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# Ovarian Cancer Biomarkers: Current Options and Future Promise

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## Abstract

In recent years, as more effective, less toxic cancer drugs reach the patient population, the need for accurate and reliable cancer diagnostics and prognostics has become widely appreciated. Nowhere is this need more dire than in ovarian cancer, where the majority of women are diagnosed late in the course of disease progression. The ability to sensitively and specifically predict the presence of early disease, its status, stage, and associated therapeutic efficacy, has the potential to revolutionize ovarian cancer detection and treatment. Here, we review current ovarian cancer diagnostics and prognostics as well as potential ovarian cancer biomarkers that are being studied and validated. We also present some of the most recent molecular approaches being used to identify genes and proteins that may represent the next generation of ovarian cancer diagnostics and prognostics.

### Keywords

Ovarian cancer; biomarkers; diagnostics; prognostics

## Introduction

Ovarian cancer accounts for over 25,000 new cancer diagnoses annually and approximately 16,000 cancer deaths in 2007 [1]. The most lethal of the gynecological cancers, ovarian cancer has a five year survival rate of approximately 45% [1]. Ovarian cancer's relatively asymptomatic nature early in disease has earned it the reputation of being a "silent killer". In fact, approximately 70% of all patients with epithelial ovarian cancer present with clinically advanced stage III and IV disease, highlighting a significant clinical need for reliable and accurate biomarkers of ovarian cancer.

The focus of this review will be on secreted, shed and circulating biomarkers either currently being utilized or in development, that are present in human body fluids such as plasma, serum and urine. We compliment this discussion with a review of the state-of-the-art molecular approaches that are currently being employed in the discovery of new ovarian cancer biomarkers.

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## Current diagnostic and prognostic approaches

Current diagnosis of ovarian cancer relies on pelvic exam, transvaginal ultrasonography (TVS), abdominal ultrasonography, and exploratory or diagnostic laparoscopy when evaluating a pelvic mass [2]. One tumor biomarker, the cancer antigen 125 (CA125) is commonly used preoperatively to help predict potential for malignancy (see below) [3].

Disease stage at diagnosis is a powerful prognostic variable for predicting patient outcome in ovarian cancer. Patients with International Federation of Gynecology and Obstetrics (FIGO) stage III ovarian cancer, indicating tumor dissemination and seeding of the peritoneal lining outside of the pelvis, have a 5-year survival rate of approximately 35%. This survival rate drops to less than 10% in patients diagnosed with stage IV ovarian cancer, where disease has spread to distant metastasis. In contrast, patients with FIGO stage I disease, classified as ovarian carcinoma confined to the ovary, and having well to moderately differentiated tumors, have a greater than 95% 5-year survival rate [4]. Development of new biomarkers of early-stage ovarian cancer has the potential to significantly improve these bleak survival statistics.

Histological subtype is also considered when evaluating patient prognosis. There are five common histological subtypes of epithelial ovarian carcinoma: serous, mucinous, clear cell, transitional cell and endometrioid. Evidence suggests that clear cell carcinoma may predict a worse outcome for patients with early-stage disease [5,6].

For patients with advanced stage III and IV ovarian tumors, the most important prognostic factor for predicting favorable outcome is success of complete cytoreductive surgery and minimal residual tumor volume [7].

## Cancer antigen 125 (CA125)

The detection of elevated levels of serum CA125 has been useful preoperatively, to predict malignant potential [3]. Increased preoperative serum CA125 is correlated with poor overall survival [8]. In combination with transvaginal sonography, CA125 is also used to screen for ovarian cancer in BRCA1 mutated and other high risk populations [9]. CA125 has been used to evaluate the response of ovarian tumors to chemotherapy and to monitor disease recurrence as CA125 levels correlate with progression or regression of established disease [10]. Post-surgery, the CA125 level is correlated to the volume of remaining disease. Although the standard use for this marker is primarily in monitoring therapeutic response, increased CA125 levels are not completely consistent with a lack of therapeutic efficacy since early fluctuations in CA125 patterns are often seen during chemotherapy treatment involving certain drugs, and treatment decisions should not be based solely on CA125 levels [11].

Elevated levels of circulating CA125 are occasionally detected months before diagnosis of late-stage ovarian cancer [12]. Up to 80% of women diagnosed with late-stage epithelial ovarian cancer have elevated CA125 levels in their serum [13]. However, it has also been shown that as many as 20% of advanced epithelial ovarian tumors do not express CA125 at all.

Unfortunately, CA125 is limited in its usefulness in detection of early ovarian carcinoma when the disease is confined to the ovary, as only 50% of early-stage I and II cases demonstrate elevated CA125 levels [12]. Many benign ovarian conditions and nongynecologic conditions are also associated with an increase in circulating CA125, suggesting that CA125 lacks both the specificity and sensitivity to be a reliable biomarker in screening for early detection of ovarian cancer [14]. Researchers are now developing

biomarkers complimentary to CA125 to aid in the early detection and prognostic evaluation of this disease [15,16]. More than thirty potential serum biomarkers have been evaluated alone or with CA125.

## Osteopontin

One such protein is osteopontin, a glycophosphoprotein secreted by activated T lymphocytes, macrophages and leukocytes, found in extracellular matrix, sites of inflammation and body fluids. Osteopontin was initially found to be differentially expressed during a cDNA micorarray study of RNA isolated from ovarian cancer cell lines and human ovarian surface epithelial cells [17]. Subsequently, osteopontin plasma levels were significantly higher in 51 ovarian cancer patients compared to 107 healthy controls, 46 patients with benign ovarian disease, and 47 women with other gynecological malignancies [18].

A study to evaluate osteopontin levels in serum collected from 234 ovarian cancer patients after debulking primary surgery and longitudinally throughout chemotherapy revealed that mean osteopontin levels of cancer patients after surgery were significantly lower than that of normal controls suggesting that serum osteopontin might be secreted by the primary tumor [19]. In post-surgery patients, osteopontin levels in the serum significantly correlated with recurrent disease and correlated specifically with bulk disease [19].

Additionally, the level of osteopontin staining in metastatic lesions of 40 women with stage III ovarian cancer has been shown to be significantly increased compared to the corresponding primary ovarian tumor and this increase was reported to be an independent prognostic indicator of metastasis [20]. Osteopontin staining in metastatic tissue correlated with extremely poor outcome in these patients (3-year survival of less than 5%), compared to stage III ovarian carcinoma patients who did not display increased osteopontin in metastatic tissue compared to primary tumor (3 year survival rate of 75%) [20].

Recently, a proteomic-based method identified a C-terminal fragment of osteopontin in the pre-surgical urine samples of ovarian cancer patients and in patients with early-stage disease, suggesting that it may have potential as a useful non-invasive biomarker for the early detection and prognosis of ovarian cancer [21].

## Kallikreins

A specific human gene locus containing 15 kallikrein genes was identified on chromosome 19q13.4 as being the largest uninterrupted gene cluster of serine proteases in the human genome. Research has focused on kallikreins as potential serum biomarkers in ovarian, breast and prostate cancers because they are expressed in epithelial and endocrine tissues, regulated by hormones in cancer, and are shed and detectable in human body fluids [22].

Many kallikreins have been demonstrated to have differential expression in ovarian carcinomas compared to normal ovary [22–24], and are present in circulation. In one study, the majority of the 20 ovarian cancer patients screened had elevated human kallikrein 14 in their serum, but kallikrein 14 was undetectable by ELISA in the serum of 28 normal controls [25].

In a larger study of 146 ovarian cancer patients compared to 97 normal women and 141 women with benign gynecologic diseases, mean human kallikrein 10 (hK10) serum level was significantly elevated in 141 ovarian cancer patients before surgery, but was not elevated in the serum of patients with benign gynecologic diseases [26]. This serum elevation of hK10 significantly correlated with unfavorable prognosis, serous subtype, late-

stage disease and poor response to chemotherapy [26]. Interestingly, hK10 was found in the serum of 35% of ovarian cancer patients who were negative for CA125. When hK10 was combined with CA125 in early-stage I and II patients, sensitivity was increased by 21% (with 90% specificity) compared to CA125 alone [26].

Detection of other human kallikreins, including kallikrein 5, 6, 8 and 11 in the serum of breast and ovarian cancer patients, is also being investigated for its prognostic potential when combined with CA125.

## Bikunin

Bikunin is a glycosylated, Kunitz-type protease inhibitor which is known to inhibit invasion and metastasis. A study of 41 ovarian cancer patients found that reduced gene expression of bikunin in 17 of the 41 ovarian cancer tissues predicted poor prognosis of ovarian cancer patients [27]. Surgically removed ovarian tumor samples were further analyzed by immunohistochemistry and found high bikunin expression in 40 of 89 ovarian tumor samples, where high bikunin expression predicted favorable outcome and longer disease free survival [28]. A larger study reported the development of an immunoassay to detect bikunin in the circulation of 200 normal healthy women, 200 patients with benign gynecological diseases and 327 pre-surgery ovarian cancer patients [29], and demonstrated that low plasma levels of bikunin were associated with late-stage disease, presence of residual tumor after surgery, poor response to chemotherapy and reduced survival time. High preoperative plasma bikunin levels have been reported to be a strong favorable prognostic marker for ovarian cancer (median survival time was 60 months) compared to low serum bikunin levels (median survival time of 26 months) [29].

## Human epididymis protein 4 (HE4)

The gene encoding HE4 is commonly amplified in ovarian tumors. While the exact function of HE4 remains uncharacterized, it is a secreted protein that is absent in normal ovarian surface epithelium, but expressed specifically in 100% of the 16 human endometrioid epithelial ovarian cancers screened and 93% of the 60 serous ovarian carcinomas stained for HE4 [30]. An analysis of serum HE4 levels by ELISA in 37 patients with ovarian cancer compared to 65 healthy controls demonstrated that HE4 was specific and sensitive a test as CA125, and detected fewer false positives than CA125 in patients without malignant disease [31]. Additionally, an analysis of a series of biomarkers including CA125, osteopontin, HE4, EGFR, ErbB2 and others, analyzed alone and in combination, in the preoperative urine and serum samples from 67 patients with invasive ovarian carcinomas, and 166 controls with benign ovarian neoplasms revealed that, as a single tumor marker, HE4 had the highest sensitivity for detecting stage I ovarian cancer [15]. When multiplexed, CA125 and HE4 demonstrated the highest sensitivity (with a specificity of 95%) in detecting disease compared to either of these proteins alone [15].

## Vascular Endothelial Growth Factor (VEGF)

Multiple angiogenic factors and cytokines in circulation have been analyzed for their potential role in evaluating ovarian cancer detection and prognosis. The most studied of these is vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF). VEGF levels have been known to be elevated in ovarian cancer patients, where it contributes to the accumulation of ascites [32]. A number of groups have since evaluated the prognostic potential of serum VEGF levels in women with ovarian cancer [33]. A study that correlated clinical outcome with VEGF levels in the pre-operative serum of 314 patients with both early and advanced stage ovarian cancer, reported that higher serum VEGF levels independently correlated with shorter survival time [34]. In a multivariate Cox analysis

regression model, high serum VEGF expression in stage I patients correlated with an 8-fold increase in cancer-related death [34].

Other studies have reported that, while elevated VEGF in ascites is important as a prognostic factor of outcome in ovarian cancer, no discernable difference in serum VEGF levels were noted between ovarian cancer patients, controls and patients with benign disease [35,36].

Given the complexity and limitations of circulating biomarkers, it is not surprising that studies reporting the multiplexing of a small panel of biomarkers present in urine or serum yielded the most powerful sensitivity and specificity to date in detecting ovarian carcinoma [15,37,38].

We and others are examining biomarkers originally discovered and validated in other cancers, for their ability to detect and monitor ovarian cancer [39–43]. Some of the urinary biomarkers that we have reported include a panel of MMPs (Matrix Metalloproteinases) [41,44–48] and ADAM12 (A Disintegrin and Metalloproteinase 12) [41,43], among others. Given that these biomarkers are involved in basic processes common to most human cancers, the possibility exists that they may prove to be useful in ovarian cancer as well.

## Molecular approaches to the discovery of new ovarian cancer biomarkers

The discovery of new biomarkers relies on state-of-the-art methods for detecting genes and proteins in human body fluid and tissues. Below, we discuss some of the approaches currently employed to identify new biomarkers of ovarian cancer.

## Whole-genome analysis

Comparative genomic hybridization (CGH) is a whole-genome assay that detects gains or losses of gene copy number. Using this assay, a number of chromosome regions with abnormal gene copy number in ovarian cancer have been identified [49,50]. Some genes in these regions have been further evaluated as potential prognostic markers.

One such study in advanced serous epithelial ovarian cancers determined that chromosome 1q22, harboring the *RAB25* gene encoding a small GTPase, was amplified in 28 of 52 ovarian cancers [51]. Gene expression analysis confirmed that *RAB25* mRNA was increased in advanced stage ovarian tumor samples. Furthermore, increased *RAB25* mRNA levels were found to associate with decreased disease-free survival.

CGH analysis from another study suggested that FGF1 (Fibroblast Growth Factor 1) may also be a potential prognostic marker for ovarian cancer [52]. In the 42 late-stage, high-grade serous ovarian cancer cases analyzed, amplification in the chromosome region 5q31–35 significantly associated with poor survival. Amplification of the *FGF1* gene, which is located in this region, was further confirmed to associate with survival. Furthermore, this study found a significant correlation between FGF-1 expression and tumor angiogenesis, providing a possible mechanism underlying the correlation between FGF-1 and patient survival.

Clear cell ovarian cancers generally have poor prognosis and are more chemoresistant than other ovarian cancer histological subtypes. Tsuda and coworkers [53] applied CGH on DNA from 30 clear cell ovarian cancer patients and 19 serous patients and identified that *ABCF2*, a member of the ATP-binding cassette (ABC) family, was amplified in clear cell cancers. ABCF2 protein levels were also shown to correlate with chemoresistance in 20 ovarian cancer patients examined.

## Transcription profiling

Since different ovarian cancer histological subtypes are associated with different prognoses, many transcription profiling studies have focused on the discovery of markers that can discriminate between subtype. Schwartz et al. [54] found a gene expression pattern that distinguished clear cell cancer from the other subtypes. Seven out of eight clear cell cancers were accurately identified using a panel of 158 genes. The uniqueness of clear cell ovarian cancer was later confirmed by another study [55]. However, both of these studies have shown that a certain overlap of gene expression signatures still existed between the subtypes, suggesting some shared mechanisms underlying ovarian carcinogenesis.

Transcription profiling studies have also identified markers that may predict patients' survival. Spentzos et al. [56] reported a 115-gene signature identified from 34 samples that could distinguish between unfavorable (median survival, 30 months) and favorable overall survival (median survival not yet reached). In another study, which profiled 54 late-stage serous ovarian cancers, a panel of genes was identified that could discriminate between short-term (<3 years) and long-term (>7 years) survival [57]. This gene expression signature achieved a 100% accuracy in classifying 11 early-stage ovarian cancers as long-term survivors in an independent test set. The prognostic value of this signature was confirmed using the independent data set previously published [56].

Several other studies have attempted to identify gene expression patterns that can predict response to chemotherapy. These findings could potentially influence treatment decisions and make individually tailored therapy possible. A 93-gene signature predictive of pathologic complete response to chemotherapy was identified in a training set of 24 ovarian cancer patients [58]. This signature distinguished, in a separate validation set, between unfavorable (median 41 months) and favorable (median not yet reached) overall survival. Interestingly, the 93-gene signature shared no genetic overlap with the 115-gene survival signature that was described above. However, the combination of these two signatures provided more powerful prognostic discrimination than either one alone. Another 85 genes were found to be differentially regulated between primary chemosensitive and chemoresistant tumors by Jazaeri et al. [59]. Dressman and colleagues [60] further evaluated the gene expression signatures that defined the status of oncogenic signaling pathways. Gene signatures consistent with activated Src and Rb/E2F pathways were identified in chemoresistant patients. This finding could potentially lead to patient-tailored therapy that specifically targets these implicated pathways.

## MicroRNA profiling

MicroRNA (miRNA) was first discovered in C. Elegans in 1993 [61]. These RNAs are 19–24 nucleotides in length and do not encode proteins. They interact with the 3′ untranslated region of target mRNAs, leading to target mRNA degradation and inhibition of translation [62].

miRNAs have been found to be differentially expressed in tumor versus normal tissues in a range of solid as well as hematopoietic tumors. In some cases, distinct miRNA signatures can accurately distinguish tumor from normal tissues and are correlated with disease outcome [62]. Furthermore, in a study that analyzed miRNA signatures in a range of tumor types, it was suggested that the expression pattern of a relatively small number of miRNAs (about 200 in total) was more accurate than cDNA arrays in classifying human cancers [63]. These studies strongly suggest that miRNA profiling may have significant potential in cancer diagnosis and prognosis.

Iorio and coworkers compared the miRNA profiles of 69 malignant ovarian tumors with 15 normal ovarian samples [64]. Thirty-nine miRNAs were identified as being differentially expressed in tumor and normal tissues. This study also identified miRNAs that were associated with specific ovarian carcinoma subtypes. Some of the most regulated miRNAs have either tumor suppressors or other cancer associated molecules as their known or potential targets. For example, all four of the most upregulated miRNAs in tumors have the tumor suppressor BAP-1 (BRCA-1 associated protein-1) as their putative target. miR-140, which is among the most downregulated miRNAs, is predicted to target MMP-13 and FGF-2.

The differentially expressed miRNAs identified in this study were, for the most part, consistent with an earlier study which focused on the alterations in the miRNA genes [65]. The four most upregulated miRNAs were also amplified at the DNA level and 12 out of the 29 downregulated miRNAs were deleted.

In both studies above, let-7 family members were found to be either downregulated at the transcript level, or deleted at the DNA level, in ovarian cancers. Consistently, miRNA let-7d was identified as a marker for less advanced ovarian cancer [66]. The combination of let-7d and its target, high-mobility group A2 (HMGA2), was shown to be a superior prognosis predictor compared with other classical markers such as E-cadherin and vimentin. The HMGA2/let-7d expression ratio inversely correlated with progression-free survival of ovarian cancer patients.

## Proteomic profiling

One significant limitation of transcription profiling studies is that changes at the mRNA level do not always translate into changes at the protein level. Therefore, proteomic profiling is the most direct approach to search for diagnostic and prognostic biomarkers for ovarian cancer, and mass spectrometry (MS) is one of the principle methods used in proteomic profiling.

Proteomic profiling in ovarian cancer has been performed using two strategies. One strategy is to identify the distinct proteomic patterns of peptides in cancer samples. Petricoin and colleagues obtained mass spectra from a training set of serum samples from 50 women without cancer and 50 women with ovarian cancer and identified a cancer-specific proteomic pattern [67]. Three panels each containing four to five protein peaks were identified in another study as differentially expressed in ovarian cancer versus normal serum samples [68]. These protein peaks were different from those reported previously [67].

An alternative strategy is to identify individual peptides that are differentially expressed between cancer samples and normal samples. Three such proteins, transferrin, haptoglobin precursor fragment and immunoglobin heavy chain, were identified by comparing the proteomic profiles of plasma samples from 43 women with cancer and 38 women without cancer [69]. The combination of these proteins and CA125 significantly improved predictive performance compared to CA125 alone. Haptoglobin-alpha was also identified in a later serum profiling study [70]. Three other proteins, apolipoprotein A1, a truncated form of transthyretin and a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4, were identified in a five-center case-control study [71]. When the sensitivity was fixed at 97%, a combination of these three biomarkers and CA125 showed increased specificity for detecting early-stage invasive epithelial ovarian cancer compared to CA125 alone (74% vs 65%). These findings were later replicated in an independent blinded study [72].

In addition to the proteomic profiling of serum and plasma samples as described above, profiling has also been performed in other body fluids. For example, glycosylated

eosinophil-derived neurotoxin and COOH-terminal osteopontin fragments were identified as potential urinary markers for detection of early-stage ovarian cancer [21]. Gortzak-Uzan and colleagues studied the ascites proteome of ovarian cancer and reported 80 potential biomarkers [73].

These genomic and proteomic profiling studies have yielded important mechanistic information regarding the development and progression of ovarian cancer and once validated in large studies, may identify new diagnostic and prognostic biomarkers. However, many profiling studies are performed on small numbers of samples, results of different studies show limited overlap, and they are not always reproducible [74]. The use of large numbers of samples, and multiple and independent sample sets will be required to validate the discriminatory power of these candidate biomarkers [75].

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