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Early detection of ovarian cancer

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Abstract

Ovarian cancer is associated with an overall mortality of 75%, but can be cured in up to 90% of cases if diagnosed while still limited to the ovaries. Given the low prevalence of ovarian cancer in the general population, an effective screening strategy must not only have a high sensitivity for early-stage disease (>75%), but must also have a very high specificity (99.6%) to prompt no more than ten operations for each case of ovarian cancer diagnosed (positive predictive value [PPV] of 10%). Attempts to develop an effective screening strategy for ovarian cancer have utilized ultrasonography and serum tumor markers. Transvaginal sonography (TVS) and the serum marker CA125 have received the most attention to date. Used individually on a single occasion, neither of these approaches provides an adequate PPV and the cost of annual TVS is significant. Recent clinical trials have focused on serial monitoring of CA125 and the sequential use of a rising CA125 to prompt TVS in a limited number of women screened. Sequential monitoring of CA125 has significantly improved specificity of the assay in women over 50 years of age. The limited sensitivity of CA125 has, however, prompted a search for multiple serum markers that, in combination, would detect more than 90% of early-stage disease. Recent developments in genomic and proteomic research have identified a number of candidate biomarkers. Platforms have been developed that can assay more than 50 analytes in a few hundred microliters of serum. Panels of biomarkers have been discovered with high sensitivity and specificity for early-stage disease, but these require prospective validation. Several biomarkers have also been detected in urine, raising the possibility of a less expensive, more convenient screening test. Imaging techniques have been improved and mathematical methods developed that, in aggregate, promise to provide an effective screening strategy for ovarian cancer. In this review, we will assess the current status and describe future directions in ovarian cancer screening.

Keywords

biomarkers; early detection; ovarian cancer; proteomics; screening

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Ovarian cancer mortality & the rationale for early detection

Epithelial ovarian cancer kills 15,000 women in the USA each year, making it the leading cause of gynecologic cancer death and the fourth most common cause of cancer death among women [1]. Although the disease responds to cytoreductive surgical resection and chemotherapy in 70% of cases, less than 20% of women with advanced ovarian cancer (stage III and IV) can be cured. Disease limited only to the ovaries (stage I) can, however, be cured in up to 90% of patients [2]. As there are few specific symptoms [3] and routine pelvic examination is remarkably insensitive, only 20% of patients are diagnosed in stage I. Effective screening methods have impacted on survival in breast and cervical cancer, but at present there is no effective screening test for ovarian cancer. Since diagnosis at an early stage is associated with improved rates of survival, an effective screening strategy that detects early-stage ovarian cancer could have a significant impact on mortality from this disease.

Biological requirements for early detection of ovarian cancer

The potential of screening for any cancer is limited by the clinical biology of the particular disease. At diagnosis, the majority of patients with advanced ovarian cancer have multiple nodules of cancer that involve different sites on the peritoneum [4]. While it has generally been assumed that each peritoneal nodule represents a metastasis from a primary ovarian cancer, the disease might, in fact, be multifocal. Should multifocal peritoneal disease occur frequently, detection of lesions within the ovary would not assure complete resection or cytoreduction. To determine how frequently ovarian cancers and their metastases share a common heritage, the molecular characteristics of primary cancers and apparent metastases have been compared and determined whether the same X chromosome had been inactivated, whether a specific p53 mutation could be detected in both lesions and whether a distinctive pattern of loss of heterogeneity on multiple chromosomes was shared. Based on this analysis, approximately 90% of sporadic ovarian cancers were clonal, consistent with their origin from the progeny of a single cell within the ovary [5,6].

Another requirement for effective screening is that the metastatic disease observed in stage II–IV must actually arise from lesions that can be detected in stage I. Cancers that grow large enough to cause symptoms or to be readily detected on pelvic examination without metastasizing could have a different genotype and phenotype from cancers that metastasize to the peritoneum. The latter might spread from very small lesions that could not be detected by currently available imaging techniques. As in other forms of cancer, several mutations are required for malignant transformation of normal ovarian epithelial cells. Oncogenes are activated and the function of tumor-suppressor genes is lost. For high-grade ovarian cancers, the same genes were dysregulated in stage I–IV, albeit more frequently in the latter. This is consistent with the possibility that clinically detectable stage I disease is a precursor of metastatic ovarian cancer, at least for the most common high-grade cancers.

A third requirement for effective screening is that there is a sufficient interval between the development of detectable stage I disease and the development of metastasis. Serial measurement of CA125 has provided one estimate of the lead time before clinical detection of disease. In healthy women, CA125 levels appear to fluctuate around a stable mean, whereas in ovarian cancer patients an exponential rise is seen in CA125 levels. Serial annual evaluations of CA125 were performed in 28 patients who eventually developed ovarian cancer [7]. Using a rising CA125 level to indicate development of disease and assuming an exponential increase in CA125 over time, the mean duration of preclinical cancer was 1.9 ± 0.4 years [7]. While it is not certain how long the cancers had remained limited to the ovaries (stage I), these data do suggest that annual screening might suffice.

Ovarian epithelial tumors have highly heterogeneous histological appearances. Morphologically, they can resemble the mullerian epithelium, endocervix or endometrium. In addition, transitional clear cell and mixed carcinomas are also seen on histology. Although it is generally accepted that ovarian cancer arises from the ovarian surface epithelial cells, there is growing evidence that at least a part of the tumors arise from secondary mullerian structures around the ovaries. Tumors arising from these areas probably represent serous tumors that present on the ovarian surface or on the peritoneal surface. Findings of dysplastic changes and carcinomas in the fallopian tube of patients with the *BRCA* mutations gave rise to the alternative origin of ovarian cancer from cells other than ovarian surface epithelial cells [8–10]. Epithelial ovarian cancer is also thought to originate from inclusion cysts, which are believed to be invaginations of the ovarian surface. These cysts have been found in increased numbers in the ovaries of women with family history of ovarian cancer, in the normal ovary of women with unilateral ovarian cancer, ovarian tissue adjacent to invasive cancer and in the ovaries of women with positive or suspicious screening results [11,12]. A better understanding of the 'cell of origin' of ovarian cancer will help in discovering effective biomarkers for early diagnosis. It is possible that because of the molecular heterogeneity of ovarian cancer a panel of markers may be more effective than a single biomarker.

Epidemiological requirements for early detection of ovarian cancer

An effective screening strategy for ovarian cancer must meet quite stringent requirements. Owing to the low prevalence of ovarian cancer in the general population, it has been estimated that a specificity of 99.6% and a sensitivity of more than 75% for early-stage disease would be required to achieve a positive predictive value (PPV) of 10% (ten surgical interventions for each case of malignant disease diagnosed), even in postmenopausal women over 50 years of age who are at higher risk than younger women [13].

Requirements would be less stringent if women at higher than average risk were screened. The incidence of sporadic ovarian cancer increases with age and the median age at diagnosis is 63 years. Thus, it is prudent to target an older population, especially postmenopausal women. Nulliparity, age at first pregnancy, early menarche, late menopause, infertility, hormone replacement therapy, lifestyle and dietary habits are definite contributing factors. Prominent protective factors have also been identified: multiparity, oral contraceptive use, hysterectomy, breast feeding and prior oophorectomy. With the possible exception of oral contraceptive use for more than 5 years, which can reduce risk of ovarian cancer by 50% or more, most of these factors have a relatively modest impact on risk and have not permitted identification of an appropriate group at sufficiently high risk to prove useful in planning clinical trials.

However, the risk of developing ovarian cancer is markedly increased in women who carry mutations of the *BRCA1, BRCA2* or the *HNPCC* mismatch repair genes [14]. Experience to date with familial ovarian cancer raises concerns that the biology of the hereditary disease may differ from that of sporadic ovarian cancer and that detection of ovarian cancer in mutation carriers may require a different screening strategy. Hereditary ovarian cancer is frequently multifocal rather than clonal [4,15]. Morphologically transformed cells with *p53* mutations indicative of metastatic potential can be detected in microscopic cysts observed in prophylactic oophorectomy specimens [16,17]. Anecdotal cases indicate that widespread disease can be diagnosed 3 months after a negative transvaginal sonography (TVS) and normal CA125 value have been obtained [18]. Consequently, separate trials and strategies may be required to detect sporadic and hereditary ovarian cancer.

Available screening strategies for ovarian cancer

Transvaginal sonography

Earlier use of transabdominal ultrasonography has largely been replaced by TVS, which provides a more precise and detailed image of the ovary [19]. Data can be obtained regarding ovarian volume, morphology, echogenicity, presence of abnormal lesions, blood flow and any other pelvic masses. To increase sensitivity and specificity, morphological indices have also been developed that incorporate cyst wall structure, thickness and complexity of septae, presence of internal papillae or solid structures. Three large studies used TVS as a screening modality in more than 70,000 women to achieve a sensitivity of 90% for stage I disease with a PPV of 7.4–9.9% [19–21]. In a more recent study, Van Nagell *et al*. screened 25,327 women using TVS. Asymptomatic women aged 50 years or over and women aged 25 years or over who had a family history of ovarian cancer were eligible for participation in this trial. They reported a sensitivity of 85% for all stages of disease with a specificity of 98.7% and a PPV of 14%. In addition, they concluded that with annual TVS screening they could detect the disease at stage I or II. In this study, 82% of women who developed cancer during screening had either stage I or II compared with 34% of women in the unscreened control group ($p < 0.0001$). Although a significantly higher fraction of early-stage cancer was detected, they also had nine patients who developed ovarian cancer within 12 months of a normal scan. These patients had minimally enlarged ovaries at surgery and eight of the nine patients (88%) had elevated CA125 at surgery [22]. It is possible that adding CA125 to the screening modality might have detected several of these nine patients at an earlier stage.

The use and role of Doppler ultrasonography as a screening technique is controversial and has been debated since the early 1990s [23–26]. In a study by Stein *et al*., both grayscale and Doppler ultrasonography had similar PPVs of 50 and 49%, respectively. A similar finding that Doppler adds little to grayscale was also noted by Valentin [27]. Data from color Doppler are processed to calculate impedance, resistive index (RI) and pulsatility index. Using an RI of less than 0.4, Kurjak *et al.* predicted ovarian cancer with 100% sensitivity and 99% specificity [28]. However, those data could not be verified in later studies [29]. It was also noted that up to 43% of benign premenopausal tumors contained vessels with an RI of less than 0.4 during the follicular phase of the menstrual cycle [30]. A new parameter in color Doppler is time-averaged maximum velocity (TAMVX). TAMVX, in conjunction with age and papillary projection score, could determine the presence or absence of disease confidently. By utilizing artificial neural network (ANN) to evaluate the above parameters, Tailor *et al.* achieved a sensitivity of 100% and a specificity of 98.1% [31]. An advanced variation of color Doppler is power Doppler and is reported to have a better diagnostic potential than conventional Doppler [32]. Power Doppler measures the energy of a returning Doppler signal rather than analyzing the flow pattern. An advantage of power Doppler is that it can evaluate low-velocity blood flow. A further improvement of power Doppler is 3D power Doppler, providing imaging and measurement of blood flow in solid areas and excrescences of complex cysts [33]. Cohen *et al.* studied 71 women with solid and complex ovarian masses to evaluate if 3D power Doppler was superior to 2D power Doppler in evaluating ovarian masses. They found that all malignancies were correctly identified by both 2D and 3D imaging; however, the specificity significantly improved with the addition of 3D power Doppler [34]. Availability of the instruments and the necessary expertise for interpretation has limited the use of both techniques.

While TVS may provide an adequate PPV, expense precludes its routine application for annual screening in women at conventional risk. At US$250–400 per procedure, calculations of the cost per year of life exceed those for other accepted screening procedures such as mammography. More cost-effective approaches might utilize serum markers alone or in combination with TVS in a more limited number of women.

CA125

To date, CA125 is the serum marker that has received the most attention in identifying methods for early detection. CA125 was originally developed to monitor patients previously diagnosed with ovarian cancer and not for screening. When used as an individual marker on a single occasion, CA125 is not sufficiently sensitive to detect all cases of early-stage ovarian cancer [35]. In most studies, CA125 is elevated in approximately 50–60% of stage I disease at the time of conventional diagnosis [36].

In addition, CA125 is not sufficiently specific to screen a general population in that a number of common benign conditions can cause elevation of CA125 levels, including endometriosis, adenomyosis, ovarian cysts, uterine fibroids, renal dysfunction and hepatic disease. Even in postmenopausal women, a single value of CA125 at a conventional cutoff of 35 U/ml can achieve 99% specificity, but this falls short of the requirement of 99.7% [13].

CA125 is elevated in serum from 90% of patients with advanced epithelial ovarian cancer and released into blood both from cancer cells and from the inflamed peritoneum. CA125 is a mucin (MUC 16) of more than 1M Da with an intracellular, transmembrane and extracellular domain. The extracellular domain is heavily glycosylated and contains multiple repeating subunits of 154 amino acids. A protease cleavage site is found in the extracellular domain, which is thought to mediate its shedding. CA125 was first detected using the OC125 murine monoclonal antibody [37]. As there were multiple repeating subunits on the CA125 molecule, the OC125 antibody could be used both to capture antigen on a bead and to quantitate the trapped antigen in a double determinant radioimmunoassay [38]. Subsequently, a second antibody reactive with CA125, M11, was developed by O'Brien *et al.* in 1991, permitting development of the CA125 II assay, which traps antigen with M11 and detects trapped antigen with OC125, with less day-to-day variation than that observed with the original CA125 assay [39].

Elevated CA125 levels were seen up to 5 years prior to diagnosis of ovarian cancer in population-based studies [40]. Bast *et al.* recorded an exponential rise in CA125 levels 10–12 months prior to clinical diagnosis [41]. Exponentially rising CA125 was also documented in studies from Stockholm and the UK [40,42,43]. In the Stockholm study, a CA125 II assay was used to examine 5550 women. Six out of the 175 women with raised CA125 levels ultimately developed cancer. Of the women with normal CA125 levels, three were later diagnosed with cancer [43]. Interestingly, only the patients with ovarian cancer displayed a progressive increase in CA125 levels, whereas in those with benign conditions, CA125 levels remained constant over time. A more recent study conducted on 9233 women with two or more serial CA125 measurements showed that serial CA125 measurements were better than a single cutoff value [44]. Receiver operating characteristic curves show that the area under the curve increases significantly from 84 to 93% when serial CA125 values are used over a single CA125 value [44]. Exponentially rising CA125 appears to be a feature differentiating cancer from noncancer patients, in whom the levels tend to remain stable over a period of time.

Two-stage strategies

To augment the specificity and sensitivity for screening, CA125 has been combined with imaging techniques in a two-stage strategy. Combinations of CA125 and imaging have been tried both concurrently as well as sequentially. Jacobs and coworkers studied a group of 4,000 healthy women, comparing the specificities of individual evaluation or a combination of CA125, ultrasound and pelvic examination [45]. Their study showed a specificity of 98.3% for CA125 alone and 97.7% for pelvic examination alone. A combination of pelvic examination and ultrasound achieved a specificity of 99.4%; with CA125 and ultrasound specificity reaching 99.9% [45]. However, performing ultrasound for screening all women is prohibitive in terms of expense and inconvenience. Hence a two-stage strategy using sequential use of

CA125 and ultrasound for ovarian cancer, analogous to strategies used for cervical (cytology and culposcopy) and breast cancer (mammography and biopsy), was evaluated. In a recent study conducted in the UK by Jacobs and coworkers, the specificity of CA125 alone or in combination with abdominal ultrasound was evaluated in postmenopausal women 45 years of age or above. The subjects were divided into a control group (10,977) and a screened group (10,985). In the screened group, CA125 was measured annually for 3 years. If the levels were more than 30 U/ml, abdominal ultrasound was performed. If abdominal ultrasound was abnormal, surgery was performed. Using these two-stage methods, 29 operations were performed to detect six cancers (three stage I and three stage III) in the screened group, providing a PPV of 21%; in other words, five operations for each ovarian cancer detected. A further 7-year follow-up in this group detected ten more cancers. A total of 21 cancers were detected in the control group during the same interval. Median survival in the screened group (72.9 months) was significantly greater than in the control group (41.8 months) [46].

The Prostate, Lung, Colon and Ovary (PLCO) screening trial is using concurrent testing of CA125 and TVS in subjects between 55 and 74 years of age. This randomized controlled trial of screening versus usual care was initiated in 1994 and has studied 37,000 men and an equal number of women. CA125 levels were checked at enrollment and then annually for 3 years. A serum and plasma bank has been created that will enable researchers to study serial samples from patients as well as normal subjects. Participants are being followed for a period of 13 years. On detection of pelvic lesions or an elevated CA125 level, the patients are referred to their local physicians for further management. An initial report of this study shows abnormal TVS was found in 4.7%, and CA125 in 1.4% of the participants. The PPV for detection of ovarian cancer for CA125 alone was 3.7% and for TVS alone was 1%. When CA125 was elevated and TVS abnormal, the PPV for both tests combined was 23.5%, but 60% of early-stage disease would have been missed [47].

Risk of ovarian cancer algorithm

Skates and colleagues developed a computer algorithm that estimates the risk of ovarian cancer based on serial values for CA125 over time [48]. In ovarian cancer patients, CA125 levels increase exponentially with time, whereas in benign conditions and in normal patients CA125 values remain constant. Using data from the Stockholm screening series, Skates and colleagues developed the risk of ovarian cancer algorithm (ROCA), which can distinguish ovarian cancer patients from individuals without the disease. A sensitivity of 83%, specificity of 99.7% and a PPV of 16.0% was obtained in the initial analysis of the serial CA125 values [43].

To make screening decisions using ROCA, CA125 values are used to calculate the risk of having ovarian cancer and subjects are grouped as normal, intermediate and elevated risk. Normal-risk subjects undergo annual CA125 II tests, intermediate-risk individuals return for a CA125 II assay in 3 months time and elevated-risk subjects are referred for TVS. Every time a subject has a CA125 II test the ROCA is re-evaluated and the individual re-triaged. In a prospective trial using the ROCA, Menon *et al.* achieved a specificity of 99.8% and a PPV of 19.0% [49]. The United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKC-TOCS) in postmenopausal women to evaluate the ROCA. In this study, 200,000 women have been randomized into a control group of 100,000 subjects who are being followed up by annual pelvic examinations, 50,000 are being followed by CA125 II to prompt TVS in 2% of women screened (ROCA), and another 50,000 are monitored by annual TVS alone. Subjects will be accrued for 3 years, and will be followed over 7 years. Jacobs *et al.* used the ROCA and TVS in a study of the first 10,000 subjects and obtained a PPV of 20%. They screened 5046 women and performed TVS in 101 subjects. Abnormal TVS led to 17 surgeries, detecting four ovarian cancers (two in stage IC and one each in stage II and borderline cancer in stage I).

Novel serum biomarkers

Requirements for additional biomarkers

At the tissue level, approximately 20% of ovarian cancers express little or no CA125. Therefore, the sensitivity of a strategy based on CA125 alone will not exceed 80%. Finding additional markers that detect cancers which do not express CA125 should help to achieve higher sensitivity. Extensive efforts to discover novel biomarkers have identified several serum, plasma and urinary markers, but at high specificity, the sensitivity of these does not equal that of CA125 when each marker is considered individually. Simultaneous evaluation of multiple markers may increase the sensitivity while maintaining high specificity.

HE4

The *WFDC2* gene, which encodes HE4, is amplified in ovarian carcinomas [50]. ELISA for HE4 showed that it is detected in the serum of early- as well as late-stage ovarian cancer patients. The HE4 test successfully identified cancer in 30 of the 37 serum samples from women known to have the disease, while the CA125 test identified 29 cases. When used together, both biomarkers detected 33 of the 37 cancer cases. HE4 may have an advantage over CA125 in that it is less frequently positive in patients with nonmalignant disease [51–53]. In a recent report, Moore *et al.* analyzed serum and urine samples from 67 patients with invasive epithelial ovarian cancers and 166 with benign ovarian neoplasm's for levels of CA125, mesothelin, HE4, CA72–4, activin, inhibin, osteopontin, EGFR and ERBB2 (Her2) [54]. Statistical analysis was carried out to obtain sensitivities at fixed specificities of 90, 95 and 98%. HE4 had the highest sensitivity as a single marker (72.9% sensitivity at 95% specificity) and HE4 and CA125 were complementary to each other in predicting ovarian cancer with a combined sensitivity of 76.4% at 95% specificity [54]. However, HE4 alone had a sensitivity of only 45.9% at 95% specificity to differentiate the benign from stage I cancer. All markers and combination of markers had low detection sensitivity for stage I disease in this study. In addition, less frequent occurrence of HE4 in nonmalignant conditions makes it more useful in evaluation of premenopausal women.

Mesothelin

Mesothelin (SMRP) is a glycosylphosphatidylinositol-linked cell surface molecule expressed in ovarian cancer cells and in the mesothelial lining of the body cavities. This novel CA125-binding protein is a promising candidate biomarker for detection of early ovarian cancer [55]. In a study by Hellstrom *et al.*, elevated serum mesothelin was detected in 60% of ovarian cancer with a specificity of 98% [56]. Interestingly, a combination of mesothelin and CA125 detected more ovarian cancers than each marker alone. Mesothelin was elevated in the sera from 48% of late-stage and 12% of early-stage ovarian cancer patients at 95% specificity in a series of cases studied by our group [57]. Our laboratory also found that glomerular filtration rate (GFR) normalized urinary mesothelin was raised in 42% of early (stage I and II) cancers and 75% of late (stage III and IV) cancers. Urinary mesothelin values provide some complementarity to serum CA125 values in the detection of early-stage ovarian cancer.

Prostasin

Prostasin is a prostatic serine protease that was identified to be a marker for ovarian cancer using gene-expression array analysis of mRNA from normal ovarian surface epithelial cells and ovarian cancers. Significantly, higher levels of prostasin were detected in the serum from ovarian cancer patients than from healthy women and stage II disease had the highest levels of prostasin [58]. However, this biomarker has low sensitivity in stage I disease [59]. In their study, Mok *et al.* obtained a sensitivity of 64.9% for CA125 and 51.4% for prostasin at a specificity of 94% for prostasin. Using a combination of CA125 and prostasin, a sensitivity of

92% and a specificity of 94% was achieved when sera from 37 patients (nonmucinous ovarian cancers) of all stages were compared with 100 healthy subjects [58]. The stage distribution of these tumors was not specified. This specificity falls far short of the values required for effective screening. However, there was lack of correlation between the two markers, suggesting that these markers may be complementary to each other.

Kallikriens

Kallikriens (KLKs) are a family of serine proteases that are overexpressed in ovarian cancer [60]. Several, but not all, of the 13 KLKs are overexpressed in the tissues and serum of ovarian cancer patients, including KLK6, -10 and -11. Using an immunoassay for serum KLK10, 56% of ovarian cancer patients had significantly elevated KLK10 serum levels compared with healthy women [61]. Similarly, elevated KLK11 was found in 70% of ovarian cancer sera at a specificity of 95% [62].

Osteopontin

Osteopontin is another potential biomarker that was detected using cDNA microarrays [63]. It is a calcium-binding glycophosphoprotein and is found in all body fluids and in the extracellular matrix. Serum osteopontin levels are significantly higher in ovarian cancer patients when compared with normal subjects [64]. However, in contrast to CA125, higher levels of osteopontin were found in mucinous cancers than in the serous histotype of ovarian cancer. In a study of sera from healthy women and ovarian cancer patients, including early-stage disease, Nakae *et al.* obtained 81 and 84% sensitivity for ovarian cancer using osteopontin and CA125, respectively. A combination of the two markers increased sensitivity to 94% but the specificity was very low (33.7%), which would not support effective screening [65]. Using osteopontin in combination with leptin, prolectin and IGF, Mor *et al.* reported a sensitivity of 96% and a specificity of 94% in 24 early-stage ovarian cancer patients. Interestingly, similar to mesothelin, a fragment of osteopontin has been detected in the urine of ovarian cancer patients [66].

Lysophosphatidic acid

Lysophosphatidic acid (LPA) is a bioactive phospholipid and has been shown to stimulate proliferation of ovarian cancer cells and increase resistance to chemotherapy [67]. Elevated LPA levels were found in ascitic fluid from most ovarian cancer patients [68]. This biomarker is measured in the plasma rather than serum, since serum contains platelets derived lipids. Xu *et al.* have shown elevated LPA levels in plasma from 90% of stage I disease and in 100% of stage II/III ovarian cancer patients [69]. Later studies evaluating the subspecies of LPA achieved better sensitivity and specificity in detecting ovarian cancer.

Other markers

Levels of OVX1, a $Lewis_y$ determinant, are raised in the serum from 48% of ovarian cancer patients at 99% specificity [70,71]. OVX1 in combination with macrophage colony-stimulating factor (M-CSF) and CA125 can detect a greater fraction of stage I patients than CA125 alone. M-CSF is a cytokine that binds to the CSF-1 receptor. Low levels of M-CSF are expressed in normal ovarian cells but elevated levels are seen in approximately 70% of women with ovarian cancer. M-CSF complements CA125 in the identification of ovarian cancer patients, and elevated levels of M-CSF are seen in patients with normal CA125 levels. Data from two separate studies evaluating these three markers in stage I disease show improved sensitivity from 69% (CA125 alone) to 84% (CA125, M-CSF and OVX1), but specificity fell from 99% (CA125 alone) to 89% (CA125, M-CSF and OVX1).

Other novel markers include IL-6 and -8 [72,73]. IL-6 and -8 in combination with CA125 were able to achieve a sensitivity of 88% and a specificity of 98% in early-stage ovarian cancer serum samples. VEGF may prove to be a complementary marker to CA125, as has been shown in studies by Gorelik *et al.* and Rosen *et al.* [72]. Analysis of ovarian cancer tissue from patients with low or absent CA125 levels VEGF was found to be present in 81% of CA125-deficient tissues. In our laboratory we used Affymetrix arrays to compare gene-expression pattern of 41,441 known genes and expressed sequence tags between 42 ovarian cancers and five pools of normal ovarian surface epithelial cells. Analysis of our samples showed at least threefold upregulation of 86 genes. Utilizing recursive descent partition analysis of known genes we found that a combination of HE4, CA125 MUC1 and VEGF expression could distinguish the tumor from normal samples [52].

Multiple marker analysis

It is unlikely that a single biomarker will be able to attain the sensitivity and specificity required for effective screening. Thus, it is important to identify a panel of biomarkers that can detect up to 90% of early-stage ovarian cancers with a specificity of 98%, requiring TVS examinations in only 2% of screened women to provide a cost-effective strategy. Several studies have demonstrated that a combination of markers will yield better results than a single marker in isolation. In general, these have required specialized mathematical analyses to maintain specificity, while increasing sensitivity. Such methods include mixed, multivariate analysis and neural network analysis. Using the classification and regression tree analysis Woolas *et al.* combined eight different markers (CA125, M-CSF, OVX1, LASA, CA15–3, CA72–4, CA19–9 and CA54/61) and obtained better results than individual assays in differentiating the benign masses from malignant ones (sensitivity of 94.3% and specificity of 90.9%) [74]. In another study, a panel of four markers, CA125, CA72–4, CA15–3 and lipid-associated sialic acid, was analyzed using artificial neural network. This combination of markers was able to achieve a sensitivity of 79% and a specificity of 87.5%, which was better than CA125 alone for distinguishing malignant from benign pelvic masses [75]. In a recent publication by Zhang *et al.*, it was shown that using ANN analysis of multiple markers yielded better result than CA125 alone for use in a two-stage screening [76]. A total of 468 serum specimens were analyzed for CA125 II, CA72–4, CA15–3 and M-CSF. Comparison of the diagnostic power of CA125 II alone and the ANN-derived index showed that the latter calculation was superior. At 98% specificity, ANN-derived index and CA125 II had a sensitivity of 71 and 46%, respectively ($p = 0.047$) for detecting early-stage ovarian cancer [76].

Lokshin *et al.* have measured multiple markers in small quantities of serum using a novel multiplex array system that utilizes miniaturized double determinant immunoassays [72,73]. A family of antibodies that recognize up to 100 different tumor biomarkers are bound to polystyrene microspheres that are internally dyed with red and infrared fluorophores. A second family of antibodies that recognize different epitopes on the same biomarkers are labeled with a green fluor. After incubation with serum specimens, beads are analyzed by flow cytometry, using the red laser to identify the biomarker and the green laser to quantitate the concentration of the biomarker. They have analyzed 93 different markers including cytokines, chemokines, adhesion molecules, cancer antigens and apoptotic markers [77]. A combination of CA125, EGF, VEGF, IL-6 and -8 was able to reach a sensitivity of 91% and a specificity of 91% [72]. Their group also used adaptive bandwidth kernel based density estimator combined with projection pursuit technique and identified four serum protein markers (CA125, HE4, sEFGR and sVCAM-1) that achieve 90% sensitivity and 98% specificity for early-stage ovarian cancer [Yurkovetsky Z, Lomakin A, Skates S *et al.*: Development of a multimarker assay for early detection of ovarian cancer (2008), Submitted].

Proteomic techniques

Current proteomic techniques are highly developed complex systems of mass spectroscopy with enhanced bioinformatics tools for data analysis [78]. Proteomic analysis of sera from patients with ovarian cancer and from healthy individuals can identify subtle differences in the pattern of mass to charge ratios among the thousands of peptides that can be detected. There are two basic approaches to utilize these data for early detection of ovarian cancer. One method attempts to identify a distinctive signature or pattern of protein expression that distinguishes sera from ovarian cancer patients. The second approach identifies those proteins and peptides that provide the greatest discriminatory power and develops individual bioassays for each protein. Each approach has strengths and limitations. Using the pattern of peptide spectra obtained with surface-enhanced laser desorption and ionization – time of flight (SELDI-TOF) or matrix-enhanced surface desorption and ionization – time of flight mass spectrometry (MALDI-TOF), investigators have reported a sensitivity of 95% or more at 95% specificity for early detection. Petricoin *et al.* compared ovarian cancer sera from all stages with controls (100 cancer, 100 normal and 16 benign disease) using SELDI-TOF to analyze the proteomic pattern [79]. This study consisted of a 'training set' containing 50 women with ovarian cancer and 50 healthy women. The aim was to identify a unique serum protein expression pattern that could distinguish the cancer patients from healthy individuals. Analysis of 116 unknown serum samples (validation set) showed 100% sensitivity and 95% specificity in differentiating the patients from normals (PPV 94%). It was very encouraging that all the early-stage cancer was identified correctly. While early reports analyzed relatively few stage I specimens, later studies have included larger numbers. Of greater concern has been the continuing evolution of the analytical computer algorithm and difficulty in reproducing work from the primary data. Using SELDI-TOF for biomarker development requires very meticulous experimental design to obtain reproducibility. Quality of the chips used, calibration protocols, machine noise, serum handling procedures and sample randomization on the chips are a few of the variables which affect outcomes of proteomic analysis [80]. Some reviewers found evidence for nonbiologic experimental bias in many of these experiments that may invalidate many results. Use of routine statistical methods along with the proprietary algorithms should be employed to minimize irreproducible findings [81].

Zhang *et al.* employed SELDI to identify putative markers and then studied the individual markers in detail [82]. They analyzed serum proteomic patterns for sera from 153 patients and 142 healthy women. ApoA1, a truncated form of transthyretin and a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4 (IATH4) were identified and studied further. The sensitivity of the three biomarkers and CA125 (74% [95% confidence interval [CI]: 52–90%]) was higher than that of CA125 alone (65% [95% CI: 43–84%]) at a matched specificity of 97% (95% CI: 89–100%) [82]. In addition to ApoA1, transthyretin and IATH4, subsequent studies have identified four other biomarkers that aid in distinguishing malignant from benign pelvic masses, including fragments of transferrin, hepcidin, β2 microglobulin and connective tissue activating protein 3. Addition of the seven proteomic biomarkers to CA125 increased sensitivity for stage I disease from 68% with CA125 alone to 80% for the combination at a specificity of 98% [83].

Other approaches to ovarian biomarker discovery

mRNA

miRNA's are recently discovered small noncoding RNA molecules (19–25 nucleotides in length) that have been shown to regulate gene expression in an sequence specific manner. They are thought to negatively target human mRNA and have been found to have a role in the development of many types of tumors and also affect various biological processes in plants and other animals. Few studies have reported the alterations of miRNA in ovarian cancer;

however, no study has documented using miRNA signature for screening ovarian cancer [84, 85]. This is probably because this is a very recent development in cancer research. Iorio *et al.* found miR-200a, miR-141, miR-200c and miR-200b were upregulated and four other miRNAs were downregulated in ovarian cancer samples. Similarly, Zhang *et al.* in their study looked at DNA copy number abnormalities of genomic regions containing 283 known human miRNA genes by using high-resolution array comparative genomic hybridization [85]. It appears that miRNA's play a critical role in ovarian carcinogenesis, but it remains to be seen whether the alterations in ovarian tumor-specific miRNA's in plasma can be used for early diagnosis or screening.

Antibody array

Antibody array is an emerging technology that is used for direct profiling of protein expression. One of its major advantages is it allows multiparametric quantification of protein levels and the analysis of post-translational modifications. Antibody arrays are available in different formats but the most commonly used is the sandwich ELISA format. It has been used for studying leukemias, breast, urinary bladder, prostate and colon cancers [86]. Sanchez-Carbayo *et al.* studied serum from bladder cancer patients and were able to discriminate cancer from controls with 93.7% accuracy [87]. Although no reports of antibody arrays are available for ovarian cancer biomarker detection, fairly good success in other tumors has been achieved. Major limiting factors are availability of good quality specific antibodies and cost [86].

DNA methylation

Other potential techniques such as DNA methylation markers in the serum, glycomic analysis and free-circulating tumor DNA have all been found to be helpful markers of diagnosis, prognosis or response to therapy. In a recent report, IGF-binding protein-3 promoter methylation was found to be a prognostic marker for disease progression and death in early-stage ovarian cancer [88]. Few methylation markers (secreted frizzled-related protein 1 and CTGF) have been associated with poor prognosis in ovarian cancer [89,90]. It is highly likely that combinations of methylated loci in plasma can function as biomarkers for early detection of ovarian cancer.

Novel urine biomarkers

Identification of sensitive and specific urine markers for early-stage ovarian cancer would preclude the need for phlebotomy, reducing cost and providing a more convenient initial screening test. The pattern of proteins in urine is less complex than serum or plasma and the proteins or peptides excreted in urine are more stable [2]. Glycosylated eosinophil-derived neurotoxin, COOH-terminal osteopontin fragments and the β-subunit core fragment of human chorionic gonadotrophin have been reported in urine from ovarian cancer patients [66]. Our laboratory has measured SMRP in the serum and urine of early-and late-stage ovarian cancer patients. Serum and urine levels correlated, but the urine assay exhibited greater sensitivity for early-stage disease. When urine values were adjusted for GFR, SMRP levels were elevated in 42% of early-stage and 75% of late-stage ovarian cancer patients [57]. Patricia Kruk at the University of South Florida has found that the anti-apoptotic protein Bcl-2 is elevated in urine from a majority of ovarian cancer patients [91]. Collaborative studies are underway to determine whether a combination of Bcl-2 and mesothelin exhibit greater sensitivity than either biomarker alone. Proteomic techniques have also been applied to the analysis of urine from ovarian cancer patients.

Conclusions

Ovarian cancer is neither a common nor a rare disease, which places severe constraints on the specificity as well as the sensitivity of a screening strategy. When completed in 2011, the UKCTOCS trial will determine whether serial measurement of CA125 alone followed by TVS will be sufficiently sensitive and specific to impact on survival. If this trial is successful, it is clear that multiple markers will be required to achieve optimal sensitivity in the first stage of a two-stage screening strategy. Work from several laboratories has identified candidate panels using a variety of techniques for biomarker discovery including gene-expression arrays and proteomics. Banks of serum are available through the UKCTOCS and PLCO trials. The four National Cancer Institute-funded Specialized Programs of Research Excellence (SPOREs) in Ovarian Cancer are collaborating with the University of Pittsburgh, PA, USA, to analyze more than 50 biomarkers from participating institutions and the PLCO serum bank where a significant number of samples have been stored from women prior to diagnosis of ovarian cancer. The ultimate goal must be to use multiple serum biomarkers and TVS or other imaging techniques to detect this preclinical disease. With the progress that has been made over the last decade, there is great promise for the development of an effective screening strategy for early detection of ovarian cancer within the next few years.

Executive summary

Rationale & requirements for early detection of ovarian cancer

- Ovarian cancer is neither a common nor a rare disease.
- Up to 90% of patients can be cured if the disease is detected in the early stages when it is limited to the ovaries.
- Currently no effective screening strategy is available for ovarian cancer.
- A specificity of 99.6% and a sensitivity of more than 75% for early-stage disease is needed to achieve a positive predictive value of 10%.

Available screening strategies for ovarian cancer

- A single determination of CA125, the only serum marker currently available for early detection, is not sufficiently sensitive or specific to be used as a biomarker for screening a general population.
- Two-stage strategies using rising CA125 and imaging sequentially have shown promising results, but trials need to be completed.

Novel serum biomarkers

- Novel markers in the serum, urine and plasma are being analyzed to complement CA125 for achieving better sensitivity at high specificity.
- HE4, mesothelin, prostasin, lysophosphatidic acid, kallikriens and proteomic signatures are promising new markers.
- A panel of multiple markers will be needed to achieve the best possible sensitivity and specificity.
- Multiplex assays using Luminex beads can measure several markers in small quantities of serum and have been used successfully.
- Analyzing multiple marker panels with mixed, multivariate analysis and artificial neural networks can yield better algorithms for biomarker discovery.

- Proteomic techniques such as surface-enhanced laser desorption and ionization (SELDI) – time of flight and matrix-enhanced surface desorption and ionization (MALDI) – time of flight mass spectrometry have been used to identify unique protein expression patterns in ovarian cancer patients.
- Serum handling procedures, ensuring proper calibration protocols, machine noise and use of different algorithms remain major concerns of SELDI- and MALDI-based assays.

Urinary markers

- Few urinary markers are currently available. Once more markers are discovered, urine can be of great value since it is noninvasive, easy to collect and contains a less complex protein pattern.

Future perspective

- With a steady progress in biomarker discovery using modern techniques and complex bioinformatics analytical tools, a reliable and successful ovarian cancer screening strategy will soon be available.

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