, esophagus, renal cell, cervix, and melanoma. In many of these malignancies, EphA2 overexpression is associated with poor survival, advanced stage, or increased metastatic potential. In an immunohistochemical study of 94 tissue specimens (EOC and control) by our group, EphA2 overexpression was significantly associated not only with higher tumor grade and advanced stage of disease, but also with significantly shorter survival (median, 3.1 years vs. 12 years for patients with low or absent EphA2 tumor expression), even after multivariate analysis in a Cox proportional hazards model.²⁸ Another study in endothelial and ovarian cancer cells revealed a strong association between EphA2 overexpression and the expression of critical tumor angiogenic and invasive factors.⁴⁷

Given the positive association of EphA2 with aggressive clinical and pathological features in human cancers and the cancer-promoting features of EphA2 overexpression,²⁸ we have examined the therapeutic potential of downregulating EphA2 expression in preclinical models. We found that silencing EphA2 alone could reduce tumor growth by up to 60% and, in combination with antibody/paclitaxel therapy, by up to 95%.^{46, 48–50}

Our recent study of the enhancer of Zeste homolog 2 (EZH2) showed the functional and biological importance of EZH2 in angiogenesis and tumor growth and the potential value of increased EZH2 expression in either tumor cells or tumor vasculature as a predictor of poor clinical outcome. This suggests the therapeutic potential of targeting EZH2.⁵¹ Many other angiogenic factors have potential prognostic value in EOC, yet, their prognostic significance has to be confirmed in further studies. These factors include FGF family members, interleukins (especially IL-6 and IL-8), cell-cell and cell-ECM (extracellular matrix) factors, including several claudin family members (claudin-3, claudin-4, and claudin-7) and matrix metalloproteinases (MMPs) (eg, MMP2 and MMP9), focal adhesion kinase (FAK), and tissue factor (TF), and their alterations in tissue, serum, and other biological fluids including cyst fluid and ascites.^{51–53}

Given the role of cytokines in the biology of ovarian cancer cells, the current interest in their diagnostic, monitory, and prognostic uses is not surprising. Interleukins are potent

endothelial-cell chemo-attractants. Current investigations have focused on IL-6 as a prognostic factor. High IL-6 levels have been associated with unfavorable clinical outcome,^{54–56} though not always consistently.⁵⁷ IL-6 level reflects a large tumor burden, and macrophage activation followed by subsequent release and accumulation of IL-6 in the serum and ascites and the expression of factors that might affect tumor burden and macrophage activation are not easy to control. In one study, IL-6 was no better than traditional markers as a disease biomarker when used alone, but was considered useful when used as part of a panel of cytokines.⁵⁸ Although IL-6-mediated tumor growth and angiogenesis has been demonstrated in ovarian cancer models,⁵⁹ the benefit of IL-6 as a marker of ovarian tumor angiogenesis remains to be determined.⁵²

Interleukin-8, one of the most widely studied cytokines in ovarian cancer, is a potent proangiogenic factor overexpressed in most human cancers, including ovarian carcinoma.^{60–63} Because IL-8 helps mediate tumor angiogenesis,⁶³ quantifying circulating IL-8 levels may be a useful biomarker of response. High IL-8 expression has been associated with advanced tumor stage, high tumor grade, and worse survival (median survival for patients with high vs. low IL-8 expression: 1.62 vs. 3.79 years). Increased IL-8 expression has also been associated with poor clinical outcome in human ovarian carcinoma and IL-8 gene silencing with decreased tumor growth via anti-angiogenic mechanisms.⁶⁴

Conversely, others have reported that IL-8 levels increase immediately after the initiation of chemotherapy in ovarian cancer patients, especially in those with residual disease.⁶⁵ This reflects not only the secretion of IL-8 by multiple sources including monocytes, neutrophils, and mesothelial cells, but also the need for further preclinical investigations of IL-8 as a tumor biomarker. The claudins are integral components of tight junctions (ie, apical cell-cell adhesions that regulate epithelial paracellular permeability and are critical for epithelial cell polarity). In tumor cells, the loss of cell-cell adhesion is central to cellular transformation and metastatic potential.^{66, 67} Certain members of the claudin gene family including claudin-3, -4, and 7 are abnormally regulated in several human cancers including ovarian cancer.^{68, 69} Claudin-3 is overexpressed in 90% of ovarian tumors, including all 4 major subtypes (serous, mucinous, clear cell, and endometrioid),⁷⁰ and is also associated with shorter survival.⁷¹ Claudin-3 expression also promotes migration, invasion, and survival of ovarian cancer cells.^{68, 72} Conversely, silencing the claudin-3 gene suppresses ovarian tumor growth and metastasis in mouse tumor models.⁷⁰ Claudin-4 overexpression in EOC apparently does not correlate with survival or other clinical endpoints.⁶⁹

Tissue MMPs are a large family of zinc- and calcium-dependent proteolytic enzymes that can degrade most components of the extracellular matrix and mediate tumor invasion and metastasis.⁷³ The MMPs associated with ovarian carcinomas include MMP-2, MMP-9, and membrane type-1 (MT1)-MMP according to majority of authors.^{74–78} MMP-2, MMP-7, MMP-8, MMP-9, and MT1-MMP have been detected in 54%, 81%, 95%, 97% and 100% of EOCs, respectively.^{78, 79} Torng et al found stromal MMP-2 expression to be a poor predictor of disease specific survival for patients with endometrioid cancer, but not for those with serous histology.⁸⁰ Davidson et al found MMP-9 mRNA expression in tumor cells to be an independent poor prognostic predictor of survival in patients with EOC.⁸¹ Others have reported that patients with weak tumor expression of stromal MMP-9 had significantly

longer survival than did those with moderate or intense expression.⁸² In a study of 90 EOC tissue specimens, Kamet et al reported that high epithelial and stromal expression of MMP-2, MMP-9, and MT1-MMP were each significantly associated with shorter disease-specific survival on univariate analysis, and that high stromal expression of MMP-9 and MT1-MMP and strong epithelial expression of MT1-MMP were poor independent predictors on multivariate analysis.⁷⁸ In contrast, Westerlund et al reported that, stromal MMP-2 expression correlated with earlier ovarian carcinoma and better prognosis.⁸³

Cell migration is an important component of the metastatic process and requires repeated adhesion to and detachment from the extracellular matrix microenvironment. These events are mediated, in large part, by integrins. FAK, an integral component of the integrin-signaling pathway, is a critical mediator of signaling events between cells and their extracellular matrix, thereby facilitating invasion and migration.⁸⁴ FAK is overexpressed in metastatic human colorectal, breast, thyroid, and prostate cancer cells and ovarian cancer.^{85–89} Its overexpression predicts poor clinical outcome by virtue of its significant association with worse survival (median, 7.6 years *versus* 2.98 years).⁹⁰ In a series of *in vitro* and *in vivo* experiments in which ovarian cancer cells were transfected with the dominant-negative construct FRNK, FAK inhibitor-TAE226, or FAK-siRNA-DOPC separately, the inhibition or silencing of FAK and its phosphorylation significantly reduced tumor burden in multiple tumor models and decreased invasion, migration, and cell spread.^{40, 90, 91} Further studies are warranted and necessary to translate these experimental findings into clinical practice. Other angiogenic markers, such as tumor cell-lined vasculature, microvessel density (MVD), and stromal compartment, are potentially prognostic and therefore potentially therapeutic in EOC.^{31, 42, 47, 92, 93} However, their utility after anti-angiogenic therapy remains to be determined. Consequently, we consider them to be microscopic level markers and outside the scope of this review.

Modern gene array-based profiling approaches have been used to develop EOC angiogenic biomarkers.^{94, 95} Using immunohistochemistry-guided laser-capture microdissection and genome-wide transcriptional profiling techniques, Buckanovich et al identified and validated 12 novel ovarian tumor vascular markers (TVMs) and found that overexpression of any one of three ovarian TVMs by vascular cells was associated with decreased disease-free interval. Using a similar approach, Mendiola et al tested 61 formalin-fixed, paraffin-embedded samples from late stage I EOC patients and generated a 34-gene profile that had independent prognostic value on multivariate analysis. This gene profiling approach will be discussed in more detail later in this review.⁹⁵

Despite the promise of anti-angiogenic therapy for EOC, its potential clinical use remains controversial.^{27, 96–98} Several of the most promising anti-angiogenic drugs, which target VEGF and VEGF related genes (eg, bevacizumab, sorafenib, sunitinib, and temsirolimus), are already approved by the FDA for use against solid tumors, such as advanced RCC and metastatic colorectal cancer, but their use in EOC remains the focus of preclinical and clinical trials.⁹⁶ Nevertheless, the promising results achieved with angiogenesis-related factors over the last few decades may soon lead to the targeted application of such factors in EOC and their use as biomarkers of clinical course and responsiveness.

3.2. Signaling pathway, apoptotic pathway, and genetic/ epigenetic related markers

Successful biomarker development depends on approaches that originate from the discovery phase and culminate in the clinical validation of an appropriately targeted biomarker. The most frequently reported molecular pathway biomarkers in ovarian cancer are EGF receptor (HER) family members, apoptotic pathway surrogates (such as p53 and cell cycle- related kinases) and DNA repair proteins (such as BRCA1/2 and ERCC1).

3.2.1. EGF receptor (HER) family—The human EGF receptor (HER) family mediates crucial cellular processes, including growth, proliferation, and survival. This receptor family consists of four transmembrane receptors: HER1 (EGFR), HER2, HER3, and HER4. Each receptor has an extracellular binding domain, a transmembrane domain, and an intracellular domain.^{99, 100} This receptor is noted for the interdependence and functional complementarity of its members and their tendency to heterodimerize with each other (eg, HER2/HER3, HER2/HER4), traits that have significant implications for the development of targeted therapies.^{101, 102} EGFR (HER) – 1,2 and 4 proteins possess tyrosine kinase activities, whereas EGFR(HER)–3 doesn't, but EGFR(HER3)-3 can dimerize with other EGFR(HER) family members and lead to activation of the EGFR(HER) pathways.¹⁰³

EGFR is widely expressed in a variety of human tumors including ovarian, head and neck, breast, and non-small-cell lung (NSCLC) cancers.¹⁰⁴ Estimates ranging from 60% to 98% of all epithelial ovarian cancers express high levels of the EGFR (HER), and extensive studies also revealed that EGFR (HER) had significant implication in tumor growth and progression.^{103, 105–108} Two separate groups have shown an inverse correlation between EGFR and survival in ovarian cancer.^{109, 110} Others have delineated the key role of EGFR in downstream signaling pathways such as the PI3K (phosphatidylinositol-3 kinase)/Akt and ERK (external signal-regulated kinase) pathways.¹¹¹ Still others have revealed a positive correlation between nuclear EGFR and cyclin D1 and K1–67.¹¹²

The role of HER2 has been studied in several tumor types, including breast, head and neck, prostate, and ovarian cancer.¹¹³ In breast cancer, HER2 overexpression varies widely (from 1.8% to 76%).¹¹⁴ In a randomized trial, Slamon found HER2 expression in breast and ovarian carcinoma to be indicative of aggressive tumor growth and poor prognosis.¹¹⁵ In a Dutch study of 208 patients, mean survival was shorter in patients with HER2-positive tumors than in those with HER-2 negative tumors (17 months vs. 26 months).¹¹⁶ Similar findings were made by Tanner et al in a study of 96 women with stage III ovarian cancer (12 months vs 25 months)¹¹⁷ and by Felip et al (62 weeks vs. 123 weeks).¹¹⁸ Other studies have shown HER2 expression to be an independent prognostic indicator of survival.^{119, 120}

In a study of patients with HER2-negative ovarian tumors, Gorden et al found that HER2 was activated in 45% of the tumors and that HER2 amplification was not required for any clinical activity associated with pertuzumab.¹²¹ Others have shown that HER2 may activate key downstream signaling pathways (eg, PI3K—Akt and RAS-Raf-MEK) by dimerizing with other ligand-activated HER receptors, particularly HER3.^{122–124} Indeed, HER2 preferentially binds with HER3, and the resulting HER2/HER3 heterodimer has a strong mitogenic signaling effect.¹²⁵ In a study of 116 consecutive patients with primary EOC, HER3 levels were associated with patient survival on both univariate and multivariate

analysis.¹²⁶ Moreover, as shown by Kaplan-Meier analysis, patients with low HER3 protein levels (n = 54) had a significantly longer median survival time than did patients with high HER3 protein levels (n = 62) (3.31 years versus 1.80 years). HER3, lacking of tyrosine kinase activity itself, however, after dimerization with other members of the EGFR family, several signal transduction cascades can be activated, including PI3-K (phosphoinositide 3'-kinase)/Akt and extracellular signal-regulated kinase (ERK1/2). In this study, they reported that HER3 overexpression was observed in 53.4% of patients.¹²⁶

HER2 and HER3, by themselves, are functionally incomplete receptors and therefore particularly dependent on each other. HER2 has an extracellular domain but no apparent ligand-binding activity, while HER3 has a non-functional kinase domain and no catalytic kinase activity. In contrast, the HER2/HER3 heterodimer is a highly functional signaling unit and is in fact the most active signaling dimer in the HER family.¹²⁷ HER4 mediates antiproliferative effects. However, its overactivity does not appear to play a major role in tumorigenesis.¹²⁸ Moreover, there exists the debate on the prognostic value of EGFR (HER) in EOC. Graeff and colleagues performed a meta-analysis on total 15 EGFR studies, 20 HER-2/neu studies and 62 p53 studies; they concluded that although p53, EGFR and HER-2/neu status modestly influences survival, these markers are, by themselves, unlikely to be useful as prognostic markers in clinical practice. Thus, further validation based on studies with well-defined, prospective clinical trials and more complete reporting of results are needed.¹²⁹

Biologically targeted antibodies and tyrosine kinase inhibitors directed toward EGFR/ ErbB1/HER1 (eg, cetuximab, erlotinib and gefitinib) and ErbB2/HER2 (eg, trastuzumab), and more recently toward ErbB3/HER3 and ErbB4/HER4, are being investigated as therapies for patients with EGFR/ERBB/HER proto-oncogene-driven malignancies.¹³⁰ Ongoing trials of these agents alone or in combination with traditional chemotherapeutic agents are underway.¹³¹ Trastuzumab, a monoclonal antibody against HER2, is the standard of care for patients with HER2-positive breast cancer in the metastatic and adjuvant settings and is associated with improved disease-free and overall survival. However, in a phase II trial of single-agent trastuzumab in patients with persistent or recurrent epithelial ovarian or primary peritoneal cancer, no relationship was found between HER2 expression and clinical response, progression-free survival, or overall survival.¹³² Interestingly, the combination of cytotoxic chemotherapy and EGFR/HER inhibitors seems to provide better clinical response and therefore warrants further evaluation. Inhibition of HER2/HER3 dimerization is a particularly attractive approach to anticancer therapy.^{124, 133}

3.2.2. Apoptotic pathway biomarkers

3.2.2.a. p53: The p53 suppressor gene has come to the forefront of cancer research because it is commonly mutated in human cancer. Thus, detection of p53 abnormalities may have diagnostic, prognostic, and therapeutic implications. p53 is mutated in all major cancers,¹³⁴ and represents the most frequent mutation in ovarian cancer.¹³⁵ p53 is one of the most important mediators of the intrinsic apoptotic pathway. Upon activation, the p53 protein acts as a tumor suppressor whose genetic effects lead to cell-cycle arrest, apoptosis, senescence, or differentiation.^{136–138} p53 is mutated in 60–80% of all sporadic EOCs and correlates

highly with high-grade tumors.^{139–143} Subtype-specific mutations in p53 have been found in almost 60% of serous tumors but only 16% of mucinous tumors,¹⁴⁴ which suggests a prominent role for p53 mutation in carcinogenesis of serous but not mucinous ovarian tumors. p53 mutation is a late event in ovarian carcinogenesis, and the loss of p53 apparently confers a more aggressive, rapidly growing phenotype. Several studies have examined the prognostic value of p53 antibodies in ovarian cancer.¹⁴⁵ One study on total 126 patients reported that the plasma p53-Ab positivity was correlated with the highest risk of cancer progression in serous EOC.¹⁴⁶ In another study of presurgical serum from 104 women with ovarian cancer, p53 antibody was associated with better overall survival. In a meta-analysis, patients with aberrant p53, EGFR, or HER2 tumor status had significantly lower odds of surviving 5 years. Moreover, Goodell and colleagues evaluated the relationship between antibody immunity to the p53 oncogenic protein with EOC outcome, and they demonstrated the presence of p53-Ab was an independent prognostic indicator of overall survival in advanced-stage patients.¹⁴⁷

Other studies have associated overexpression of mutant p53 with worse survival, FIGO stages,^{148–151} poorer histological grade of differentiation, and high proliferative fraction. Shahin et al observed an association between worse prognosis and p53 mutation status in patients with EOC. Overall, 48.5% and 57.3% of tumor samples from those patients showed p53 overexpression and p53 mutation, respectively; and even though neither p53 overexpression nor p53 mutation affected overall survival, the combination did predict worse survival in both univariate and multivariate models. When p53-nonresponsive and p53-null tumors were grouped together as functionally null, the worst prognosis was associated with missense mutations on univariate analysis. Functionally null p53, stage, and optimal cytoreduction were independent prognostic factors on multivariate analysis. More detailed characterization of p53's mutational spectrum and classification of mutations according to their effect on p53 functionality have led some groups to hypothesize that distinct types of mutations can shorten survival.^{152, 153} However, there is no consensus to date, and regardless of tumor stage, the effect of p53 mutation status on overall survival remains questionable. Some studies have found no association between any p53 alteration and prognosis.^{154–156}

3.2.2.b. Cell cycle-related kinases: Cell cycle deregulation is a hallmark of carcinoma.³⁷ CCRK (cycle-related kinase) is frequently overexpressed in brain, renal cell, and ovarian cancers and is expressed either weakly or not at all in normal ovarian epithelial tissue.^{157, 158} In a study by Wu et al, overexpression of CCRK in ovarian carcinomas correlated strongly with ascending histopathological grade, poor differentiation, and/or advanced clinical stage and thus served as an independent predictor of shorter overall survival. Upregulated expression of CCRK in ovarian carcinomas might involve the cyclin D1-associated pathway. Cyclin D1 is essential for G1 phase progression and is a candidate proto-oncogene.¹⁵⁹ Cyclin D1 overexpression is associated with tumor characteristics and clinical outcome in many cancers including ovarian cancer.^{160–166} In a study of 134 patients with serous EOC,¹⁶⁷ cyclin D1 overexpression (>10% versus 10%) was an independent predictor of poor survival. Such findings, however, are not fully consistent with those by other groups.^{168, 169} In a Cox regression analysis, Barbieri et al found that women with

cyclin D1-overexpressing ovarian adenocarcinomas and residual disease > 2 cm after surgery had significantly shorter survival than those who did not (relative risk(RR) of death: 2.48 and 3.7, respectively). These investigators did not, however, note any significant difference in cyclin D1 overexpression between histologic subtypes.¹⁶⁸ On the other hand, Masciullo et al¹⁶⁵ found that overexpression of cyclin D1 mRNA in EOC was significantly associated with well-differentiated and moderately well-differentiated tumor grade (grade 1–2) but not with clinical outcome and other parameters. Moreover, no association has been found between the presence of cyclin D1 expression and clinical outcome.^{161, 164, 168} These contrasting results are likely due to differences in analytical techniques and scoring systems used in those studies.¹⁷⁰

The role of cyclin E in oncogenesis and its function as a clinical prognostic indicator in cancer patients have been the focus of many reports in recent years. Cyclin E has been consistently associated with disease progression in various malignancies and with poor prognosis in patients with breast, bladder, colorectal, and ovarian carcinoma.^{171–178} Cyclin E gene amplification has been detected in 12% to 21% of ovarian tumors,^{177–179} RNA overexpression in up to 30% cases,^{177–179} and protein overexpression in up to 70%.^{180–182} Together, these findings suggest that cyclin E is an important mediator of survival and its expression an independent predictor of poor prognosis in patients with ovarian carcinoma. Overexpression of the cyclin E protein has been linked to shortening of the G1 phase of the cell cycle, decreased requirement for growth factors, enhanced cell proliferation, induction of chromosomal instability, and polyploidy.^{172, 183–186} These processes contribute to the oncogenic potential of cyclin E. Cyclin E's prognostic value is debatable. Rosen et al demonstrated that a high level of cyclin E is an independent predictor of poor prognosis.¹⁸⁷ Sui et al correlated increased cyclin E expression with tumor progression and poor prognosis in EOC patients.¹⁸⁰ In contrast, others have found no correlation between cyclin E expression and survival in patients with ovarian carcinoma^{167, 188} or only a trend toward an association with a poor prognosis.¹⁸⁹

3.2.3. BRCA1 and BRCA2—The BRCA1 and BRCA2 tumor suppressor genes help regulate cellular proliferation, chromosomal stability, and DNA repair via homologous recombination. They help maintain genomic stability by recognizing and mediating repair of DNA damage, regulating transcription, and controlling cell cycle checkpoints through p53-dependent and independent mechanisms.^{190–192} Approximately 10% of breast and ovarian carcinomas are due to a genetic predisposition (mainly germline mutations in the BRCA1 and BRCA2 genes).¹⁹⁰ Females carrying BRCA1 or BRCA2 mutations have a 16%–60% and a 16%–27% lifetime risk, respectively, of developing EOC.^{190, 193} Studies in high-risk families indicate that mutations in BRCA1 or BRCA2 genes confer as much as an 87% risk of breast cancer and 44% risk of ovarian cancer by the age of 70 (with mean age of onset 5–10 years earlier than in non-carriers) and that the vast majority of these patients will have serous adenocarcinomas.^{194–196} Interestingly, however, most studies indicate that women with a BRCA1 or 2-related ovarian cancer have better survival than non-carriers,^{197–204} particularly if they receive platinum-based therapy.^{201–204} In vitro studies have shown that some BRCA1 mutated ovarian cell lines have increased sensitivity to various chemotherapeutic agents.¹⁷¹ These findings are supported by an unbiased historical cohort

study of Ashkenazi Jews in which ovarian cancer outcomes were better for BRCA1 or 2 mutation carriers than for non-carriers¹⁹⁹ and by studies showing a more favorable response to chemotherapy among patients with hereditary ovarian cancer.^{199, 200, 205, 206}

3.2.4. ERCC—ERCC1 (excision repair cross-complementation group 1) is a major mediator of nucleotide excision repair (NER), a process that can correct most bulky lesions in DNA.²⁰⁷ ERCC1 overexpression is a potential marker of prognosis and of tumor resistance to platinum-based chemotherapy regimens in many cancers, including EOC.^{207–214} In patients with advanced EOC, higher levels of serum ERCC1 protein are often associated with widespread metastasis, enhanced DNA damage repair, and tumor aggressiveness. On the contrary, lower levels of ERCC1 (and resulting lower rates of DNA repair and increased early senescence and apoptosis of tumor cells) are often associated with slower metastasis and fewer lesions, which might render such tumors more amenable to optimal debulking surgery.²¹¹ Studies in EOC patients support this view. ERCC1 was shown to be an independent prognostic marker of debulking outcome, and ERCC protein expression was associated with survival or response to platinum compounds (but not always with debulking outcome).^{215, 216}

3.3. Epithelial ovarian cancer biomarkers with immune signatures

Given the well-established importance of the immune system in tumor development,^{217, 218} researchers are striving to find novel immune-related prognostic biomarkers. Zhang and colleagues reported that the presence of intratumoral T cells correlated with improved clinical outcome in advanced ovarian carcinoma from a study on 186 ovarian cancer specimens. They found that the five-year overall survival rate was 38% in patients with positive intratumoral T cells, compared with 4.5% in patients without T cells in tumor islets. They also found significant differences in progression-free survival and overall survival according to the presence or absence of intratumoral T cells in 74 patients with a complete clinical response after debulking and platinum-based chemotherapy (the five-year overall survival rate: 73.9% vs. 11.9%). The presence of intratumoral T cells independently correlated with delayed recurrence or delayed death on multivariate analysis and was associated with increased intratumoral expression of interferon, interleukin-2, and lymphocyte-attracting chemokines. The absence of intratumoral T cells was associated with increased levels of vascular endothelial growth factor.²¹⁹

Several novel immune-related biomarkers of EOC have independent prognostic value. One of these is the antigen processing machinery (APM) components. Han and colleagues were the first to report that APM downregulation and lack of intratumoral T-cell infiltrates are independent prognostic markers for death from ovarian carcinoma. In their study of 150 tumor samples from patients with invasive EOC, they found that most tumors expressed TAP1, TAP2, HLA-HC, and β_2 microglobulin either heterogeneously or positively; 67% possessed intratumoral CD3⁺ or CD8⁺ cells; and 88% possessed peritumoral CD3⁺ or CD8⁺ cells.²²⁰ Another prognostically valuable immune-related biomarker is B7-H3. Recently, Zang et al reported that B7-H3 (one of the B7 family members of immunoregulatory ligands), positive tumor vasculature was associated with significantly shorter survival time

and a higher incidence of recurrence.²²¹ Together, these findings imply the critical role of immune surveillance and immune escape in the clinical course of EOC.

3.4. MicroRNA, Dicer, and Drosha --- EOC biomarkers on the frontier of discovery

Recently, a new epigenetic regulatory program was identified with the discovery of small non-coding microRNAs (miRNAs) in *Caenorhabditis elegans*.²²² MiRNAs are endogenous, small non-coding RNAs (approximately 22 nucleotides) that negatively regulate gene expression at the posttranscriptional level in a sequence-specific manner.^{223–226} miRNAs may play a role in cancer development and progression. For instance, loss of miRNA-mediated gene regulation may be a tumorigenic mechanism,²²³ impaired miRNA processing may enhance cellular transformation and tumorigenesis,²²⁷ and miRNAs may act as oncogenes or tumor suppressor genes.^{228, 229} These insights have stimulated investigations into miRNA's role in ovarian cancer. An early study showed that approximately 40% of miRNA genes exhibit altered DNA copy numbers.²³⁰ More recent studies have shown that deregulation of miRNAs affects survival and drug resistance.^{226, 231, 232} In a study by Nam et al, higher expression of miR-200, miR-141, miR-18a, miR-93, and miR-429, and lower expression of let-7b, and miR-199a correlated significantly with poor prognosis.²⁴⁶ In a study by Yang et al, miR-214, miR-199*, and miR-200a were associated with high-grade and late-stage ovarian tumors.⁶³ Other studies have shown that miRNAs may be useful in predicting ovarian carcinoma outcome.²³³ For example, many members of the let-7 protein family act as tumor suppressors, and patients with low let-7a-3 methylation levels have worse overall survival than those with high methylation levels.²³³ In addition, even though most miRNAs are downregulated in cancer tissues, some are upregulated in recurrent as opposed to primary tumors.

Increased knowledge of the RNA-interference machinery and its 2 main components—the miRNAs Dicer and Drosha—has led to investigations of their use as diagnostic, prognostic, and therapeutic biomarkers in cancer.^{234–238} In mammalian miRNA biogenesis, the primary transcripts of miRNA genes (pri-miRNAs) are cleaved into hairpin intermediates (pre-miRNAs) by Drosha (nuclear RNase III) and further processed into mature miRNAs by cytosolic Dicer (another RNase III-related enzyme).²³³ Dysregulation of miRNA secondary to the defects in RNA silencing machinery has been observed in several types of tumors including ovarian cancers.^{238, 239} We were the first to report that low Dicer expression independently predicted poor outcomes in ovarian cancer patients.²³⁸ In line with our results, Faggad et al provided evidence that the decreased expression of Dicer has functional consequences for miRNA expression and leads to a global downregulation of miRNA expression and significant changes in gene expression.²⁴⁰ When we extended our analysis to other tumor types using mRNA expression profiling data, we confirmed a similar relationship between Dicer expression and outcomes in lung, breast, and other cancers.^{239, 241} Dicer expression appears to be upregulated in non-invasive precursors of invasive lung adenocarcinoma.²³⁹ Contrary findings in other tumor types have been reported (ie, correlation of high Dicer and Drosha expression with poor prognosis in prostate cancer²³⁹ and no correlation of Dicer expression with outcome in acute myeloid leukemia),²⁴² although these discrepancies might be explained by tissue specificity.

Suzuki et al have forged a link between the tumor suppressor p53 and posttranscriptional maturation of miRNAs by demonstrating that p53 interacts with the Drosha microprocessor complex through DEAD-box RNA helicase p68 (DDX5) to facilitate the processing of pri-miRNAs into pre-miRNAs.²⁴³ Recently, Wu et al reported that TAp63, a p53 family member, suppresses tumorigenesis and metastasis through the coordinate regulation of Dicer and miR-130b by directly binding and trans-activating the Dicer promoter, thereby demonstrating direct transcriptional regulation of Dicer by TAp63.²⁴¹

3.5. Plasma cell free DNA

Circulating plasma cell free nucleic acids, now known to be mainly tumor derived,^{244, 245} were discovered by Mandel in 1948.²⁴⁶ They are detected in the blood of both healthy and sick individuals, and their concentration is generally related to tumor load and disease extent. Patients suffering from malignant diseases like EOC usually have increased amounts of circulating cell free nucleic acids. Tumors release genomic DNA into the systemic circulation, probably through cellular necrosis and apoptosis.^{247, 248} Such tumor-specific cell free DNA may be detected in the plasma by the presence of genetic and epigenetic alterations specific to the primary tumor.^{249–251} This makes tumor-specific cell free DNA potential tumor biomarkers and therapeutic targets.

Fiegl and colleagues have found that persistence of *RASSF1A* DNA methylation of cell free DNA 1 year after primary surgery and adjuvant tamoxifen therapy is an independent predictor of poor outcome in breast cancer.²⁵² In an orthotopic EOC mouse model, we have shown that cell free DNA levels correlate closely with tumor load and that those levels decline appreciably with chemotherapy.²⁵³ Consequently, cell-free DNA is a potential prognostic marker in several solid tumors, including ovarian cancer.^{254–256} We have reported that cell-free DNA at concentrations >22,000 GE/mL is significantly associated with decreased patient survival. After adjusting for other clinical variables, preoperative cell-free DNA >22,000 GE/mL was an independent predictor for disease-specific survival. This suggests that serial monitoring of plasma nucleic acids may be useful for monitoring disease status and predict outcome. Zachariah and colleagues found higher levels of cell-free nuclear and mitochondrial DNA in ovarian cancer patients than in healthy controls but no correlation between cell-free DNA and prognosis.²⁵⁷ Further prospective studies on larger numbers of patients are needed to validate the prognostic value of cell-free DNA.

4. Combination of EOC prognostic biomarkers

The continuing discovery of new biomarkers of EOC raises the question of how to use them effectively. As already stated above, CA125 has often been combined with other biomarkers.^{26, 258–260} Jacob et al have included it in their Risk of Malignancy Index (RMI), which combines serum CA125 level, ultrasound results, and menopausal status to help identify and triage high risk patients by estimating background cancer risk. They found that patients with RMI scores >200 had 42 times the background risk and that patients with lower scores had 0.15 times the risk.²⁶¹

The development of effective biomarker combinations will ultimately rely on the proper design and appropriate use of systemic models and analyses.^{262, 263} One clinical trial in

200,000 women in the United Kingdom is now utilizing an algorithm to evaluate the ability of a 2-stage screening strategy to improve survival in ovarian cancer. More than 30 serum markers besides CA125 will be required to detect all patients in an initial phase of screening and will then need to be evaluated alone and in combination with CA125 by different investigators.²⁵

5. Challenges and future directions

Despite years of research and mushrooming reports of new tumor biomarkers, the number of clinically useful markers is pitifully small.²⁶⁴ This is partly due to the complex nature of the tumor biomarkers themselves—especially those at the DNA, RNA, and protein levels—and partly due to the lack of acceptable standards for effectively evaluating and incorporating these newer biomarkers in the clinic. Thus, further validation and effective translation are needed. In addition, most prognostic studies in cancer are not protocol driven or prospective but retrospective.²⁶⁵ Retrospective studies offer the distinct advantage of large cohorts whose follow-up periods are long enough to allow assessment of a substantial number of outcomes of interest. Conversely, their disadvantages include unclear inclusion criteria, cohort completeness and follow-up; incomplete baseline data; lack of standardization of diagnostic and therapeutic procedures; and statistical analytical issues.³ Thus, validation and randomized clinical trials are needed to determine the true value of the new biomarkers for early diagnosis and improved survival and quality of life.

The first challenge is to establish acceptable criteria for evaluating new biomarkers and for standardizing the information that is gained in an integrated and clinically useful setting.²⁶⁶ In 2005, REMARK proposed guidelines meant to encourage more clarity and comprehensiveness in biomarker studies.²⁶⁷ It is imperative to establish integrated, clinically feasible criteria for tumor biomarker studies and to design and conduct prospective studies that use a reliable model, and enroll sufficient numbers of patients, and are properly and comprehensively analyzed statistically.²⁶⁸ Although it is important to seek a convergence of findings across multiple, independent data sets, it is only through the accumulation of evidence that tumor biomarkers in EOC can begin to be validated clinically.^{258, 259, 269}

An important obstacle to the development of clinically useful tumor biomarkers is the availability of techniques for detecting them. Cancer-associated biomarkers in blood exist at low concentrations within complex mixtures of very abundant proteins, such as albumin and immunoglobulins, and there can be serious physiologic challenges to separating them out. Advances in nanotechnology are providing a completely new approach to this problem. For example, Liotta created a nanoparticle for biomarker “harvesting” that rapidly concentrates and amplifies scarce proteins for mass spectrometry analysis, multiple reaction monitoring, or immunoassay-based analysis.²⁷⁰

During the past decade, a growing knowledge of tumor biomarkers has been translated into targeted and personalized clinical therapies. One successful example is anti-angiogenesis therapy based on the developing knowledge of angiogenic biomarkers.^{28, 46, 48, 49, 50, 96} Another is the use of targeted drugs such as trastuzumab to target the HER2 proto-oncogene and EGFR inhibitors to block the EGFR signaling pathway.^{262, 263} In 2007, in an attempt to

speed up the drug development process, improve safety and efficacy, and increase the cost efficiency of using tumor biomarkers to identify potentially useful new cancer drugs, the AACR-FDA-NCI Cancer Biomarkers Collaborative launched a national effort to clearly delineate the barriers to such research, develop recommendations for integrating biomarkers into the cancer drug development enterprise, and set in motion the necessary action plans and collaborations.²⁷¹ Further efforts are needed to advance the use of biomarkers in cancer drug development and in the improvement of patient survival and quality of life.

In the near future, continued collaboration between clinicians, statisticians, and others should lead to establishment of clinically feasible criteria for biomarker studies, integration of reliable and statistically robust biomarker information into clinical treatment planning guidelines, and the development of more effective targeted strategies based on the knowledge gained from tumor biomarkers.

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Table 1

Prognostic Biomarkers of Epithelial Ovarian Cancer

Biomarkers	Clinical Utility
<u>Classic plasma/serum markers</u>	
CA 125	Postoperative levels of CA 125 >35 U/mL (no residual) or >65 U/mL (residual) are independent prognostic factors for survival ¹⁴
HE4	High serum level predicts unfavorable prognosis. HE4 combined with CA(125) assay can improve diagnosis ²⁷² A change in HE4 level of 25% is considered significant ⁸ (an increase of this magnitude suggests recurrence or disease progression; a decrease suggests therapeutic response)
Mesothelin	High mesothelin expression is associated with chemoresistance and with shorter disease-free survival and worse overall survival. ²⁷³
M-CSF	Markedly elevated levels of M-CSF1 in serum and ascites are associated with a poor prognosis ²⁷⁴
Osteopontin (OPN)	Osteopontin level in metastatic lesion may be a prognostic indicator in ovarian cancers ²⁷⁵
Expression of kallikrein (KLK)-related peptidases	Several kallikrein-related peptidases (such as KLK6, KLK13), in addition to CA125, can provide a significant advantage to detect early-stage ovarian cancer ²⁷⁶
Preoperative high plasma bikunin level	There is a 2.2 fold increased risk of death for patients with lower plasma bikunin levels ²⁷⁷
<u>Novel plasma/serum markers</u>	
Plasma cell-free DNA	Tumor-specific CFDNA levels correlate with increasing tumor burden and decline following therapy ¹⁵¹
<u>Angiogenic markers</u>	
VEGFs	Heterozygous genotype in VEGF +405G/C or VEGF -460C/T; higher serum expression of VEGF is associated with a shorter overall survival; elevated VEGF ascites levels negatively correlate with patient survival ^{278, 279} VEGF +405G/C, VEGF -460C/T, and VEGF +936C/T do not correlate with any of the investigated clinicopathological parameters ²⁸⁰
EphA2 expression	Overexpression is associated with poor prognosis ⁴⁶
FGF-1	Overexpression of FGF-1 is significantly associated with overall survival ²⁸¹
EZH2	Overexpression of EZH2 promotes the proliferation and invasion of human EOC cells ²⁸²
Claudin family members	Claudin-3 and claudin-7 expression in effusions independently predicts poor survival ²⁸³
<u>Apoptotic/signaling pathway and a genetic related markers</u>	
EGFR and HER2	Tumors with increased EGFR protein tend to grow more aggressively, are more likely to metastasize, and are more resistant to chemotherapy ²⁸⁴ ; HER2 overexpression is associated with poor overall survival ²⁸⁵ ; HER-2/neu expression does not appear to be an important prognostic factor in patients with advanced epithelial ovarian cancer ²⁸⁶
p53 mutation	p53 antibodies have shown mixed results with regard to prognostic value ^{287,288}
Cyclin D1, cyclin E	Overexpression of cyclin D1 is significantly related to a more aggressive tumor phenotype and poor prognosis in ovarian carcinoma ²⁸⁹ p16-cyclin D1/CDK4-pRb pathway is a prognostic factor for patients with abnormal G1 pathway. ³¹⁰ Overexpression of cyclin E is associated with poor outcome ¹⁸⁷
Serum sFas levels	Survival rates decrease as serum sFas levels increase ³¹¹ ; Serum sFas level is also a useful biomarker for predicting response to platinum-based chemotherapy ³¹²

Biomarkers	Clinical Utility
ERCC1	ERCC1 protein overexpression may act as a prognostic marker for poor survival of high-grade OC patients and as indicator for debulking outcome in advanced EOC ²⁹⁰⁻²⁹² Low ERCC1 expression correlates with improved survival in advanced OC ²¹²
BRCA1/2 carriers	The BRCAness profile correlates with responsiveness to platinum and PARP inhibitors ²⁹³ Patients with BRCA1-2 mutations represent only a subgroup within the group of patients with double primary breast and ovarian cancer ²⁹⁴ Low BRCA1 expression correlates with improved survival in advanced OC ²¹²
<u>Immune-related markers</u>	
IL-6, IL-7, IL - 8	Elevated IL-6 serum levels correlate with poor prognosis ⁵⁵ Increased IL-6 and IL-8 expression correlates with poor initial response to paclitaxel chemotherapy; elevated expression of IL-6 correlates with poor final outcome ²⁹⁵ IL-7 levels are strongly associated with ovarian cancer and might be used in combination with CA-125 to distinguish between malignant and benign ovarian tumors ²⁹⁶
Ascitic-fluid IL-12 levels	High ascitic-fluid IL-12 levels are associated with disease progression ²⁹⁷
APM (antigen processing machinery) component	APM (antigen processing machinery) component (TAP1, TAP2, HLA-HC, and beta 2 microglobulin) downregulation is significantly associated with improved survival ²²⁰
B7-H3	B7-H3 expression in tumor vasculature may reflect tumor aggressiveness ²⁹⁸
B7-H4	High B7-H4 levels correlate with poor prognosis; however, the effect is not significant when outcome adjusted for other clinicopathological variables ²⁹⁹
Intratumoral CD3+, CD8+ T cells infiltration	Intratumoral infiltration of CD3+ or CD8+ T-cells is significantly associated with improved survival ²²⁰
Gamma - interferon expression	Elevated interferon-gamma expression correlates with improved clinical outcome in patients with ovarian cancer ³⁰⁰ Loss of IFN gamma receptor independently predicts poor prognosis ³⁰¹
<u>Tissue-based markers</u>	
Claudin-3	High claudin-3 expression is associated with shorter survival
Claudin-4	Claudin-4 overexpression in epithelial OC does not correlate with survival or other clinical endpoints
MMP-2, MMP-9, and MT1-MMP	High epithelial and stromal expression of MMP-2, MMP-9, and MT1-MMP are each significantly associated with shorter disease-specific survival and are poor independent predictors of survival
FAK	Overexpression is associated with significantly worse survival
Levels of miR-200, miR-141,miR-18a, miR-93, miR-429, let-7b, miR-199a	Low-level expression of miR-200 mRNA predicts poor survival ³⁰² Higher expression of miR-200, miR-141, miR-18a, miR-93, and miR-429, and lower expression of let-7b, and miR-199a correlates significantly with poor prognosis ²³²
Dicer, Drosha expression	Dicer and Drosha mRNA levels in ovarian-cancers is associated with patient survival ²³⁸ Decreased Dicer expression correlates significantly with reduced patient survival in serous cancers and advanced disease ²⁴⁰