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EARLY DETECTION OF OVARIAN CANCER: NEW TECHNOLOGIES IN PURSUIT OF A DISEASE THAT IS NEITHER COMMON NOR RARE

ROBERT C. BAST, JR., M.D.

HOUSTON, TEXAS

ABSTRACT

Elimination of cancer in the 21st Century is likely to depend not only on more effective individualized treatment, but also upon earlier detection and prevention of different malignancies. Screening strategies for ovarian cancer have centered on the serum tumor marker CA 125, transvaginal sonography (TVS), or sequential use of the two modalities. A single determination of CA 125 is neither sufficiently sensitive nor specific to be used as an initial stage in screening. Specificity can be improved by monitoring CA 125 over time with an algorithm that estimates risk of ovarian cancer. Sensitivity of CA125 can be improved by use of multiple markers in combination. Gene expression array analysis, proteomics and lipomics are being utilized to identify markers that can be used in combination with CA 125 to detect >95% of early stage ovarian cancers. To maintain high specificity, values for different markers are being combined using novel approaches of neural network analysis and mixed multivariate analysis. Sequential use of multiple markers and TVS could provide a cost-effective strategy to detect a disease of intermediate prevalence.

Introduction

Ovarian cancer is neither a common nor a rare disease. Overall, some 25,400 women will develop ovarian cancer in our country this year and 14,300 deaths will occur. (1) The lifetime risk for a woman in the United States is, however, only 1 in 70, compared to 1 in 9 for breast cancer. Even in women over 50 years of age who are at highest risk for epithelial ovarian cancer, the prevalence is 1 in 2,500. Over the last two decades, 5-year survival has increased from 37% in 1974–1976 to 52% in 1992–1998. Improved 5-year survival has related to advances both in cytoreductive surgery and in combination chemotherapy. Using a taxane in combination with a platinum compound, 70% of ovarian cancer patients with advanced disease will respond to treatment in the short run, but less than 30% survive long-term.

Eliminating the threat of epithelial ovarian cancer might be accomplished through several strategies. Treatment of advanced disease

could be individualized using molecular diagnostics and molecular therapeutics. Ovarian cancer might be prevented using oral contraceptives, fenretinide, vaccines and other chemo-preventive agents, provided that subsets of women at increased risk could be identified more readily. Alternatively, strategies for prevention of ovarian cancer might be bundled with strategies that prevent a more prevalent disease such as breast cancer. Perhaps the most promising approach involves detection of ovarian cancer at a stage when it can still be cured with conventional treatment.

Ovarian cancer has a distinctive pattern of spread. Like other epithelial cancers, ovarian malignancies can spread intravascularly as well as through lymphatics. More frequently, however, ovarian cancer spreads over the peritoneal surface, producing a myriad of nodules on the serosal and parietal peritoneum. When cancer is limited to the ovaries (stage I), 90% of patients can be cured with currently available treatment. Conversely, when disease has spread from the pelvis (stage III–IV) less than 30% of patients survive long-term. At present only 25% of ovarian cancer are diagnosed in stage I. Detection of preclinical disease at an earlier stage in a larger fraction of patients might improve survival (2).

Requirements for Effective Screening

There are several biological requirements for effective screening. Most ovarian cancers must be clonal, rather than multifocal. Several studies document that 90% of epithelial ovarian cancers are indeed clonal and arise from the progeny of single cells (3,4,5). In addition, most advanced stage disease must develop from clinically detectable stage I lesions. Multiple genetic changes are required to transform normal ovarian surface epithelial cells. Substantial heterogeneity has been observed in the pattern of alterations in oncogenes and tumor suppressor genes among ovarian cancers from different individuals. It is possible that disease diagnosed currently in stage I could exhibit a different genotype and phenotype than that observed in metastatic stage III–IV disease. Studies with gene expression arrays indicate that a similar pattern of abnormally regulated genes is observed in stage I and stage III high-grade ovarian cancers (6). Finally, an adequate interval must be observed between the development of a clinically detectable ovarian cancer and metastasis. Based on elevation of serum tumor markers, Skates, et al, estimated that 1.9 years elapsed between the appearance of shed tumor products and clinical diagnosis (7).

The prevalence of ovarian cancer in the post-menopausal population

(1 in 2,500) places stringent requirements on any strategy for early detection. As definitive diagnosis requires operative intervention, most gynecologic surgeons and patient advocates feel that no more than 10 operations should be performed to diagnose a single ovarian cancer. To achieve this positive predictive value of 10%, a screening strategy must have sensitivity greater than or equal to 75% and a specificity of 99.6%.

Approaches to Screening for Epithelial Ovarian Cancer

Three approaches have been utilized to detect early stage ovarian cancer: peripheral blood markers, ultrasonography and a sequential combination of these two modalities.

Ultrasonography. Early studies utilized transabdominal sonography (TAS) (8), but more recent trials have employed transvaginal sonography (TVS) that permits more precise imaging of each ovary. Potentially, TVS could detect small malignant lesions that have not metastasized. In practice, it can be difficult to distinguish malignant from benign abnormalities and surgery may be required to achieve a diagnosis. In three large trials of TVS, 67,620 women have been screened in the United Kingdom (9), United States of America (10) and Japan (11). Some 565 operations were performed to find 45 cancers with 35 (78%) in stage I. Consequently, sensitivity for stage I ovarian cancer may not exceed 90%, particularly when screening prevalent disease. In these three trials, screening was associated with a positive predictive value 7.4 to 9.9%, at the margin of that required to perform 10 operations for each case of ovarian cancer detected. In addition to limitations in specificity, the current expense of the procedure argues against using annual TVS for cost-effective screening of a population at normal risk.

CA125. Of the serum markers for ovarian cancer, CA125 has received the greatest attention. CA125 is an epitope on a large mucin glycoprotein molecule (MUC16) of greater than 1 million Daltons (12). CA125 was first detected using a murine monoclonal antibody, OC125 that had been raised against a human ovarian cancer cell line (13). Multiple CA125 determinants are expressed on each MUC16 molecule. Consequently, a double determinant radioimmunoassay could be developed (14). CA125 antigen was trapped on a bead coated with OC125 antibody and trapped antigen was then detected using radiolabeled OC125. Over the last two decades, the CA125 assay has been applied to the management of epithelial ovarian cancer in several settings (15). CA 125 has been used for monitoring response to primary treatment, determining prognosis based on apparent half-life, predicting residual

disease at the time second look laparotomy, and detecting clinically occult ovarian cancer at the time of recurrence, as well detecting primary disease in early stage.

CA125 and TAS. CA125 can be elevated 10 to 60 months prior to conventional diagnosis of ovarian cancer (Figure 1) (16,17,18). CA125 levels are elevated in sera from 50 to 60% of patients with stage I disease (19). An individual value of CA 125 is not sufficiently specific to permit effective screening. Specificity can be improved by combining CA 125 with ultrasonography and by sequential monitoring of CA125 values over time. Jacobs, et al (20), combined CA125 with TAS, in a sequential two stage strategy. Post-menopausal women were randomized to a control group (10,777) or to a screened group (10,985). If CA125 levels were elevated on an annual screen, transabdominal ultrasonography was performed. If abnormalities were detected, surgery was undertaken. Among women screened, 29 operations were performed to detect 6 cancers, yielding a positive predictive value of 21%. Median survival in the screened group (72.9 months) was significantly greater ($p = 0.0112$) than in the control group (48.1 months) (Figure 2). Diagnosis of a disproportionate number of cases in the

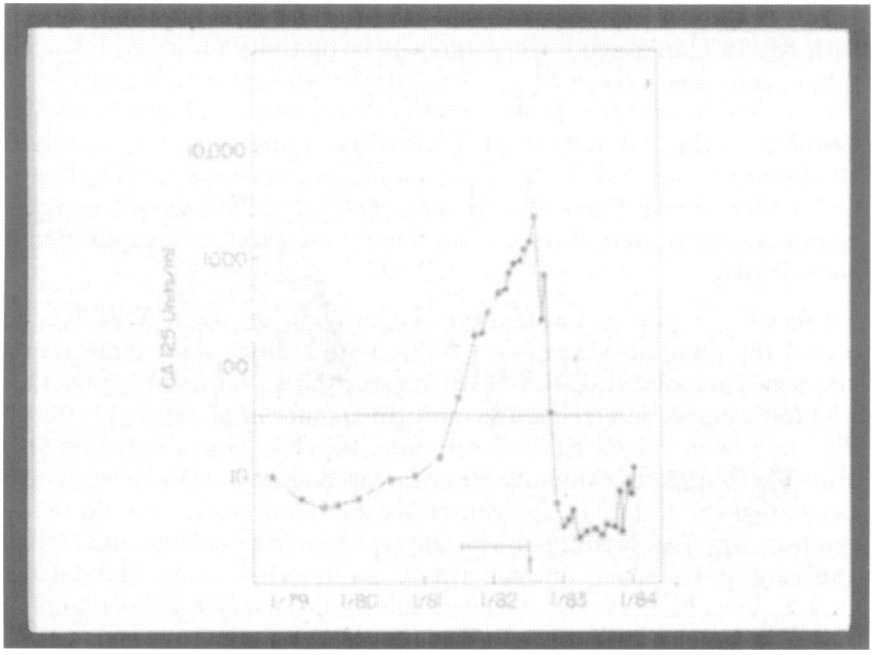

FIG. 1. CA 125 Values from a patient who was diagnosed with ovarian cancer in July, 1982, >10 months after elevation of the marker. Bast, et al., Ref 16.

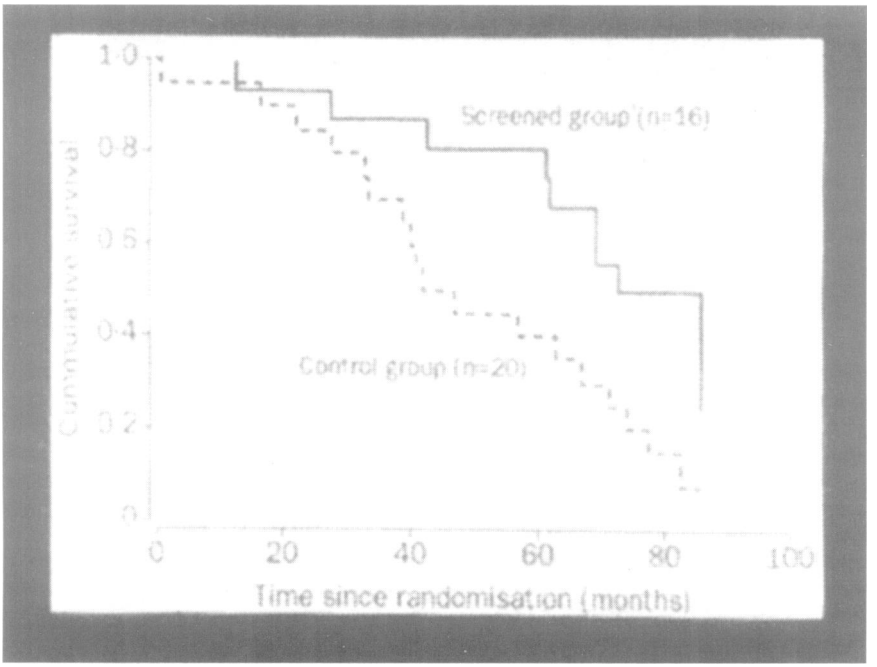

FIG. 2. Survival of women screened for ovarian cancer with annual CA 125 and transabdominal ultrasound or followed by conventional methods (p = 0.0112). Subjects were randomized to a screened group (10,985) or to a control group (10,777). Jacobs, et al., ref 20.

two-arms after screening had been discontinued has mandated a confirmatory study.

Measurement of CA125 over time. Serial measurement of CA125 can also improve specificity. In patients with ovarian cancer, CA125 values rise exponentially, whereas in patients with benign disease sequential CA125 values tend to remain essentially constant even when values are initially elevated (Figure 3A, B). A new format for the CA 125 assay, CA 125II, decreases day-to-day variation. The M11 antibody that recognizes a distinct epitope on MUC16 is used to trap MUC16 and labeled OC 125 is still used as a probe to detect the antigen that has been trapped (21). An algorithm has been developed that distinguishes patients with ovarian cancer from those with benign disease or with no disease (Figure 3C–D) (22). Using this algorithm, analysis of data from a screening trial in Stockholm achieved a sensitivity of 86%, a specificity of 99.7% and a positive predictive value of 16%. In subsequent iterations, change-point analysis has been applied

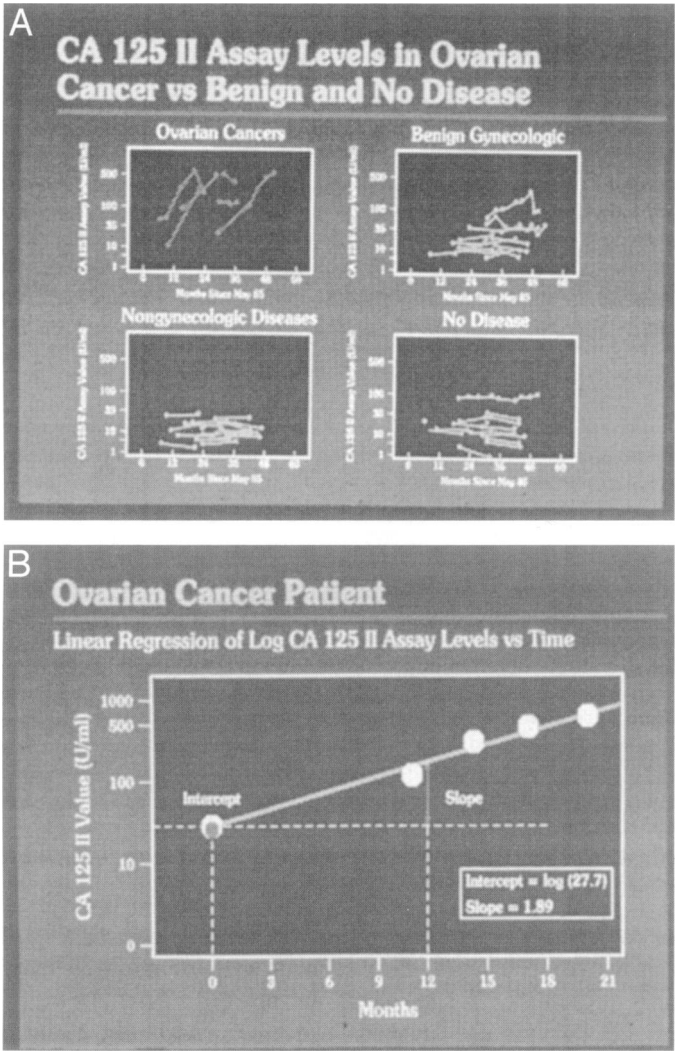

FIG. 3. A. CA 125II levels in patients with ovarian cancer, other cancers, benign disease and no disease. Skates, et al. ref 21. B. Linear regression of log CA 125II assay levels over time for a hypothetical woman with ovarian cancer. Skates, et al, ref 21.

to the same data to estimate risk of ovarian cancer (ROC), achieving even greater precision.

CA 125 Algorithm and TVS. With this improved algorithm, Jacobs et al, have randomized 10,000 volunteers to a control and a screened group (Figure 4). Among the 5046 women screened, TVS was

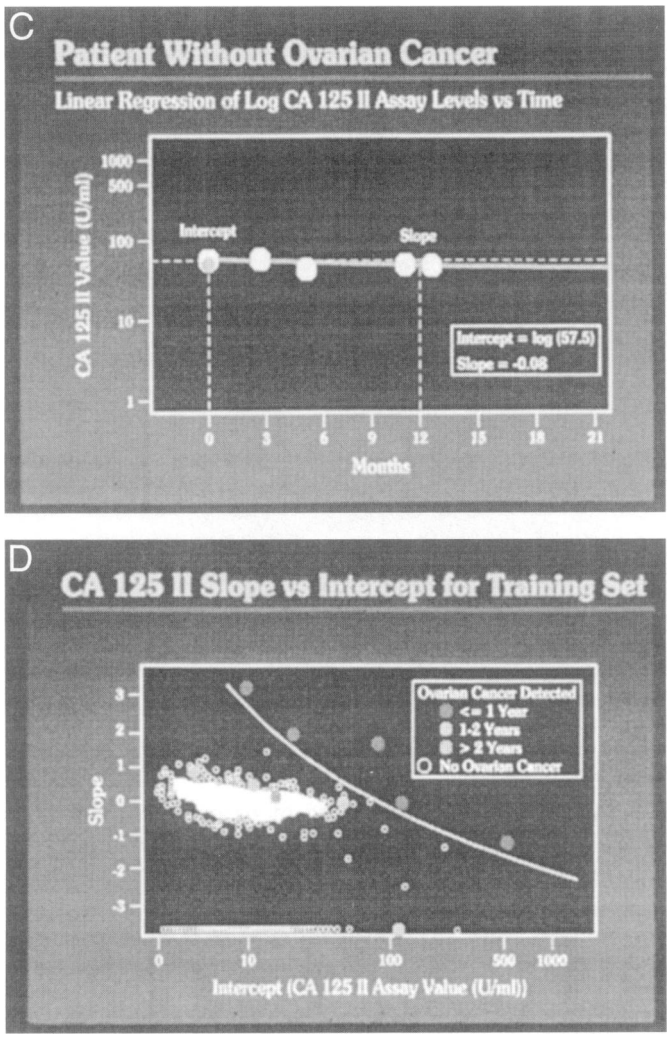

FIG. 3. C. Linear regression of log CA 125II assay levels versus time for a hypothetical woman without ovarian cancer. Skates, et al. ref 21. D. Slopes and intercepts for CA 125II values from patients with ovarian cancer and from controls. The line separates 5 of 6 ovarian cancers from all controls. Subsequent application of this algorithm to a test set yielded an apparent sensitivity of 86%, a specificity of 99.7% and a positive predictive value of 16%. Skates, et al, ref 21.

conducted in 101. Abnormal TVS prompted 17 operations, detecting 4 ovarian cancers with two invasive cancers in stage Ic and one in Stage II, as well as one borderline cancer in stage I (23). Thus, use of the CA125 algorithm and TVS has achieved a positive predictive value of

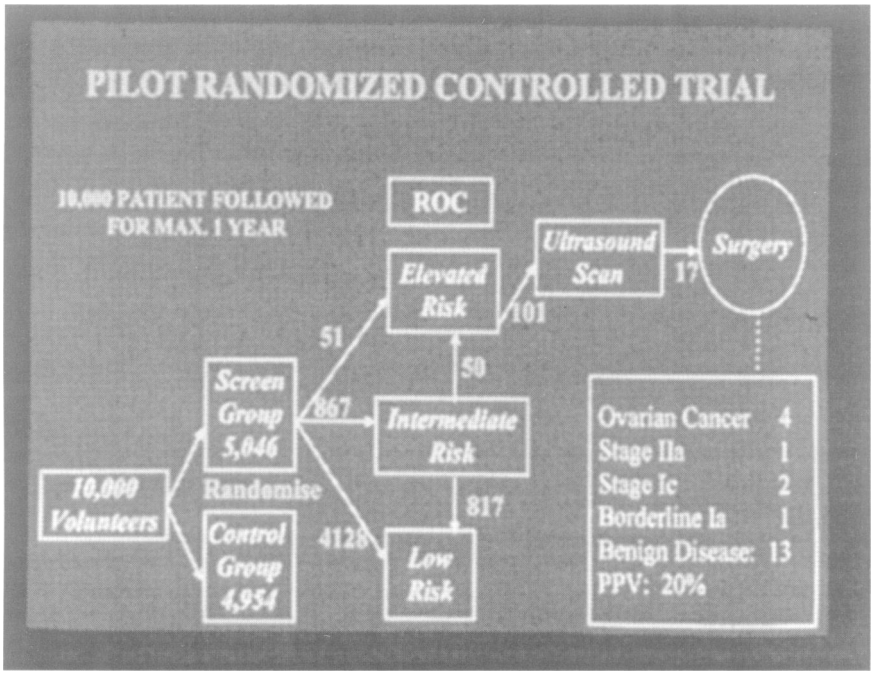

FIG. 4. Pilot randomized controlled trial of the CA 125 algorithm and TVS in 10,000 women. Rosenthal, et al, ref 22.

20% with 5 operations for each case of ovarian cancer detected. At present, a trial has been initiated in the United Kingdom that will include 200,000 post menopausal women who will be randomized to three groups: a control group of 100,000, a group evaluated annually with TVS of 50,000, and a third group that will receive CA125 values judged by the ROC algorithm, prompting TVS. Individuals with abnormal TVS will undergo surgery. Women will be screened and followed for 7 years. This trial should test the value of CA 125 for improving survival of patients with epithelial ovarian cancer.

Multiple Serum/Plasma Markers For Ovarian Cancer

Need for additional markers. Regardless of the outcome of the large screening trial in the United Kingdom, CA125 is not likely to be optimally sensitive. CA125 levels are greater than 35 units per ml in 50–60% of patients with stage I ovarian cancer. Using the ROC algorithm, disease may be detected when a rising CA125 is less than 35 units per ml, increasing sensitivity above 60%. In 20% of ovarian

cancers, however, CA125 cannot be detected in tissue sections. Consequently, the sensitivity of CA 125 alone is not likely to exceed 80%. Greater sensitivity might be achieved with multiple markers, provided that specificity is not compromised.

Over the last twenty years, 27 markers have been reported to increase sensitivity of CA125 for detecting ovarian cancer (2). Markers have generally been analyzed only 2 or 3 at a time. The increased sensitivity achieved with markers in combination has generally been associated with a marked decrease in specificity. To date, no combination of markers has been found that exhibits >95% sensitivity while retaining 98% specificity. Our group has utilized several new technologies to discover novel markers that might improve sensitivity, including gene expression array analysis, proteomics and lipomics.

Gene expression array analysis. To identify potential markers for epithelial ovarian cancer, gene expression in 42 invasive ovarian cancers has been compared to that in 5 pools of normal ovarian epithelial cells using Affymetrics U95 gene arrays (24). Strongly upregulated genes were considered as potential markers. Five-fold upregulation was observed with 26 distinct genes and three-fold upregulation was found with 105 distinct genes. Expression of several of these genes has been previously reported in ovarian cancer, including MUC1, VEGF, mammoglobin, osteopontin, and HE4. Many genes, however, were novel. Recursive descent partition (CART) analysis was performed with the 105 genes upregulated at least three-fold to identify genes that could distinguish different histotypes of cancers from normal ovarian epithelium. A combination of claudin 3 and VEGF distinguished cancers from normals. CART analysis was then performed with 11 genes that encode known serum markers for ovarian cancer including MUC1, MUC16 (CA125), mesothelin, HE4, kallikrein 6, kallikrein 10, MMP2, prostasin, osteopontin, tetranectin and inhibin. A combination of just three markers—HE4, CA125 and MUC1—could distinguish ovarian cancers from normal ovarian epithelial cells at the tissue level. Thus, five genes were identified by CART analysis that could distinguish ovarian cancers of different histotypes from normal ovarian epithelial cells: CA125, HE4, MUC1, VEGF and claudin 3. When 158 ovarian cancers were stained in tissue arrays with antibodies against the proteins encoded by these genes, a combination of antibodies against claudin 3 and MUC1 detected 155/158 (98%) of cases, a combination of claudin 3, CA125 and MUC1 stained 157/158 (99.4%), and all 158 ovarian cancers were detected with a combination of claudin 3, CA125, MUC1 and VEGF.

In collaboration with Dr. Jinsong Liu, normal ovarian surface

epithelial cells have been successfully transformed after transfection with viral T antigen, tolemerase and activated human H-ras or K-ras (25). Transformed cells exhibit papillary histology and grow in soft agar or as nodules in the abdominal cavity of immunosuppressed mice. Similar transformants have been prepared with human breast epithelial cells after transfection with viral T antigen, telomerase and human H-ras or K-ras. When genes upregulated in ovarian transformants were compared to those upregulated in breast transformants, 30 of 34 genes were distinctively upregulated in transformed ovarian epithelial cells. Of the 30 genes distinctively upregulated in ras-transformed OSE, 3 where strongly upregulated (3.6—seven fold) in ovarian cancers compared to normal ovarian surface epithelium: versican, interleukin-8 and small inducible cytokine—sub family B.

Proteomics. Several proteomic techniques have been used to distinguish proteins in sera from ovarian cancer patients from those in healthy individuals. One report suggests that surface enhanced laser desorption and ionization (SELDI) analysis can provide high specificity and sensitivity by analyzing the pattern of proteins expressed in sera from ovarian cancer patients and healthy individuals (26). Isolation of individual peaks and identification of aberrantly expressed proteins should permit development of immunoassays for critical markers. With Drs. Zhen Zhang and Dan Chan at Johns Hopkins, SELDI analysis has been performed following systematic fractionation of 90 sera from patients with Stage I ovarian cancer (27). Levels of two proteins (apolipoprotein A1 and transthyretin) are reduced in sera from early stage ovarian cancer patients. A third peak (fragment of inter-alpha-trypsin inhibitor—heavy chain 4) is increased in sera in patients with stage I disease. Using the three peaks and CA 125, 83% sensitivity and 94% specificity can be achieved for stage I disease with an independent test set. Assays have been developed for 2 of the 3 proteins.

Lipomics. Distinctive lipids have been detected in the plasma of the patients with ovarian cancer. More than 70% of stage I epithelial ovarian cancers are associated with elevations of LPA in plasma (28). Assay of LPA requires plasma rather than serum and has a narrow range. LPA is generated by soluble phospholipase A2 (SPLA2) and lysophospholipase D (lysoPLD) and is catabolized by liposyphospholipid phosphatase-1 (LPP-1) (29). The balance of these enzymes should determine LPA levels in plasma. Each of these proteins can be assayed in serum and may provide more convenient markers for detecting early stage ovarian cancer. In collaboration

with Dr. Gordon Mills, assays have been established for SPL2 and lysoPLD. Methods are being developed to measure LPP-1.

Analysis of multiple markers to improve sensitivity. At present we are analyzing some 18 potential markers (Table 1). Nine are shed into serum and immunoassays have been developed. Three potential markers are shed and immunoassays are under development. Six markers are strongly upregulated in ovarian cancers at the tissue level, but we do not yet know whether they are shed in sufficient quantities to be useful for detecting early stage disease.

In collaboration with Drs. Steven Skates and Zhen Zhang, statistical techniques are being developed that permit utilization of multiple markers to increase sensitivity without sacrificing specificity. Artificial neural network analysis (ANN) (30) and mixtures of multivariate normal distribution (31) have been evaluated using panels of sera from patients with stage I ovarian cancer. Either technique can combine four markers including CA125 to detect stage I disease, retaining specificity of 98% and increasing sensitivity from 48% to

TABLE 1
Candidate Markers for Early Detection of Ovarian Cancer

Marker	Shed/ Secreted	Antibody	Immuno-assay
Shed/secreted with immunoassay			
CA125 (MUC-16)	+	+	+
MUC-1	+	+	+
HE4	+	+	+
IL-8	+	+	+
VEGF	+	+	+
Apolipoprotein A1	+	+	+
Truncated form of transthyretin (pre-albumin)	+	+	+
SPLA2 (Synovial form)	+	+	+
Lyso PLD	+	+	+
Shed/secreted without immunoassay			
Small inducible cytokine-sub family B (MCP-1)	+	+	–
Versican	+	+	–
Cleavage fragment of inter-α-typsin inhibitor heavy chain H4	+	–	–
Shedding/secretion unknown (in order of potential development)			
LPP1	?	+	+
Claudin 3	?	+	–
Notch Homolog 3 (Drosophila)	?	–	–
E2F transcription factor 3	?	+	–
Rac GTPase activating protein	?	+	–
Hematological and neurological expressed 1	?	–	–

72–75%. Greater sensitivity should result from assaying additional markers, whereas greater specificity might be achieved by developing mathematical techniques to analyze the trend of multiple markers over time.

To obtain serum and plasma for evaluating multiple markers over time, a screening trial is being conducted at 5 Centers in Houston, Texas, Des Moines, Iowa, Providence, Rhode Island, and Vero Beach, Florida. In this trial, women aged 50–75 are evaluated with CA 125 assays at annual intervals for 3 or more years. If the trend of CA 125 values indicates normal risk, CA 125 is repeated in one year. If the trend of CA 125 values predicts a moderate increase in risk, the antigen is measured once again in three months. If the trend is consistent with higher risk, TVS is performed immediately. If the ultrasound Is consistent with malignant disease, exploratory laparotomy is undertaken. Overall, serum and plasma will be obtained on 12,500 occasions. This study should permit us to test whether the CA 125 algorithm achieves 98% specificity among women in the United States and whether the positive predictive value is at least 10%. Serum and plasma samples obtained from this study will also permit an estimate of the year-to-year variation in the levels of multiple markers in women who do not have ovarian cancer.

Progress Toward Effective Screening

If current strategies succeed, ovarian cancer screening will have required more than 3 decades to develop. In the future, advances in technology will almost certainly accelerate progress in detecting diseases of intermediate prevalence. A better understanding of human genetics should identify individuals at increased risk, facilitating cost-effective screening. Coordinated efforts of academe, NIH, industry and the FDA will be required to optimize progress and to develop individualized strategies for cancer detection and prevention.

Conclusion

Early detection of ovarian cancer could have a major impact on the disease. Two stage strategies are likely to be most effective. Multiple serum markers will be needed for an optimal initial stage. TVS is a reasonable second stage. Novel markers and computational methods are currently being evaluated. Sequential use of multiple markers and TVS could provide a cost-effective strategy to detect a disease of intermediate prevalence.

ACKNOWLEDGMENT

Over the years, studies of CA 125 and other markers for ovarian cancer have been the work of an extended team of collaborators that has included Drs. Robert Knapp, Vince Zurowski, Yinhua Yu, Feng-Ji Xu, Ian Jacobs, Karen Lu, Jinsong Liu, Steven Skates, Zhen Zhang, Dan Chan, Eric Fung, Gordon Mills, David Gershenson, Andrew Berchuck, David Smith, Vigee Shridhar, Lynn Hartmann, David Fishman, Edward N. Atkinson, Kieth Baggerly, Carolien van Haaften-Day, Neville Hacker, Henk WA de Bruijn, Atte van der Zee, Rosemarie Schmandt and Ingegard Hellstrom. The efforts of Rebecca Marquez, Andrea Patterson and Lance Ramoth have been particularly important for the expression array studies.

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DISCUSSION

Berlin, North Miami Beach: A few things Bob. Earlier this week I was at Northwestern University and heard a talk by Lance Liotta. Proteomics is here, the data that he showed with respect to detection of ovarian cancer, I think is pretty much the same that you have. Next I've never seen nor never heard a better presentation of the development of a cancer detection or cancer screening process, and I thank you very much.

Bast, Houston: Thank you for your kind comment. Proteomics is a work in progress. The technology has tremendous potential, but to date only one paper has been published by Dr. Liotta's group in Lancet 2 years ago utilizing proteomics to distinguish sera of ovarian cancer patients from the sera of healthy individuals. This study included both early and late stage disease. Over the last two years, the proteomic technology has evolved and the informatic algorithms for analyzing proteomic data have also changed. The reproducibility of these techniques has not been optimal and, to my knowledge, no published study has actually set the right algorithm and then evaluated a large number of sera from patients with Stage 1 disease and from healthy individuals. Whether the pattern of protein fragments will provide the most reliable test or whether a combination of individual assays will be most convenient, I think is still an open question. Whatever the outcome, I am optimistic that assay of sera will provide an initial step for an effective screening strategy to detect ovarian cancer at an early stage.

Billings, Baton Rouge: Bob, have you looked at BRCA-1 and BRCA-2 patients as they relate to your tumor markers and how do they fit? Any other familial clusterings?

Bast: About 10% of ovarian cancer is familial. For familial ovarian cancer, in contrast to familial breast cancer, almost all cases can be accounted for by BRCA1 or BRCA2 mutations. The biology of the 10% of familial ovarian cancers appears to differ from the biology of 90% of sporadic cases. On the one hand, familial ovarian cancer may be a little more sensitive to chemotherapy than the sporadic form. On the other, familial ovarian cancer may spread from the ovary to the peritoneal cavity at a much earlier interval. Familial ovarian cancers express CA125. Dr. Beth Karlan has used both CA125 and transvaginal sonography to track patients at high-risk in a screening clinic at Cedar-Sinai Medical Center in Los Angeles. Dr. Karlan has reported patients who had a normal CA125 and a normal ultrasound three months before the discovery of widespread intra-peritoneal disease. That doesn't seem to be the case for the sporadic ovarian cancer where CA125 can be elevated on an average 1.9 years prior to the presentation of clinically apparent disease.

Several biological observations support the feasibility of early detection, at least for sporadic ovarian cancer. For effective screening, ovarian cancer should be a clonal

disease that begins in the ovary and spreads to the peritoneum. More than 90% of sporadic ovarian cancers have been shown to start from the progeny of single cells in the ovary. Familial ovarian cancer is more frequently a multi-focal, polyclonal disease. A second requirement for effective screening is that late stage diseases should arise from detectable lesions that share a common genotype and phenotype. If late stage ovarian cancers exhibit a distinct profile of genetic changes associated with more aggressive disease and early stage cancers had a different spectrum of genetic alterations associated with a more indolent course, screening might have little impact. In collaboration with investigators at the Mayo Clinic, our group has examined gene expression in early stage and late stage ovarian cancer of high grade. A similar spectrum of genes are up or down regulated in stage I–II and in Stage III–IV disease.

Similar studies have not been done so far as I know in patients with familial ovarian cancer. In ovarian cancers that have been found at prophylactic oophorectomy in high-risk patients, p53 mutations that correlate with metastatic potential have been detected in the smallest cancers. So a different screening strategy may be required for high-risk patients.

Carey, Charlottesville: Thank you for that talk. You reported that VEGF was increased in some of your work. My question concerns angiogenic factors in the early detection of ovarian cancer and the specificity of that kind of approach. Was VEGF the only angiogenic factor that was increased?

Bast: Interleukin 8 was also on the list. Josh Fidler at MD Anderson has shown that for ovarian cancer, as for a number of other cancers, VEGF, IL-8 and basic FGF are important angiogenic factors. We are currently attempting to stimulate expression of IL-8, IL-6 and GRO-1 in ovarian neoplasms by treatment with lysophophosphatidic acid analogs to develop a stimulartory assay to detect small volumes of occult cancer. An inductive assay similar to that utilized in the past to detect medullary carcinoma of the thyroid could prove useful in ovarian cancer.

Angiogenic factors might also serve as targets for therapy in ovarian cancer. About two thirds of patients with advanced disease will have circulating levels of VEGF, IL-8 or basic FGF. With Dr. Fidler and Dr. Judith Wolf we are testing the ability of pegylated interferon to reduce secretion of these angiogenic factors in a clinical trial based on pre-clinical results in a human ovarian cancer xenograft model. Attempts to target angiogenic factors for diagnosis or for therapy are clearly works in progress and it remains to be seen whether these approaches will prove of value in the clinic.