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14. ABSTRACT
The purpose of this study is to elucidate the pathogenesis of serous carcinoma by identifying the molecular genetic changes and preferentially expressed genes of different histological types of serous neoplasms. We hypothesize that the development of serous carcinoma proceeds along two main pathways: one is rapid progression from ovarian surface epithelium to high-grade serous carcinoma without well-established morphological precursors ("de novo" pathway) and the other is a gradual development from borderline tumors, to non-invasive micropapillary serous carcinomas then to low-grade carcinomas (stepwise pathway). The first pathway results in a high-grade neoplasm (conventional serous carcinoma) and the second leads to the development of a low-grade indolent tumor. Both types of carcinomas and the putative precursor lesions of invasive MPSC are characterized by distinctive molecular genetic alterations and specific gene expression. We identified that mutations in KRAS and BRAF genes characterized the development of low-grade serous carcinomas. Expression of HLA-G, apoE and membralin molecules were confined to high-grade serous carcinomas. This project, designed to test our proposed model of diverse pathways in the pathogenesis of ovarian serous carcinoma, provides an etiologic basis for the other two projects.

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INTRODUCTION - Project 1

The objective of this proposal is to elucidate the pathogenesis of serous carcinoma by identifying the molecular genetic changes and preferentially expressed genes of different histological types of serous neoplasms. We hypothesize that the development of serous carcinoma proceeds along two main pathways: one is rapid progression from ovarian surface epithelium to conventional serous carcinoma without well-established morphological precursors ("de novo" pathway) and the other is a gradual development from serous borderline tumor (atypical proliferative tumor), to non-invasive micropapillary serous carcinoma then to invasive micropapillary serous carcinoma (stepwise pathway). The first pathway results in a high-grade neoplasm (conventional serous carcinoma) and the second leads to the development of a low-grade indolent tumor (invasive micropapillary serous carcinoma). Both types of carcinomas and the putative precursor lesions of invasive micropapillary serous carcinoma are characterized by distinctive molecular genetic alterations and specific gene expression. This project, designed to test our proposed model of diverse pathways in the pathogenesis of ovarian serous carcinoma, provides an etiologic basis for the other two projects in this proposal. Although many genes are altered during tumorigenesis, only a few are truly critical for tumor progression. Identifying these genes holds promise for the development of new diagnostic assays and immunology-directed treatment for patients with different types of serous carcinoma.

BODY

There are no substantial changes or modifications of the original statements. The accomplishments associated with each task outlined in the approved statement of work are detailed, point by point in the followings.

Task 1: To characterize the molecular genetic alterations of ovarian serous tumors developing along two different pathways.

In the grand funding period, we have conducted a systematic clinicopathologic and molecular analysis of a large number of ovarian serous borderline tumors (SBTs) and invasive epithelial ovarian tumors of all histologic types in an effort to delineate their pathogenesis and behavior [Shih, 2004 #4718]. By careful examination of histopathology of SBTs, we identify a unique "variant" of SBTs- non-invasive micropapillary serous carcinoma (or intraepithelial low-grade serous carcinoma) which are more frequently associated with extraovarian disease (implant) and poor clinical outcome as compared to the conventional SBT (called atypical proliferative tumor) [Burks, 1996 #4258; Riopel, 1999 #4340; Seidman, 2002 #4482; Seidman, 1996 #3113]. We define the morphological criteria of "good" versus "bad" implants to predict the clinical outcome in SBT patients [Sehdev, 2003 #4373]. Based on mutational analysis and the patterns of allelic imbalance, we provide the molecular evidence of tumor progression from cystadenoma to atypical proliferative tumor, intraepithelial low-grade serous carcinoma (or non-invasive micropapillary serous carcinoma) then to invasive low-grade serous carcinoma [Singer, 2002 #4255]. We demonstrate that mutations in BRAF and KRAS characterize SBT and low-grade serous carcinoma [Singer, 2003 #4490; Singer, 2002 #4255] and that mutations in both genes occur early in the tumor progression, i.e., the cystadenoma stage, preceding the development of SBTs [Ho, 2004 #4861]. We show that mutations of BRAF and KRAS correlate with the activation of mitogen activated protein kinase (MAPK), the downstream target of KRAS-BRAF signaling pathway, in SBT and low-grade serous carcinomas [Hsu, 2004 #4792] and have defined the downstream molecular targets regulated by activated MAPK in SBT using serial analysis of gene expression [Pohl, 2005 #5175]. We further show that in contrast to BRAF and KRAS, mutations in p53 gene are significantly less frequent in SBTs and low-grade serous carcinoma than in conventional high-grade serous carcinoma [Singer, 2004 #4815; Katabuchi, 1998 #3111]. Furthermore, we have reported the distinct pattern in sequence mutations and amplification of PIK3CA and AKT2 genes in purified ovarian serous high-grade and low-grade tumors. We have studied the chromosomal imbalance in SBTs using CGH and demonstrated that the pattern of chromosomal imbalance was similar between APST and non-invasive MPSC (intraepithelial low-grade serous carcinoma) but distinct from conventional high-grade serous carcinoma [Staebler, 2002 #4497]. Based on our studies, we have extended our "dualistic pathway" model into a modified hypothesis in the development of ovarian cancer [Shih, 2004 #4718]. Besides, we have developed a new technology to detect KRAS mutations based on homogeneous point mutation detection by quantum dot-mediated two-color fluorescence coincidence analysis.

Task 2: To identify the genes preferentially expressed in the serous carcinomas.

Based on serial analysis of gene expression (SAGE), we have identified and characterized differential genes expressed in either low-grade (micropapillary) or high-grade serous carcinomas. We have finished SAGE library construction, sequencing and analysis in 5 high-grade and 3 low-grade ovarian serous carcinomas. We then combined the SAGE results and Affymetrix microarray analysis (as an alternative approach to complement SAGE as described in the original aim), we have identified several high-grade associated and low-grade associated genes in ovarian cancer. More specifically, we have identified HLA-G, membralin and apolipoprotein E overexpression in high-grade but not in low-grade serous carcinoma. The validation and clinical application of HLA-G and apolipoprotein E expression will be described in the next section (Task 3). Besides, we have identified two low-grade associated genes, p21 and anti-trypsin, based on analysis of SAGE data we have collected. In addition, we have identified mesothelin as an ovarian cancer associated gene and the expression levels of mesothelin correlated with patient's overall survival.

Task 3: To validate the candidate genes and assess their biological significance in the development of serous carcinoma.

A total of four genes among many candidate genes were validated and characterized for their clinical significance and they include HLA-G [Singer, 2003 #4551;Davidson, 2005 #5266] apolipoprotein E [Chen, 2005 #5004], mesothelin [Yen, 2006 #5408] and membralin [Chen, 2005 #5316]. The summary of these three studies are summarized as follows.

The HLA-G immunoreactivity ranging from focal to diffuse was detected in 45 of 74 (61%) high-grade ovarian serous carcinomas but in none of the 18 low-grade serous carcinomas or 26 serous borderline tumors (atypical proliferative tumors and non-invasive micropapillary serous carcinomas) that were studied. The differential expression of HLA-G in high-grade but not low-grade serous carcinomas may have biological

significance as HLA-G appears to facilitate tumor cell evasion of the immune system by protecting the malignant cells from lysis by natural killer cells. This finding is similar to the HLA-G expression observed in large cell carcinoma of the lung that is associated with a poor outcome as compared with absence of expression in carcinomas with a better prognosis. HLA-G staining was not detected in a wide variety of normal tissues including ovarian surface epithelium and normal breast tissue. RT-PCR demonstrated the presence of HLA-G5 isoform in all tumor samples expressing HLA-G. ELISA was performed to measure the sHLA-G in 42 malignant and 18 benign ascites supernatants. sHLA-G levels were significantly higher in malignant ascites than in benign controls ($p<0.001$). We found that the area under the receiver-operating characteristic (ROC) curve for sHLA-G was 0.95 for malignant versus benign ascites specimens. At 100% specificity, the highest sensitivity to detect a malignant ascites was 78% (95% CI, 68% - 88%) at a cutoff of 13 ng/ml. In summary, our findings suggest that measurement of sHLA-G is a useful molecular adjunct to cytology in the differential diagnosis of malignant versus benign peritoneal ascites.

Apolipoprotein (Apo) E has been recently identified as a potential tumor-associated marker in ovarian cancer by serial analysis of gene expression. ApoE has long been known to play a key role in lipid transport and its specific isoforms participate in the atherosclerogenesis. However, its role in human cancer is not known. In this study, apoE expression was frequently detected in ovarian serous carcinomas, the most common and lethal type of ovarian cancer. It was not detected in serous borderline tumors and normal ovarian surface epithelium. Inhibition of apoE expression using an apoE specific siRNA led to G_2 cell cycle arrest and apoptosis in an apoE expressing ovarian cancer cell line, OVCAR3, but not in an apoE negative cell line, SKOV3. Furthermore, the phenotype of apoE-siRNA treated OVCAR3 cells was not reversed by exogenous apoE in the culture medium. Expression of apoE in nuclei was significantly associated with a better patient survival who had advanced stage serous carcinomas at the 5-year follow-up ($p=0.004$). This study suggests a new role of apoE in cancer as apoE expression is important for the survival in apoE expressing ovarian cancer cells and its expression is associated with a favorable clinical outcome in ovarian cancer patients.

A novel gene, membralin, was cloned from a human ovarian cancer cell line. Human membralin is unique without significant sequence homology to other human genes except the membralin from the other species. The gene contains 11 exons which encodes at least two spliced variants in human cancer. The long form of membralin is composed of 11 exons, encoding a protein of 620 amino acids and the short form contains all exons except the exon 10, encoding a protein of 408 amino acids. Expression of different membralin isoforms depends on the tissue type. Long form of membralin is expressed in ovarian and colorectal carcinomas but not in breast or pancreatic carcinomas which express only the short from. Membralin-GFP fusion protein demonstrates its exclusive cytoplasmic localization. Based on quantitative real-time PCR, in situ hybridization and Western blot analysis, membralin was highly expressed in ovarian serous carcinomas as compared to serous borderline tumors and ovarian surface epithelium ($p< 0.001$). Furthermore, the expression level of membralin correlated with a worse 5-year survival in patients with previously untreated stage III ovarian serous carcinomas ($p< 0.05$). In conclusion, these findings provide the first characterization of human membralin and suggest that membralin is a novel tumor-associated marker in ovarian serous carcinomas with potential prognostic significance.

Mesothelin expression levels were compared among 81 publicly available SAGE libraries of various carcinoma and normal tissue types. Immunohistochemistry using a well-characterized mesothelin monoclonal antibody (5B2) was performed to evaluate mesothelin expression in 167 high-grade and 31 low-grade ovarian serous carcinomas. Immunohistochemistry staining scores were correlated with patient survival, tumor site, tumor grade, *in vitro* drug resistance and differentiation status of tumor cells. SAGE analysis demonstrated that mesothelin was overexpressed in 50% of ovarian and pancreatic carcinomas but rarely in other cancer types including liver, colon, kidney, prostate and breast. Mesothelin immunoreactivity (>5% of tumor cells) was present in 55% of ovarian serous carcinomas with no difference in expression between high-grade and low-grade serous tumors ($p = 0.82$). Based on Kaplan-Meier analysis, we found that a diffuse mesothelin staining (> 50% of tumor cells) in primary high-grade ovarian carcinomas correlated significantly with prolonged survival in patients who had advanced stage disease and had received optimal debulking surgery followed by chemotherapy ($p = 0.023$). Mesothelin expression did not correlate significantly with patient age, tumor site, *in vitro* drug resistance or tumor differentiation status ($p > 0.10$).

Our results provided new evidence that mesothelin expression is associated with prolonged survival in patients with high-grade ovarian serous carcinoma.

KEY RESEARCH ACCOMPLISHEMEHNTS

- We have provided molecular genetic evidence to support the hypothesis of the dualistic pathway in the development of ovarian serous carcinoma. We further modify and extend our hypothesis to include all the major types of ovarian carcinomas.
- We have concluded that mutations of *KRAS* and *BRAF* characterize the development of low-grade (invasive micropapillary) serous carcinomas while mutations of p53 characterize high-grade (conventional) serous carcinomas.
- We have demonstrated that mutations of *KRAS* and *BRAF* occur very early in the tumor progression of serous borderline tumors, suggesting "gate-keeper" roles of both genes in the development of serous borderline tumors. Furthermore, we have identified the downstream gene, cyclin D1, that is regulated by the activation of the KRAS-BRAF-MEK-MAPK pathway.
- Based on SAGE, we found that HLA-G, apoE and membralin overexpression is associated with high-grade (conventional) serous carcinomas and p21 and anti-trypsin overexpression is associated with low-grade serous tumors. Further studies reveal their clinical relevance and biological functions in ovarian serous carcinomas.
- We have identified a new amplified ovarian cancer associated gene, Rsf-1, in high-grade ovarian serous carcinomas but not in low-grade serous tumors. Amplification and overexpression of Rsf-1 correlate with the aggressiveness of high-grade ovarian serous carcinomas.
- We have completed the generations of ovarian tumor tissue arrays including a whole spectrum of lesions and normal tissues that could serve as an important research tool for this program project and others in ovarian cancer research. The tissue microarray set is currently ready to be used in the entire program project.

REPORTABLE OUTCOMES

Articles published or in press in the funding period:

(A total of 27 peer-reviewed papers related to Project 1)

Singer G, Kurman RJ, Chang H-W, Cho SKR, Shih IM. Diverse tumorigenic pathways in ovarian serous carcinoma. Am J Pathol, 160:1223-1228, 2002.

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Nakayama K, Nakayama N, Davidson B, Katabuchi H, Kurman RJ, Velculescu VE, Shih IM, Wang TL. Homozygous deletion of MKK4 in ovarian serous carcinoma. Cancer Biol Therapy, in press.

Park, JT, Li M, Nakayama K, Mao T-L, Davidson B, Zheng Z, Kurman RJ, Eberhart CG, Shih IM, Wang TL. Notch-3 gene amplification in ovarian cancer. Cancer Res, accepted.

Article submitted for review:

Research resource:
Ovarian tumor tissue microarrays- TMA 64, 65, 66, 209, 210, 211, 212, 213, 311, 312.
Protocols of digital PCR analysis for allelic imbalance.

Trainees who received awards using this funding resource:

- **HERA Research Award,** 2005**,** Kentaro Nakayma, MD, PhD, a research fellow
- **First Place Award for Research Fellow in Basic Research, Johns Hopkins Oncology,** 2005, Jim Sheu, PhD, a research fellow
- **International Union Against Cancer Technology Transfer Fellowship,** 2004, Gudun Pohl, MD, a research fellow
- **HERA Research Award,** 2003**,** Brant Wang, MD, PhD, a research fellow
- **Yong Investigator Award of the International Society of Gynecologic Pathologists**, 2004, Gad Singer, MD, a clinical fellow
- **Howard Hughes Undergraduate Research Award**, 2003, Robert J. Oldt III, JHU undergraduate student

CONCLUSIONS

Summary of the accomplished research findings: Ovarian epithelial tumors are the most common type of ovarian cancer and are the most lethal gynecologic malignancy. The main purpose of the Project 1 is to delineate the molecular basis of ovarian serous tumors and identity the genes that are associated with tumors that develop along different pathways in tumor progression. The overall DoD project 1 has made progress to meet the objectives of the proposal as we have finished all the tasks proposed in the specific aims. Furthermore, we have extended the aims and have obtained new and related findings to the Project 1. Based on clinicopathological and molecular observations, we propose a new model for their development. In this model, ovarian serous tumors are divided into two categories designated low-grade and high-grade tumors which correspond to two main pathways of tumorigenesis. Low-grade neoplasms arise in a stepwise fashion from borderline tumors whereas high-grade tumors arise from ovarian surface epithelium or inclusion cysts for which morphologically recognizable precursor lesions have not been identified, so-called “de novo” development. Low-grade tumors are associated with distinct molecular changes that are rarely fond in high-grade tumors, such as *BRAF* and *KRAS* mutations. Gene expression profiling has shown that the two types of ovarian serous tumors are characterized by distinct gene

expression patterns. Some of the genes have been selected for further studied to reveal their clinical and biological significance. As a result of DoD funding, we have generated a total of 27 peer-reviewed papers related to the aims of the Project 1.

Implications and significance of the accomplished research findings: Our proposed model of carcinogenesis in ovarian serous tumors reconciles the relationship of borderline tumors to invasive carcinoma and provides a morphologic and molecular framework for studies aimed at elucidating the pathogenesis of ovarian cancer. Identification and characterization of the panoply of molecular changes associated with ovarian carcinogenesis will facilitate development of diagnostic tests for early detection of ovarian cancer and for the development of novel therapies aimed at blocking key growth-signaling pathways.

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APPENDICES

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Introduction - Project 2

The immune system constantly surveys the body for 'non-self' antigens, and generates a response in the appropriate context. A key finding of cancer biology is that cancer patients often generate antibody to neoantigens specifically expressed in their tumor (*1*). Autologous antibodies have been documented in patients afflicted with a variety of different cancers, (*2-4*) including ovarian cancer (*5*). Autologous antibodies generated by cancer patients have been used to screen expression libraries for tumor antigens. This technique, originally described by Sahin *et al* and termed SEREX (serologic analysis of recombinant cDNA expression libraries of human tumors with autologous serum), has been used to obtain tumor antigen cDNA clones. SEREX has been applied to tumors of many organs (*6*) and antibody specific for antigens identified by SEREX in other cancer types have been demonstrated in ovarian cancer patients (*7, 8*). Tumor-specific autoantigens that are common among ovarian cancer patients but not recognized by sera of healthy volunteers have been identified by us e.g. HOXA7 and HOXB7, and others e.g. cathepsin D and GRP78 (*9*). Although expressed, Cathepsin D and GRP78 derived from normal tissue were not recognized by sera from ovarian cancer patients implying that they contain tumor-specific epitopes (*9*). Detection of these autologous antibody responses to ovarian cancer antigens appears to have prognostic significance (*10*). SEREX antigenes derived from early stage serous carcinoma may represent useful biomarkers for the dissection of molecular pathways of serous carcinoma (Dr Shih, Project 1). SEREX antigens are also potential targets for cancer immunotherapy (*11*) (Dr Wu, Project 3).

Body

<u>Objective 1:</u> Obtain cDNAs of autologous tumor antigens recognized by sera of patients with early stage serous carcinoma, but not controls.

Task 1.1. Screen sera of patients (n=12) with early stage serous carcinoma by Western blot analysis to identify those patients with high titer autologous tumor-reactive antibody (months 0-2).

We have performed Western blot analysis to select sera suitable for immunoscreening of a cDNA expression library. We have screened with sera from patients with micropapillary serous carcinoma, which has been proposed as a precursor/intermediate of serous carcinoma. We also tested immunopreciptation, rather than Western blot, as a method to identify autologous tumor antigens. We derived a cell line from a micropapillary serous carcinoma of patient JH514. Affinity purified serum immunoglobulin from the same patient was covalently coupled to Sepharose beads, and these beads used to immunoprecipitate antigens from detergent lysate of the autologous JH514 cell line. The serum antibody from this patient recognized two autologous tumor associated antigens of approximately 100kDa and 200kDa (Fig. 1).

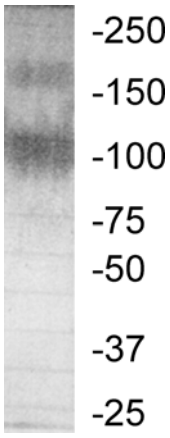


Figure 1. Immunoaffinity purification of autologous tumor antigens for micropapillary serous carcinoma of the ovary. Serum from a patient with MPSC, JH514, was passed over a protein-G spin column. After washing, the antibody was covalently linked with a cross-linker. The column was then washed with low pH elution buffer followed by PBS. A detergent lysate was prepared from a MPSC cell line derived from the same patient. This lysate was passed through the column. After washing, the column was eluted in low pH. The eluate was separated by SDS-PAGE on an 8% gel. The proteins were stained with Coomassie Blue. Antigens of ~100kDa and ~200kDa were excised for mass spectrometry.

Task 1.2. Generate serous carcinoma cDNA expression libraries and immuno-screen with autologous patient (n=3) serum antibody to identify SEREX antigens (months 2-14).

The generation of a cDNA expression library derived from the serous carcinoma cell line OVCAR-3 in ongoing. During the first year of funding we have also developed a complementary technology for the identification of autologous tumor antigens that is based upon immunopreciptation and antigen identification using mass spectrometry. This approach has a key advantage over SEREX screening in that the antigen is expressed in its native conformation and with the appropriate post-translational modifications. Therefore we excised the

bands from the gel shown in Figure 1, subjected the antigens to trypsin digest and determine the charge/mass ratio for the peptides present by mass spectrometry (Fig 2 and not shown).

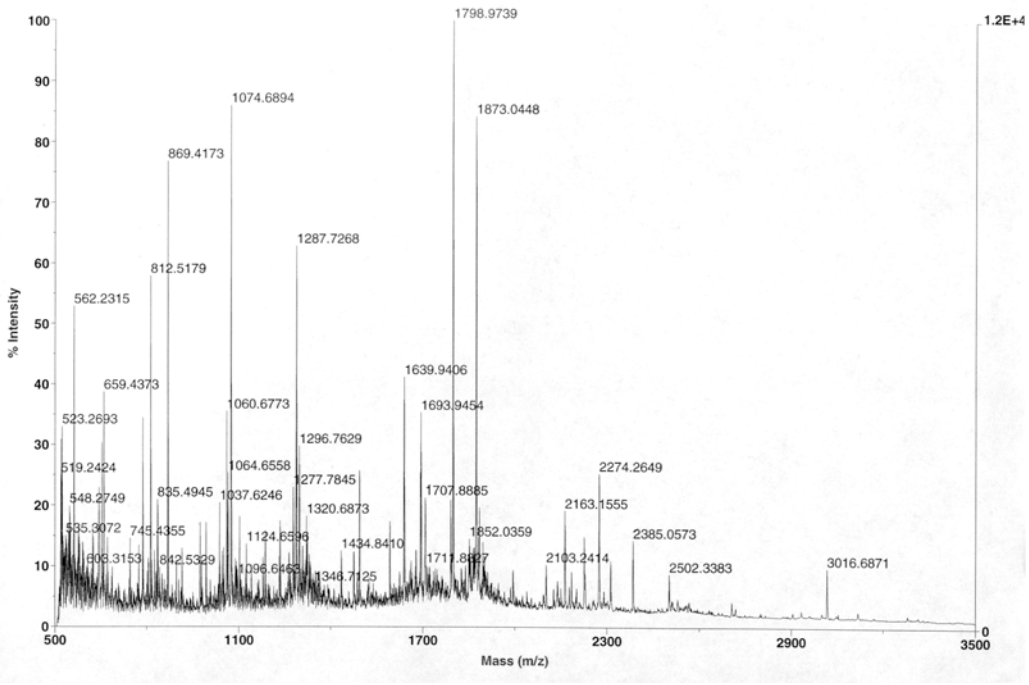


Figure 2. Mass spectrometry of tryptic peptides derived from the ~100kDa ovarian cancer-associated antigen.

Task 1.3. Sequence SEREX antigen cDNAs identified in screen and analyze sequences using BLAST searches (months 14-15).

The prospector website was used to search for proteins with matching peptides. The ~100kDa and ~200kDa antigens were identified by their peptide signatures as SMAP-1 (101kDa) (shown for SMAP–1 in Table 1) and KIAA1529 (195kDa). Nothing is known about the KIAA1529 antigen and no significant homologies or domains were identified. SMAP-1 has three TPR domains, and a recent report suggests that this protein is a chaperone that interacts with HSP90 and facilitates myosin motor assembly.

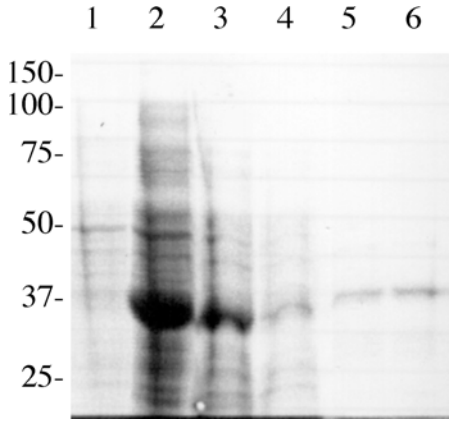
Table 1. Expected and observed M/z for peptides of SMAP-1 and the 100kDa antigen. Matched peptides cover 12% of the SMAP-1 protein, with 53 unmatched masses. 13/66 peptides match SMAP-1(AB014729), estimated MW of 101676.3Da, and SMAP-1b(AB014736).

M/z submitted	MH+ matched	ΔDa	Missed cleavages	Database Sequence
523.2691	523.2186	0.050	0	(K)CSEK(D)
562.2315	562.3201	-0.089	1	(K)GTEKK(Q)
1074.6897	1074.5618	0.13	1	(R)CVSLEPKNK(V)
1179.6649	1179.5618	0.085	0	(K)SWFEGQGLAGK(L)
1186.7039	1186.5414	0.16	0/1Met-ox	(K)LGSAGGTDFSMK(Q)
1287.7267	1287.7749	-0.048	1	(R)TVATLSILGTRR(V)
1320.6865	1320.7527	-0.066	0	(R)ASFITANGVSLLK(D)
1661.9771	1661.8606	0.12	0	(R)LLDMGETDLMLAALR(T)
1677.9307	1677.8556	0.075	0/1Met-ox	(R)LLDMGETDLMLAALR(T)
1693.9454	1693.8505	0.095	0/2Met-ox	(R)LLDMGETDLMLAALR(T)
1797.9774	1797.9063	0.071	0	(R)WAVEGLAYLTFDADVK(E)
2225.2600	2225.1119	0.15	0	(R)EIASTLMESEMMEILSVLAK(G)
2273.2726	2273.0966	0.18	0/3Met-ox	(R)EIASTLMESEMMEILSVLAK(G)

Task 1.4 Express SEREX antigens and purify from bacteria (months 15-21).

We obtained the full length cDNAs for human (MGC-999) and mouse SMAP-1 (MGC-7875). We expressed full length human SMAP-1 with a 6His tag in bacteria and purified the protein. Similarly, we expressed a central conserved region of murine SMAP-1 with a 6His tag and purified this protein from bacteria (Fig 3).

Figure 3. Purification of recombinant SMAP-1 conserved peptide from E.coli. A central portion of murine SMAP-1 was subcloned into pProExHT. Transformed E.coli DH5α were induced for 6h in 1mM IPTG. Affinity purification on a Ni-NTA Sepharose column was performed according to Qiagen's protocol. Fractions were separated in a 12% SDS-PAGE gel and stained with Coomassie Blue. Lane 1: Uninduced E.coli, Lane 2: Induced E.coli, Lane 3: Lysate of induced E.coli, Lane 4: Column flow through, Lanes 5 and 6: Elution fraction.



Task 1.5 Determine the prevalence of serum antibody specific to each SEREX antigen by direct ELISA in a case/control study (months 21-27). (100 ovarian carcinoma patient sera, 102 sera from patients with benign ovarian tumors and 200 control sera will be tested.)

To explore the utility of autologous serum antibody specific to particular TAAs as an additional tool for discrimination of sera from cancer patients and healthy women, we generated recombinant protein from 13 markers previously identified as candidate TAAs (see Table 6) and selected 5 presumptive control reagents (uncoated beads and beads coated with anti-human IgG, human IgG, vector alone extracts and calmodulin, see Table 6). Sera from both healthy women and ovarian cancer patients derived from two different academic centers were tested in parallel for reactivity to all 18 panel elements. Initially we tested sera of 23 healthy patients as controls and sera from 86 stage III/IV ovarian cancer patients. This set of sera was analyzed for reactivity to microspheres of discrete sizes coated with individual recombinant TAA or control protein. The presence of antibody bound to the beads was detected with PE-labeled anti-human IgG and the fluorescence quantified using a Luminex plate reader. Since 5 presumptive control elements were included in the panel, absolute MFI values without background subtraction were employed.

Our analytical strategy consisted of fitting a logistic regression model for cases and controls. A Bayesian Model/Variable Selection approach using a Markov Chain Monte Carlo (Gelfand and Smith, 1990) computations was implemented in the WinBugs (Spiegelhalter et al., 2003) programming environment. A full description of the model selection and details of our Bayesian Computations using Gibbs Sampling are provided in Appendix 3. The MCMC variable (here the 13 markers and 5 controls) selection approach is a stochastic search algorithm that effectively visits all 2^{18} = 216,144 different models obtained from including/excluding any of the markers or controls in a logistic regression for the probability of ovarian cancer. For numerical stability, we transformed the measured MFI levels of the markers and controls to logarithmic scale. Our approach adjusts for the associations amongst the 13 markers, and effects of the 5 controls. Given the limited number of cases and controls, we focused on an additive model assuming no interactions among the markers, controls, and between markers and controls. A priori, each specific marker or control is assumed to be equally likely to be included/excluded (i.e., with probability of 0.5) in the model. This corresponds to an equally likely prior probability of $1/2^{18}$ for each possible configuration in the model space. The inclusion probability for a specific marker or control is then updated by their posterior probability using all the available information about the other markers and controls, conditional on the observed ovarian cancer status. Although, in principle, one can compute the posterior probability of each of the 2^{18} models using the Bayesian analysis, a simpler alternative is to use the Bayes factor (BF) (Carlin and Louis, 1996), defined as the posterior and prior odds ratio, to select relevant markers or controls for future analyses. For a specific marker or control, larger values of BF provide evidence in favor of inclusion, while smaller values support exclusion from the model. Usually, a value between 1 and 3 is considered weak, between 3 and 10 as substantial, between 10 and 100 as strong, and greater than 100 as very strong (Kass and Raftery, 1995).

By using BF>=3 as a selection criterion, we picked the best model and computed multiple sensitivity, specificity, ROC curve and AUC by varying a probability cutoff between 0.01 to 1.00 (Table 2, Figure 4). The

AUC was calculated as 0.86 (95% confidence interval 0.78-0.90) for this best fitting model (Table 2). We also compared the best model for discrimination of cases and controls with detection of absolute level of CA125 or p53-specific antibody or both. The AUC was calculated as 0.83 (95% confidence interval 0.81-0.83) for the standard CA125 assay alone. Detection of p53 antibody alone was not useful for discrimination of cases and controls in this serum set (AUC of 0.52 with 95% confidence interval 0.20-0.58) and combination of absolute CA125 level and serum reactivity to p53 provided an AUC of 0.81 (95% confidence interval 0.79-0.83).

Table 2. **Modeling of serum responses to the 18 element panel in cases and controls.** Identification of the best fitting model for discrimination between sera of 23 healthy patients as controls, versus sera from 866 stage III/IV ovarian cancer patients as cases using the 18 element panel either without CA125 (A) or with CA125 (B). The table shows the posterior estimates (mean and standard deviations) of $\Pr(d_j = 1|Data)$, the regression coefficients $\{\theta_j = d_j b_j\}$, and the Bayes Factors for 13 markers and 5 controls using the data set of ULSM cases, and MDACC cases and controls. The regression coefficients θ_j are assumed to be statistically independent, and each have a two-component mixture prior distribution with a point mass at zero (when $d_j = 0$) with probability of 0.5, and a normal distribution $N(0, \sigma_j^2)$ (when $d_j = 1$) with probability 0.5.

A

Marker	$\Pr(d_j = 1\|Data)$	θ_j	BF_j
FLJ21522	0.49 (0.50)		
NY-CO-8	0.98 (0.15)	-1.49 (0.62)	49
NY-CO-16	0.45 (0.49)		
ABC7	0.65 (0.48)		
αHsIgG	0.42 (0.49)		
Calmodulin	0.42 (0.49)		
HOXB7	0.92 (0.27)	1.42 (0.76)	11.5
Hsp70	0.57 (0.49)		
Hsp90	0.49 (0.50)		
HsIgG	0.43 (0.49)		
No Antigen	0.60 (0.49)		
NY-ESO-1	0.48 (0.50)		
p53	0.96 (0.20)	1.21 (0.58)	24
Ubiquilin-1	0.61 (0.49)		
ZFP161	0.28 (0.45)		
Vector	0.40 (0.49)		
HOXA7	0.42 (0.49)		
Hsp27	0.42 (0.49)		
CA125			
	Post. Mean (Std D.)	95% CI interval for AUC	
AUC	0.86 (0.03)	0.78	0.90

B

$\Pr(d_j = 1\|Data)$	θ_j	BF_j
0.37 (0.48)		
0.90 (0.31)	-1.18 (0.67)	9
0.44 (0.50)		
0.53 (0.50)		
0.35 (0.48)		
0.42 (0.49)		
0.87 (0.33)	1.17 (0.73)	6.7
0.33 (0.47)		
0.41 (0.49)		
0.36 (0.48)		
0.52 (0.50)		
0.41 (0.50)		
0.76 (0.43)	0.73 (0.62)	3.2
0.52 (0.50)		
0.49 (0.50)		
0.41 (0.50)		
0.43 (0.49)		
0.37 (0.48)		
0.99 (0.07)	0.90 (0.37)	99
Post. Mean (Std D.)	95% CI interval for AUC	
0.89 (0.03)	0.84	0.92

We tested whether addition of absolute serum level of CA125 as an element of the panel conferred additional predictive value upon re-analysis of the serum set (Table 6B). The posterior distributions of the regression parameters are displayed in Figure 3C and the ROC curve is given in Figure 3D. Although there is improvement in terms of the accuracy of the fitted model (AUC with CA125 goes slightly up from 0.86 (95% confidence interval 0.78-0.90) to 0.89 (95% confidence interval 0.84-0.92), the same markers NY-CO-8, HOXB7, and p53 are again selected as most informative along with CA125. Our study examines the applicability of multiplex detection of autologous antibody to TAAs as a diagnostic tool for cancer. The detection of autologous antibody to this 3 member panel of TAAs, in addition to CA125, shows merit as an approach for ovarian cancer detection. However, much larger studies are required to assess its sensitivity at the high specificity required for screening healthy women. In particular it warrants further examination in high-risk populations such as female first-degree relatives of ovarian cancer patients or women carrying mutations that predispose toward cancer. The application of longitudinal screening may also significantly increase the

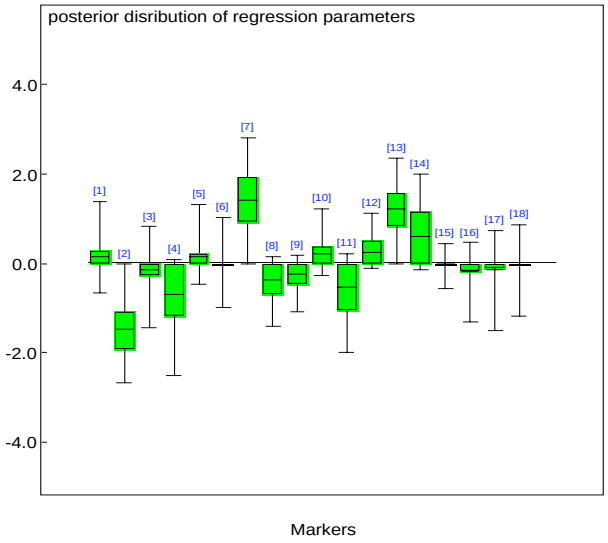
positive predictive value of the test (McIntosh and Urban, 2003; McIntosh et al., 2002). Sera collected over four time points from individual patients with ovarian (n=5) or breast (n=5) cancer were analyzed for the stability of TAA reactivity. The reactivity were generally stable over several years despite therapy indicating that this approach is not useful to monitor therapy (not shown).

Our modeling of TAA-specific antibody poorly discriminates those patients with ovarian cancer from patients with cancer of other organ sites (not shown). This reflects the use of p53 as a diagnostic marker and its importance in cancers in addition to ovarian cancer (Soussi, 2000). However, other investigators have made progress to this end (Koziol et al., 2003; Zhang et al., 2003). Three of the seven TAAs tested proved useful in detecting particular cancer types (Koziol et al., 2003; Zhang et al., 2003). Over 2000 TAAs have been entered into the SEREX database (http://www.licr.org/SEREX.html). Several other approaches have also been used to identify candidate TAAs including mass spectrometric analysis of immunopreciptiates (Chinni et al., 1997) and screening of phage display libraries (Fossa et al., 2004; Mintz et al., 2003). Inclusion of other TAAs using similar, highly multiplexed approaches (Scanlan et al., 2002), in large case/control studies may provide better discrimination between cancer types.

Pattern recognition analysis of proteomic profiles has been used to discriminate sera of ovarian cancer patients from those of healthy women, but the biological underpinning of these patterns is as yet unclear (Petricoin et al., 2002). By contrast the significance of the TAAs p53 and HOXB7 in cancer biology has been studied extensively for ovarian and other cancers (Care et al., 1998; Care et al., 1996; Care et al., 1999; Naora et al., 2001b; Shih Ie and Kurman, 2004). A partial cDNA sequence NY-CO-8 was initially identified as a colon cancer antigen. The full length gene was recently cloned and its product, CCCAP, shown to associate with centrosomes (Kenedy et al., 2003). Subsequent analysis of the serologic reactivity to NY-CO-8 suggests that it is a naturally occurring autoantigen (Scanlan et al., 2002). The presence of NY-CO-8-reactive antibody in healthy controls may account for its negative association with ovarian cancer in our study. The possibility of a protective effect warrants further investigation as described recently for MUC1 (Cramer et al., 2005).

Figure 4 (Next Page). A. Posterior distributions of $\{\theta_j\}$ in the variable selection algorithm for data of Table 6A. B. Posterior estimate of ROC curve for markers NY-CO-8, HOXB7 and p53 with 95% Credible Interval for cut off values ranging from 0.01 and 1.00 for data of Table 6A. C. Posterior distributions of $\{\theta_j\}$ for all 18 candidate markers plus CA125 levels (marked as [19] above) in the variable selection algorithm for data of Table 6B. D. Posterior estimate of ROC curve for training set using NY-CO-8, HOXB7, p53, and CA125 with 95% Credible Interval for cut off values ranging from 0.01 and 1.00 for data of Table 6B.

Figure 4 A



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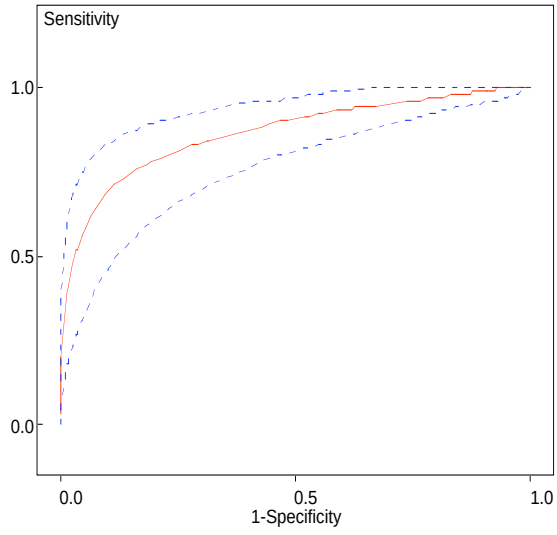

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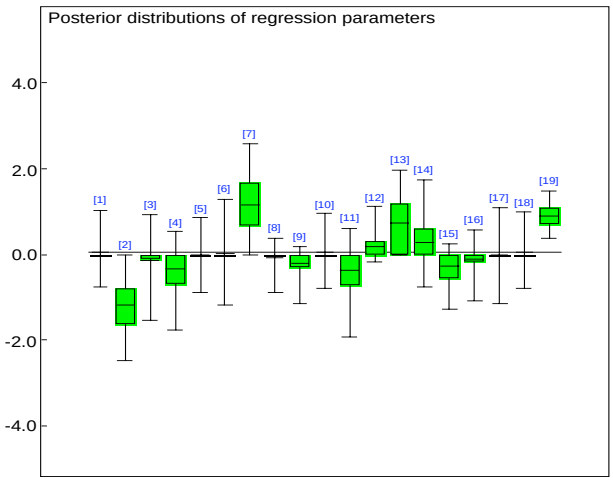
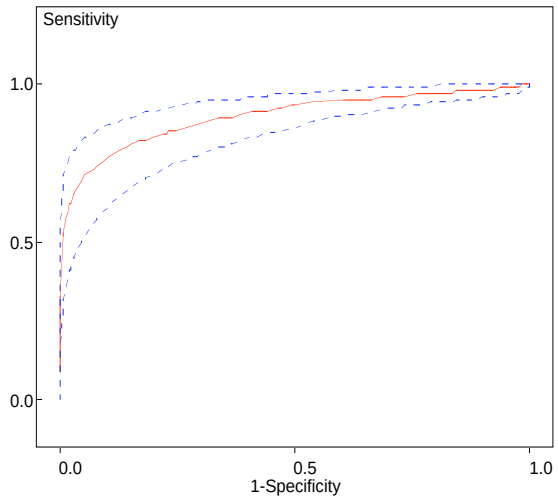




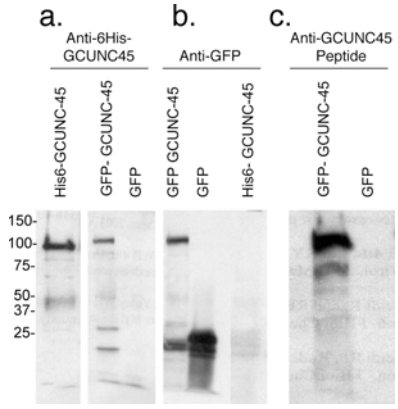
<u>Objective 2:</u> Select autologous tumor antigens expressed in early stage serous carcinoma but absent from, or a low level in normal tissue.

Task 2.1. Generate rat antiserum to SEREX antigens (months 27-29)
Task 2.2. Affinity purify and validate specificity of antibodies (months 29-32).
Task 2.3. Examine the expression pattern of each SEREX antigen by immunohistochemical staining in normal ovary and a spectrum of ovarian tumors (months 32-36)

In order to evaluate GC-UNC-45 expression in ovarian tumors and normal tissue we generated rabbit antisera against KLH coupled to a peptide comprising the final 18 residues of human GC-UNC-45. Peptide binding antibody was affinity purified on a peptide column. The specificity of the affinity purified peptide antibody was analysed by Western blot using 6His-Tagged human GC-UNC-45 purified from E.coli (not shown) and 293T cells transfected with pEGFP-C1-GC-UNC-45or pEGFP-C1 (Figure 5c). The antibody specifically recognized recombinant GC-UNC-45 with either the 6His or GFP tags (Figure 5c). We optimized conditions for detection of GC-UNC-45 in paraffin-embedded human tissue using the affinity purified peptide antibody. Immunohistochemical staining suggested a cytoplasmic localization for GC-UNC-45 (Figure 6), consistent with our in vitro studies using GFP-GC-UNC-45 (not shown). A similar immunohistochemical staining pattern was observed using the rabbit antiserum to full length GC-UNC-45 (not shown).

In order to assess whether GC-UNC-45 is differentially expressed in ovarian serous carcinoma, we performed immunohistochemical staining of tissue microarrays using GC-UNC-45-specific antibody for low grade (14 cases) and high grade (32 cases) serous carcinoma. Staining was scored blind as absent=0, weak=1, intermediate=2, and intense=3. Using this scoring system we observed that high grade serous carcinomas stained more intensely than low grade serous carcinoma ($p=0.027$, Figure 6e). Conversely, staining of normal ovarian surface epithelium (12 cases) and benign serous cystadenoma (n=4) were not significantly different from each other ($p=0.29$, Figure 6e) or from low grade serous carcinoma ($p=0.48$ and $p=0.21$ respectively). In contrast, staining of normal ovarian surface epithelium (12 cases) and benign serous cystadenoma (n=4) was significantly weaker than for high grade serous carcinoma (32 cases, $p=0.0028$ and $p=0.0065$ respectively, Figure 6e). The GC-UNC-45 antibody staining of low stage (stages 1-2) serous carcinoma (18 cases) was also significantly weaker than for high stage (stages 3-4) serous carcinoma (32 cases, $P=0.0021$, Figure 6f).

Figure 5. **Specificity of GC-UNC-45 antisera.** Rabbits were inoculated with either full length 6His-tagged human GC-UNC-45 or KLH-coupled peptide HTSAASPAVSLLSGLPLQ of human GC-UNC-45. Western blot analysis using antiserum to full length human GC-UNC-45 (a), rabbit antiserum to GFP (b) or rabbit serum against GC-UNC-45 peptide (c). Reactivity has been tested against 6His-tagged human GC-UNC-45 purified from E. coli and 293T cells transfected with pEGFP-C1-GC-UNC-45or pEGFP-C1 vector alone.



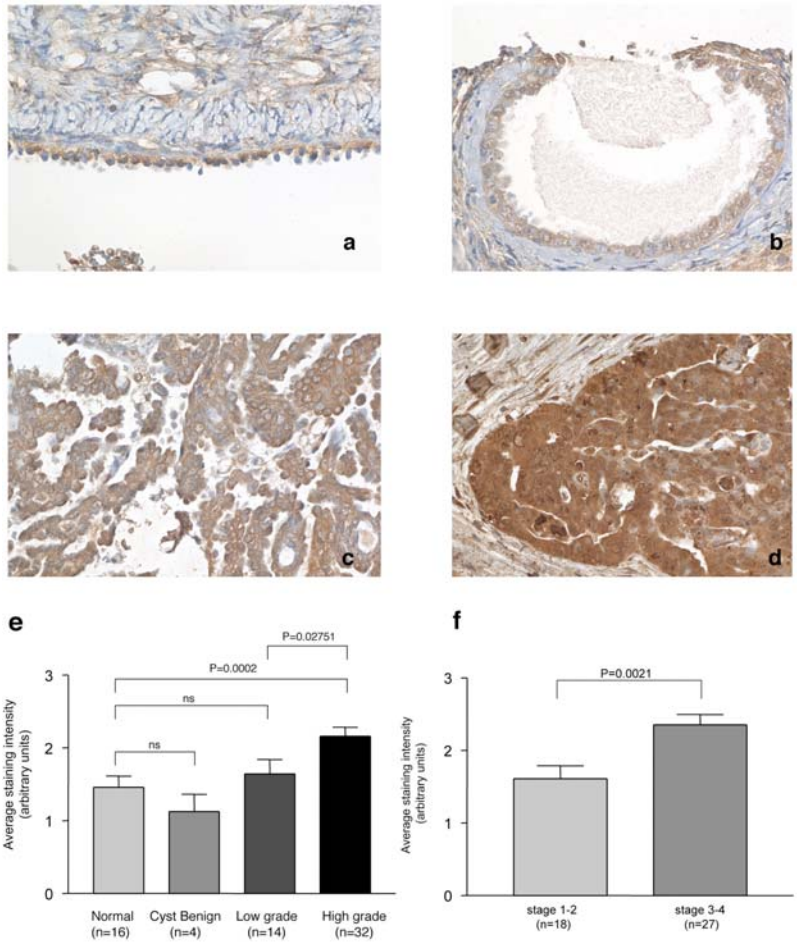


Figure 6. **GC-UNC-45 expression in normal tissue and ovarian tumors by grade and stage.**Representative histological sections showing immunohistochemical staining for GC-UNC-45 and hematoxylin counterstaining of **a.** Normal ovarian surface epithelium (160X), **b.** benign serous cystadenoma (160X), **c.** Low grade ovarian carcinoma (160X), and **d.** High grade ovarian carcinoma (100X) specimens on an ovarian tissue microarray. **e.** Immunohistochemical staining intensity for GC-UNC-45 in normal ovarian surface epithelium (12 cases), benign serous cystadenoma (4 cases), low grade (14 cases) and high grade (32 cases) serous carcinoma specimens was scored. GC-UNC-45 staining is not statistically different in normal or cystic epithelium or low grade ovarian carcinoma. However GC-UNC-45 staining is significantly elevated in high grade ovarian cancer (p=0.0002) versus low grade versus high grade ovarian cancer (p=0.0275). **f.** Immunohistochemical staining intensity for GC-UNC-45 in early (18 cases) and advanced stage (32 cases) serous carcinoma specimens was scored. GC-UNC-45 staining was significantly higher in advanced stage (3-4) versus early stage (1-2) ovarian carcinoma.

Key Research Accomplishments

- Developed a new, complementary approach for the identification of tumor-associated antigens
- Identification of 2 new tumor associated antigens for serous carcinoma using the new methodology
- Expression analysis for SMAP-1 transcripts in normal tissue and ovarian tumors
- Finding of over-expression and mislocalization of the SEREX antigen HOXB7 in serous carcinoma
- An autoantibody model. The best model generated an AUC of 0.86 (95% CI: 0.78-0.90) for discrimination between sera of EOC patients and healthy patients using antibody specific to p53, NY-CO-8 and HOXB7.

Reportable Outcomes

- Cell line derived from a micropapillary serous carcinoma
- Antisera to HOXB7 and SMAP-1/GC-UNC-45
- Two manuscripts

Conclusions

We have made significant progress in the period of grant funding, and have performed all of the tasks in our Statement of Work. We have developed a methodology for the identification of ovarian cancer-associated antigens that is complementary to SEREX. While we are generating the cDNA expression library for SEREX screening, we have used this alternate immunoprecipitation and mass spectrometry-based approach to identify two new ovarian cancer associated antigens. Almost nothing is known about the function of either antigen. For one of these new antigens, SMAP-1, we have performed a survey of transcript expression and have developed a specific antibody reagent. Furthermore, we have built upon our previous work in which HOXB7 was identified by SEREX as an ovarian cancer antigen. We have demonstrated over expression of the HOXB7 protein in ovarian cancer, as compared to normal surface epithelium. This immunohistochemical data supports our previous analysis of HOXB7 transcript expression. We also observed a mislocalization of HOXB7 in ovarian cancer. The significance of this exciting finding is under investigation. We tested multiplex detection of antibodies to candidate ovarian TAAs and statistical modeling for discrimination of sera of EOC patients and controls. Binding of serum antibody of women with EOC or healthy controls to candidate TAA-coated microspheres was assayed in parallel. A Bayesian model/variable selection approach using Markov Chain Monte Carlo computations was applied to these data, and serum CA125 values to determine the best predictive model. The selected model was subjected to area under the receiver-operator curve (AUC) analysis. The best model generated an AUC of 0.86 (95% CI: 0.78-0.90) for discrimination between sera of EOC patients and healthy patients using antibody specific to p53, NY-CO-8 and HOXB7. Inclusion of CA125 in the model provided an AUC of 0.89 (95% CI: 0.84-0.92), as compared to an AUC of 0.83 (95% CI: 0.81-0.85) using CA125 alone. However, using TAA responses alone, the model discriminated between independent sera of women with non-malignant gynecologic conditions and those with early advanced or early stage EOC with AUCs of 0.71 (95% CI: 0.67, 0.76) and 0.70 (95% CI: 0.48-0.75) respectively. We conclude that serum antibody to p53 and HOXB7 is positively associated with EOC, whereas NY-CO-8-specific antibody shows negative association. Bayesian modeling of these TAA-specific serum antibody responses exhibits similar discrimination of patients with early and advanced EOC from women with non-malignant gynecologic conditions, and may be complementary to CA125.

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Research Article

Application of Bayesian Modeling of Autologous Antibody Responses against Ovarian Tumor-Associated Antigens to Cancer Detection

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Abstract

Biomarkers for early detection of epithelial ovarian cancer (EOC) are urgently needed. Patients can generate antibodies to tumor-associated antigens (TAA). We tested multiplex detection of antibodies to candidate ovarian TAAs and statistical modeling for discrimination of sera of EOC patients and controls. Binding of serum antibody of women with EOC or healthy controls to candidate TAA-coated microspheres was assayed in parallel. A Bayesian model/variable selection approach using Markov Chain Monte Carlo computations was applied to these data, and serum CA125 values, to determine the best predictive model. The selected model was subjected to area under the receiver-operator curve (AUC) analysis. The best model generated an AUC of 0.86 [95% confidence interval (95% CI), 0.78-0.90] for discrimination between sera of EOC patients and healthy patients using antibody specific to p53, NY-CO-8, and HOXB7. Inclusion of CA125 in the model provided an AUC of 0.89 (95% CI, 0.84-0.92) compared with an AUC of 0.83 (95% CI, 0.81-0.85) using CA125 alone. However, using TAA responses alone, the model discriminated between independent sera of women with nonmalignant gynecologic conditions and those with advanced-stage or early-stage EOC with AUCs of 0.71 (95% CI, 0.67-0.76) and 0.70 (95% CI, 0.48-0.75), respectively. Serum antibody to p53 and HOXB7 is positively associated with EOC, whereas NY-CO-8-specific antibody shows negative association. Bayesian modeling of these TAA-specific serum antibody responses exhibits similar discrimination of patients with early-stage and advanced-stage EOC from women with nonmalignant gynecologic conditions and may be complementary to CA125. (Cancer Res 2006; 66(3): 1-7)

Introduction

Conventional treatment has limited efficacy against advanced-stage epithelial ovarian cancer (EOC), whereas >80% patients with early-stage disease survive 5 years after diagnosis. However, because there is no diagnostic tool for reliable screening and detection of premalignant or localized ovarian cancer, 70% of patients with ovarian cancer have advanced disease on initial diagnosis (1).

Measurement of serum CA125 levels was approved as a prognostic indicator to monitor disease recurrence (2). Normal healthy donors (~1%) have serum CA125 levels greater than 35 units/mL. Elevated levels of CA125 are detected in >90% of sera of disseminated ovarian cancer cases (stages II-IV) but in only 50% of patients with stage I disease (2). Thus, the CA125 assay is inappropriate as a "stand-alone" population screen for early-stage ovarian cancer, although its positive predictive value can be improved by combination with other screening tools (e.g., serial measurements, transvaginal sonography, or combinations with other markers and statistical modeling).

The immune system constantly surveys the body for "nonself" antigens and generates a response in the appropriate context. Significantly, cancer patients often mount a humoral response to autologous tumor-associated antigens (TAA; ref. 3). Autologous antibodies have been documented in patients afflicted with a variety of different cancers, including those of the breast, head and neck, colon, lung, kidney, and melanoma (4–6). Ovarian tumor-reactive antibodies have been detected in patient serum and ascites (7) and their antigens are identified by mass spectrometry of immunoprecipitates (8) or by SEREX (refs. 9–11; e.g., ubiquilin-1, ZFP161, FLJ21522, ABC7, HOXA7, and HOXB7). Antibodies to many TAAs are present in several cancer types (e.g., p53 and NY-ESO-1; ref. 12).

Several studies indicate that autologous antibodies specific for TAAs are prevalent in cancer patients but are absent from or infrequent in healthy volunteers. This suggests that autologous antibodies specific to relevant TAAs may have potential as serum biomarkers (13). Therefore, we sought from the literature TAAs associated with ovarian cancer and colon cancer, because the latter often resembles mucinous carcinomas of the ovary. Perhaps the autoantigen best studied in ovarian cancer patients is p53 (14). In stage I/II ovarian disease, 22% of patients had p53 antibody, 31% in stage III, and 50% in stage IV (15). Although there was no association of p53 antibody with clinical stage, tumor histologic type, or overall patient survival (16, 17), detection of autologous

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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antibody to some ovarian cancer antigens seems to have prognostic significance (18). Notably, detection of serum antibody to p53 has been shown to predict subsequent development of cancer (19).

The relevance to carcinogenesis of most TAAs is unclear, with the exception of known cell cycle regulators, such as p53, ras, c-myc, c-myb, and HER-2/*neu* (20). The ovarian TAA HOXB7 is overexpressed in ovarian and several other cancers and is associated with enhancement of fibroblast growth factor production and angiogenesis (9, 21). The mechanisms that trigger antibody responses to these autologous TAAs are not known. Although p53 is frequently mutated in cancer, many of these TAAs are not. Many TAAs are overexpressed in the cancer relative to normal tissue or not normally expressed in the tissue, such as the cancer/testis family (22). One study noted that many ovarian TAAs are encoded on chromosome arm 17q (e.g., HER-2/*neu* and HOXB7; ref. 11). Autoantibody to heat shock proteins (Hsp), notably Hsp27 and Hsp90 (23–25), has also been associated with ovarian cancer, and this may reflect the ability of certain Hsps, such as Hsp70, to bind and activate dendritic cells (26).

Although many other TAAs have been identified (http://www.licr.org/SEREX.html), the percentage of ovarian cancer patients with reactivity to individual TAAs is generally low. We hypothesized that detection of antibodies to a panel of known TAAs could discriminate sera from ovarian cancer patients and healthy women and potentially improve on the performance of the CA125 assay. However, a statistically rigorous approach to marker selection is required to develop such a clinically applicable diagnostic test by avoiding problems arising from high correlations among potential markers. Herein, we describe the application of multiplex detection of autologous antibodies to a panel of previously described ovarian TAAs and the Bayesian model/variable selection approach using Markov Chain Monte Carlo (MCMC) computations to determine the relevant TAA biomarkers and the most predictive model. MCMC variable selection is a model-based approach with a specified statistical model that puts no distributional restriction on the predictors (markers). Our model-based approach provides probabilistic assessments of uncertainty through Bayesian learning. A unique feature of the Bayesian approach is the easy incorporation of previously described markers in a natural way into existing models, which cannot be achieved by ad hoc procedures, such as recursive partitioning (27–29). Our application of multiplex detection of autologous antibodies to ovarian TAA and Bayesian model selection for detection of EOC complements the CA125 test and implicates p53, HOXB7, and NY-CO-8 in the biology of EOC.

Materials and Methods

Samples. Sera were collected as part of informed consent protocols approved by the local institutional review boards, and the study was approved by the Johns Hopkins University Institutional Review Board. Blood samples were allowed to clot at room temperature and then centrifuged at 400 × *g* to remove clot and cells. Clarified sera were stored at −70°C. A learning set of 59 sera and a validation set of 37 sera were obtained at the University of Louisville School of Medicine (ULSM; Louisville, KY) from women with stage III/IV ovarian cancer and 32 sera from women attending a gynecology clinic for conditions other than ovarian cancer as controls. Sera were also obtained at the University of Texas M. D. Anderson Cancer Center (MDACC; Houston, TX). This set was obtained from women with breast cancer (n = 18), colon cancer (n = 6), lung cancer (n = 10), and stage III/IV ovarian cancer (n = 27) and from healthy women (n = 23). Finally, a set of sera of women with early-stage ovarian cancer (n = 14) was provided by the Gynecologic Oncology Group (GOG; Bethesda, MD).

Preparation of recombinant TAA. TAAs were cloned from PCR products into the prokaryotic expression vector pBADgIII (Invitrogen, Carlsbad, CA). ABC7 (AF133659), HOXA7 (AF032095; ref. 10), HOXB7 (NM_004502; ref. 9), NY-ESO-1 (U87459; ref. 30), ubiquilin-1 (NM_013438), ZFP161 (Y12726), FLJ21522 (AK025175; ref. 11), calmodulin (Invitrogen), and p53 (X02469; ref. 14) were amplified from published constructs, whereas NY-CO-8 (AF039690) and NY-CO-16 (AF039694; ref. 6) were amplified directly from commercial cDNA libraries. Constructs were validated using Automated Laser Fluorescent Sequencing. Bacterial cultures were grown in Terrific broth supplemented with 1% (v/v) glycerol and 100 μg/mL ampicillin at 37°C to mid-log phase (A_{650}, 0.6-0.7) and induced with 0.02% (w/v) L-(+)-arabinose for 2 to 3 hours. Induction was done at 30°C with 0.002% (w/v) L-(+)-arabinose for HOXA7 and HOXB7. Cell pellets from 1 L cultures were solubilized by sonication in 20 mL of 8 mol/L urea, 3.7 mL of 10% (w/v) sodium *N*-lauroyl-sarcosinate and brought to 50 mL with 20 mmol/L Tris-HCl (pH 8.0)/0.2 mol/L NaCl/10% (v/v) glycerol and 0.1% (w/v) sodium *N*-lauroyl-sarcosinate. After centrifugation at 12,000 × *g* for 30 minutes at 4°C, the supernatant was loaded onto a Ni-NTA Superflow (Qiagen) column. The column was washed with a step gradient of 10, 20, 50, 100, and 0.5 mol/L imidazole in 20 mmol/L Tris-HCl (pH 8.0)/0.2 mol/L NaCl/10% (v/v) glycerol and 0.1% (v/v) Triton X-100. The purity and size of the purified proteins was determined by staining SDS-PAGE gels for total protein (Sypro Ruby) and performing Western blotting on duplicate gels for His_6-labeled antigen using horseradish peroxidase–labeled anti-His_6 and chemiluminescent substrate. Q2

Coupling to microspheres and Luminex analysis. Monoclonal antibody to His_6 was coupled to 11 distinct sets of LabMAP carboxylated microspheres (following the manufacturer's protocol on http://www.luminexcorp.com), which were then individually bound overnight at 4°C with 30 μg each purified His_6-tagged recombinant TAA. Similarly, a further six distinct sets of LabMAP carboxylated microspheres were directly coupled to 25 μg purified Hsp27, Hsp70, and Hsp90 (Stressgen, Victoria, British Columbia, Canada; refs. 25, 31, 32) or, as controls, 25 μg glutathione *S*-transferase (GST) or pBAD vector alone, 5 μg/mL anti-human IgG (Sigma Chemical Co., St. Louis, MO), or 50 μg/mL human IgG. Equivalent counts of each set of protein-coupled microspheres were mixed to a concentration of 5,000 per set per 50 μL/well in PBS containing 10% normal mouse serum (The Jackson Laboratory, West Grove, PA). The beads were shaken with 50 μL patient serum diluted 1:25 in a 96-well filter-bottomed microtiter plate for 1 hour in the dark at ambient temperature. The beads were washed thrice with 100 μL buffer by filtration and then shaken in 100 μL/well R-phycoerythrin (PE)–conjugated donkey anti-human IgG diluted 1:200 in PBS/bovine serum albumin [BSA; 10 g/L BSA, 1.4 g/L $NaH_2PO_4 \cdot H_2O$, 8.77 g/L NaCl, 0.5 g/L NaN_3 (pH 7.4)] for 45 minutes in the dark. After three washes, the beads were resuspended in 100 μL/well PBS/BSA and their mean fluorescence intensity (MFI) was assayed on a Luminex 100 plate reader. The MFI of 100 of each set of microspheres was determined for each well. A small panel of 10 patient sera was run on all assay plates to allow an assessment of interassay variability and bead variability or stability. Several presumptive positive and negative control antigens were included within the bead set, including human IgG (HsIgG to monitor the addition of PE-conjugated secondary antibody), anti-human IgG (αHsIgG to show addition of the human sera to each well), calmodulin (a presumptive irrelevant autoantigen associated with viral hepatitis, or vector alone (controlling for bacterial contaminants in the antigen preparations) and uncoated beads (for assessment of nonspecific binding).

Statistical analyses. A Bayesian logistic regression model/variable selection approach using MCMC (33) computations was implemented in the WinBUGS (34) programming environment. A full description of the model selection and details of our Bayesian computations using Gibbs sampling are provided in the Supplementary Materials.

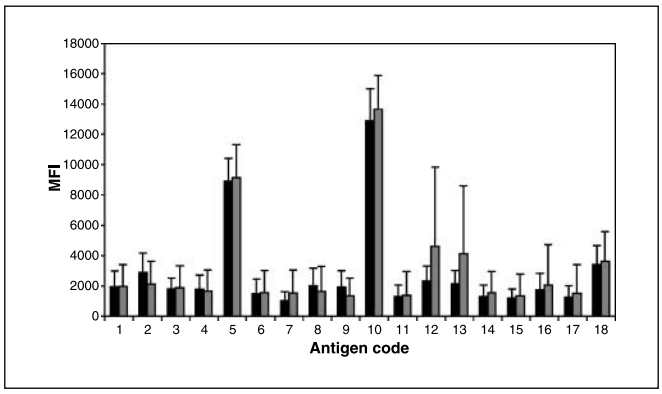

Figure 1. MFI of the beads coated with individual markers (mean ± SD) in normal volunteers (*black columns*) and ovarian cancer patients (*gray columns*). Antigen codes: *1,* FLJ21522; *2,* NY-CO-8; *3,* NY-CO-16; *4,* ABC7; *5,* αHsIgG; *6,* calmodulin; *7,* HOXB7; *8,* HSP70; *9,* HSP90; *10,* HsIgG; *11,* no antigen; *12,* NY-ESO-1; *13,* p53; *14,* ubiquilin-1; *15,* GST; *16,* ZFP161; *17,* HOXA7; *18,* HSP27.

The motivation behind the variable selection approach was the fact that all the markers and controls were highly correlated (data not shown), which is known as multicollinearity and likely reflects background effects of using serum at high concentration. Thus, simultaneous use of all the markers and controls in a statistical model will obscure the statistical significance of the core variables that are functions of the others. Consequently, the information contained in all the variables is already represented in the core variables, and a dimension/variable selection technique can be use to identify them. In preliminary analyses (data not shown), we ran a logistic regression model that contained all the 13 TAAs and 5 controls and found out that none of the markers and controls were significant, which resulted in poor discrimination of EOC.

The MCMC variable selection approach is a stochastic search algorithm that effectively visits all 2^{18} = 216,144 different models obtained from including/excluding any of the 18 TAAs or controls in a logistic regression for the probability of ovarian cancer. The outcome of this procedure is the model with the highest number of visits (the most probable model) supported by the data. For numerical stability, we transformed the measured MFI levels of the markers and controls to logarithmic scale. Our approach adjusts for the associations among the 13 markers and the effects of the 5 controls. Given the limited number of cases and controls, we focused on an additive model assuming no interactions among the markers, controls, and between markers and controls. *A priori*, each specific marker or control is assumed to be equally likely to be included/excluded (i.e., with probability of 0.5) in the model. This corresponds to an equally likely prior probability of $1/2^{18}$ for each possible configuration in the model space. The inclusion probability for a specific marker or control is then updated by their posterior probability using all the available information about the other markers and controls, conditional on the observed ovarian cancer status. Although, in principle, one can compute the posterior probability of each of the 2^{18} models using the Bayesian analysis, a simpler alternative is to use the Bayes factor (BF; ref. 35), defined as the posterior and prior odds ratio, to select relevant markers or controls for future analyses. For a specific marker or control, larger values of BF provide evidence in favor of inclusion, whereas smaller values support exclusion from the model. Usually, a value between 1 and 3 is considered weak, between 3 and 10 as substantial, between 10 and 100 as strong, and >100 as very strong (36).

Results

To explore the utility of autologous serum antibody specific to particular TAAs as an additional tool for discrimination of sera from cancer patients and healthy women, we generated recombinant protein from 13 markers identified previously as candidate TAAs and selected 5 presumptive control reagents. Sera from both healthy women and EOC patients were tested in parallel for reactivity to all 18 panel elements. Initially, we tested sera of 23 healthy women from MDACC as controls and sera from 59 stage III/IV ovarian cancer patients obtained at ULSM and 27 from MDACC as cases, respectively. This set of sera was analyzed for reactivity to microspheres of discrete sizes coated with individual recombinant TAA or control protein. The presence of antibody bound to the beads was detected with PE-labeled anti-human IgG and the fluorescence was quantified using a Luminex plate reader. Because 5 presumptive control elements were included in the panel, absolute MFI values without background subtraction were employed (Fig. 1). F1

Our analytic strategy consisted of fitting the logistic regression model in Eq. 1 (see Supplementary Materials) for ULSM and MDACC cases and controls. By using BF $\geq$ 3 as a selection criterion, we picked the best model and computed multiple sensitivity, specificity, ROC, and area under the receiver-operator curve (AUC) by varying a probability cutoff between 0.01 and 1.00 (Table 1; Fig. 2). The AUC was calculated as 0.86 [95% credible interval (Bayesian analogue of confidence interval), T1F2

Table 1. Modeling of serum responses to the 18-element panel in cases and controls

Marker	$\Pr(d_j = 1 \mid Data)$	θ_j	BF_j
FLJ21522	0.49 (0.50)		
NY-CO-8	0.98 (0.15)	−1.49 (0.62)	49
NY-CO-16	0.45 (0.49)		
ABC7	0.65 (0.48)		
αHsIgG	0.42 (0.49)		
Calmodulin	0.42 (0.49)		
HOXB7	0.92 (0.27)	1.42 (0.76)	11.5
Hsp70	0.57 (0.49)		
Hsp90	0.49 (0.50)		
HsIgG	0.43 (0.49)		
No antigen	0.60 (0.49)		
NY-ESO-1	0.48 (0.50)		
p53	0.96 (0.20)	1.21 (0.58)	24
Ubiquilin-1	0.61 (0.49)		
ZFP161	0.28 (0.45)		
Vector	0.40 (0.49)		
HOXA7	0.42 (0.49)		
Hsp27	0.42 (0.49)		
	Post. mean (SD)	95% CI for AUC	
AUC	0.86 (0.03)	0.78	0.90

NOTE: Identification of the best-fitting model for discrimination between sera of 23 healthy patients from MDACC as controls and sera from 59 stage III/IV ovarian cancer patients obtained at ULSM and 27 from MDACC as cases using the 18-element panel. Data are posterior estimates (mean and SD) of $\Pr(d_j = 1 \mid Data)$ for all the variables, the regression coefficients $\{\theta_j = d_j b_j\}$, and BFs for the best markers and controls (selected by the Bayesian approach) using the data set of ULSM and MDACC cases and controls. The regression coefficients θ_j are assumed to be statistically independent, and each have a two-component mixture prior distribution with a point mass at 0 (when $d_j = 0$) with probability of 0.5 and a normal distribution $N(0, \sigma_j^2)$ (when $d_j = 1$) with probability 0.

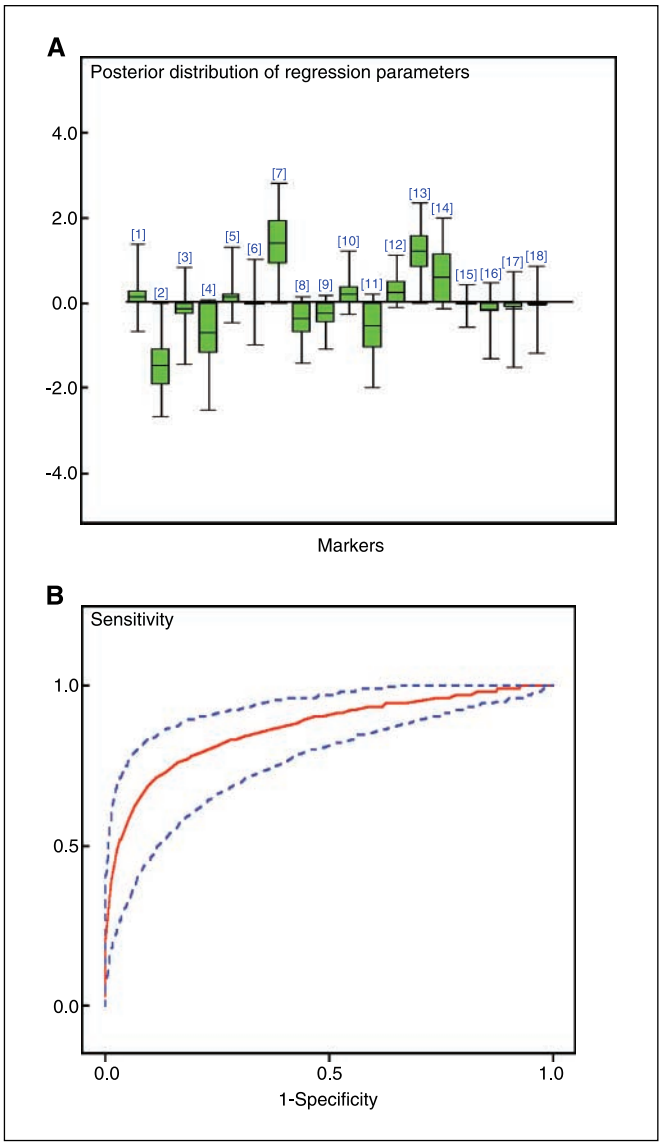

Figure 2. Posterior distributions and estimate of ROC for markers NY-CO-8, HOXB7, and p53. *A,* posterior distributions of $\{\theta_j\}$ in the variable selection algorithm for data of Table 1. *Numbers inside brackets,* elements in Table 1 (i.e., *[1]* corresponds to FLJ21522, etc.). *B,* posterior estimate of ROC for markers NY-CO-8, HOXB7, and p53 with 95% credible interval for cutoff values ranging from 0.01 to 1.00 for data of Table 1.

0.78-0.90] for this best-fitting model (Table 1). The best model used three TAA markers: p53 and HOXB7, which showed a positive association, and NY-CO-8, which showed a negative association (Table 1).

We also compared the best model for discrimination of cases and controls with detection of absolute serum level of CA125 or p53-specific antibody or both. The AUC was calculated as 0.83 [95% confidence interval (95% CI), 0.81-0.85] for the standard CA125 assay alone. Detection of p53 antibody alone was not useful for discrimination of cases and controls in this serum set (AUC, 0.52; 95% CI, 0.20-0.58) and combination of absolute CA125 level and serum reactivity to p53 provided an AUC of 0.81 (95% CI, 0.79-0.83). We tested whether addition of absolute serum level of CA125 as an element of the panel conferred additional predictive value on reanalysis of the serum set (Table 2). The

T2

posterior distributions of the regression variables are displayed in Fig. 3*A* and the ROC is given in Fig. 3*B* (along with 95% credible bands). Although there is improvement in terms of the accuracy of the fitted model [AUC with CA125 goes slightly up from 0.86 (95% CI, 0.78-0.90) to 0.89 (95% CI, 0.84-0.92)], the same markers NY-CO-8, HOXB7, and p53 are again selected as most informative along with CA125.

F3

We conducted a cross-validation analysis to determine the self-consistency of the best model. Here, a leave-one-out approach was used where each data point D_i was removed from the data set and predicted (using the best-fitting model in Table 1 and posterior predictive distribution $[D^*_i|D_{\{-i\}}]$) by the remaining cases $D_{\{-i\}}, i = 1, 2, \ldots, n$ with $\{-i\}$ denoting the data, except for the ith observation and D^*_i is a predicted value of D_i. The resulting cross-validated ROC in Fig. 4*A* was very close in shape to ROC given in Fig. 2*B*. The posterior mean of the cross-validated AUC was 0.85, with a slightly wider 95% credible interval of 0.76 to 0.92 reflecting additional uncertainty arising from predictions. Therefore, for this data set, our model is self-consistent.

F4

Several conditions unrelated to ovarian cancer result in an elevated serum CA125 level. Furthermore, the sensitivity of the CA125 for detection of early-stage disease is limited. To validate our 18-element panel and modeling in a serum set in which the CA125 assay would perform poorly, we studied sera of 32 women attending a gynecology clinic at ULSM (including 3 with CA125 levels >35 units/mL) but who had no evidence of ovarian cancer as controls. As cases, we tested sera from either 37 women with

Table 2. Modeling of the serum responses to the 18-element panel and CA125 level in cases and controls

Marker	$\Pr(d_j = 1\|Data)$	θ_j	BF_j
FLJ21522	0.37 (0.48)		
NY-CO-8	0.90 (0.31)	−1.18 (0.67)	9
NY-CO-16	0.44 (0.50)		
ABC7	0.53 (0.50)		
αHsIgG	0.35 (0.48)		
Calmodulin	0.42 (0.49)		
HOXB7	0.87 (0.33)	1.17 (0.73)	6.7
Hsp70	0.33 (0.47)		
Hsp90	0.41 (0.49)		
HsIgG	0.36 (0.48)		
No Antigen	0.52 (0.50)		
NY-ESO-1	0.41 (0.50)		
p53	0.76 (0.43)	0.73 (0.62)	3.2
Ubiquilin-1	0.52 (0.50)		
ZFP161	0.49 (0.50)		
Vector	0.41 (0.50)		
HOXA7	0.43 (0.49)		
Hsp27	0.37 (0.48)		
CA125	0.99 (0.07)	0.90 (0.37)	99
	Post. mean (SD)	95% CI for AUC	
AUC	0.89 (0.03)	0.84	0.92

NOTE: Identification of the best-fitting model for discrimination between sera of 23 healthy patients from MDACC as controls and sera from 59 stage III/IV ovarian cancer patients obtained at ULSM and 27 from MDACC as cases using the 18-element panel and absolute CA125 level. Statistical analysis was done as described for Table 1.

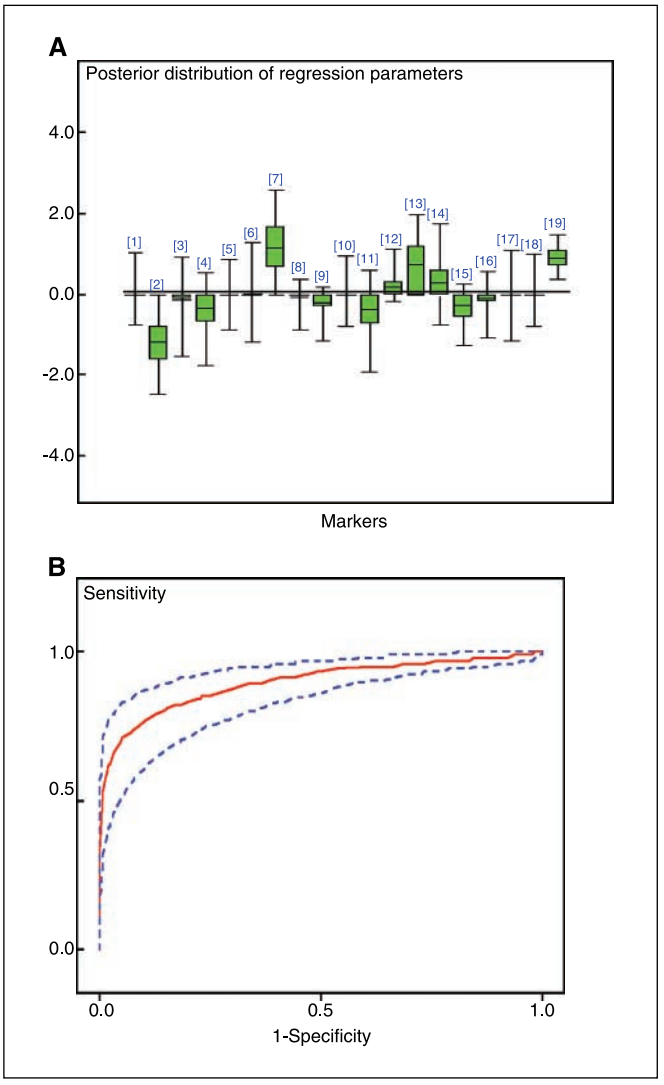

Figure 3. Posterior distributions and estimate of ROC for markers NY-CO-8, HOXB7, p53, and CA125. *A,* posterior distributions of $\{\theta_j\}$ for all 18 candidate markers plus CA125 levels (*[19]*) in the variable selection algorithm for data of Table 2. *B,* posterior estimate of ROC for training set using NY-CO-8, HOXB7, p53, and CA125 with 95% credible interval for cutoff values ranging from 0.01 to 1.00 for data of Table 2.

advanced-stage (III/IV) ovarian cancer or 14 women with early-stage (I/II) ovarian cancer (Fig. 4*B* and *C*). These test sets of independent sera were analyzed in parallel for reactivity to the panel of TAAs and controls, and the data were analyzed using the previously determined best-fitting model excluding CA125 (described in Table 1). The ROC analyses are provided in Fig. 4*B* and *C*. The model provided an AUC of 0.71 (95% CI, 0.67-0.76) for discrimination between women with advanced-stage ovarian cancer and those attending the gynecology clinic at ULSM and an AUC of 0.70 (95% CI, 0.48-0.75) for discrimination between early-stage ovarian cancer and women attending the gynecology clinic at ULSM.

Discussion

Our study examines the applicability of multiplex detection of autologous antibody to TAAs as a diagnostic tool for cancer. The

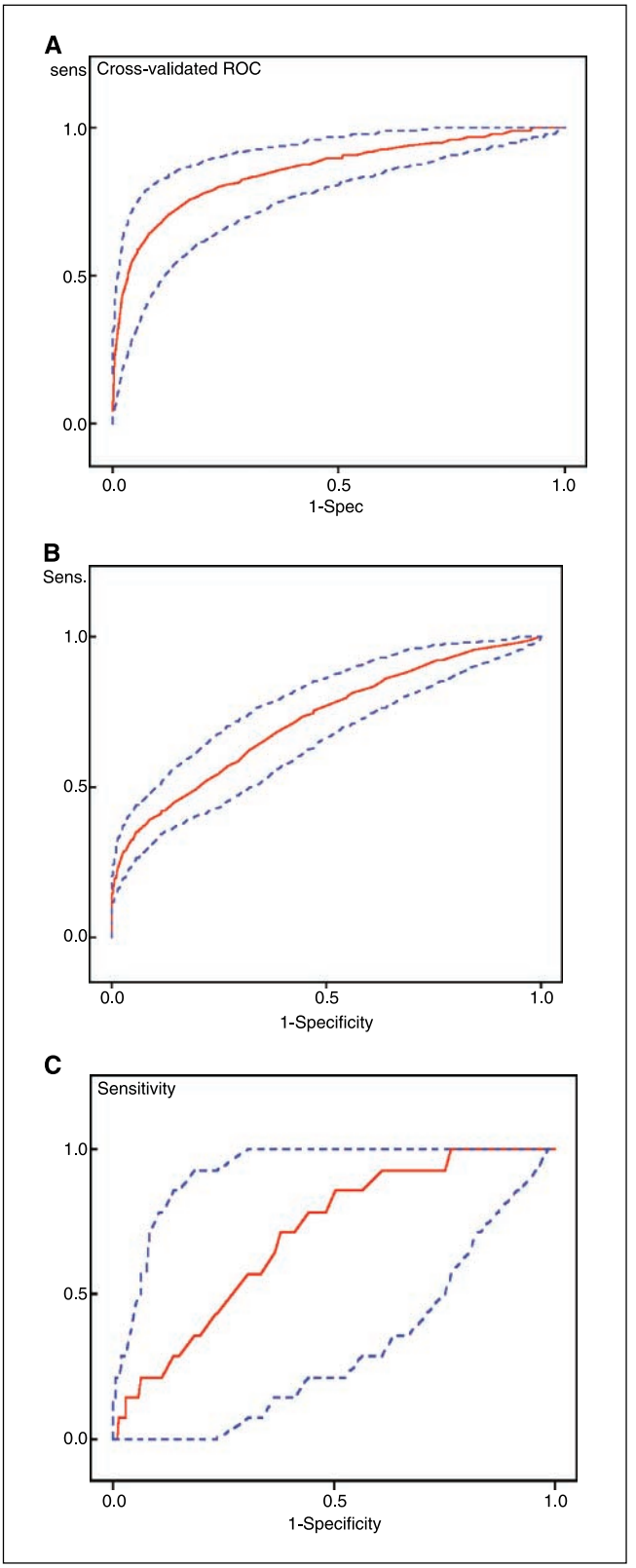

Figure 4. Validation steps. *A,* cross-validated estimate of ROC for the best-fitting model summarized in Table 1. *B* and *C,* cross-validation of the best-fitting model for p53-, NY-CO-8-, and HOXB7-specific antibody reactivity (see Table 1) using an independent set of sera from 37 advanced-stage ovarian cancer patients (*B*) or 14 early-stage ovarian cancer patients (*C*) as cases and 32 women attending a gynecology clinic for conditions other than ovarian cancer as controls in (*B* and *C*).

detection of autologous antibody to this three-member panel of TAAs, in addition to CA125, shows merit as an approach for ovarian cancer detection. However, much larger studies are required to assess its sensitivity at the high specificity required for screening healthy women. In particular, it warrants further examination in high-risk populations, such as female first-degree relatives of ovarian cancer patients or women carrying mutations that predispose toward cancer. The application of longitudinal screening may also significantly increase the positive predictive value of the test (37, 38). Sera collected over up to four time points from individual patients with ovarian (n = 5) or breast (n = 5) cancer were analyzed for the stability of TAA reactivity. The reactivity to individual TAAs was generally stable over several years despite therapy, indicating that this approach is not useful to monitor therapy (data not shown).

Our modeling of TAA-specific antibody discriminates poorly the patients with ovarian cancer from those with cancer of other organ sites, notably women with breast cancer (n = 18), colon cancer (n = 6), and lung cancer (n = 10; data not shown). This reflects the use of p53 as a diagnostic marker and its importance in cancers in addition to ovarian cancer (14). However, other investigators have improved specificity for a particular cancer type by the inclusion of additional markers (29, 39). Over 2,000 TAAs have been entered into the SEREX database (http://www.licr.org/SEREX.html). Several other approaches have also been used to identify candidate TAAs, including mass spectrometric analysis of immunoprecipitates (8) and screening of phage display libraries (40, 41). Inclusion of other TAAs using similar, highly multiplexed approaches (42) in large case-control studies may provide better discrimination between ovarian and other cancer types.

A more conventional approach to data analysis using logistic regression where all the 13 markers and 5 controls were forced to stay in the model (with prior probability of inclusion being 100% for each) failed to provide a useful model (data not shown), as an AUC of only $\sim$0.60 was obtained. Here, we describe the application of Bayesian model/variable selection approach using MCMC computations, implemented in the freely available WinBUGS program, to determine the best predictive model. The MCMC variable selection is a model-based approach with a specified statistical model on the cases and controls (in our case, binomial distribution with logistic link) but puts no distributional restriction on the predictors (markers). It represents an alternative to the existing ad hoc statistical approaches, such as recursive partitioning (27–29), and provides formal assessment of uncertainty (in probabilistic terms) through Bayesian learning and updating. A unique feature of our methods is the easy incorporation in a natural way into existing models of prior information obtained from earlier clinical studies or scientist's experience that cannot be used in other procedures.

We conducted a cross-validation analysis initially using a leave-one-out approach to determine the self-consistency of our best model for discrimination between healthy volunteers and women with advanced-stage ovarian cancer. The model has proven to be internally consistent; therefore, we sought further validation using an independent serum set. Unlike advanced-stage disease, ovarian cancer diagnosed while still confined to the ovaries is highly curable using current interventions. Thus, we examined the ability of our model to discriminate sera from patients with advanced-stage and early-stage disease. The model was tested in another set of sera that included 14 from patients with stage I/II ovarian cancer or 37 sera from advanced-stage ovarian cancer patients. Furthermore, as a more rigorous test, we used sera from women with nonmalignant gynecologic conditions as controls. The model achieved an AUC of 0.70 (95% CI, 0.48-0.75) for discrimination between sera of early-stage ovarian cancer patients and controls. The wider credible intervals partially reflect the relatively small number of early-stage cases (n = 14). This outcome represents a significantly poorer performance than for advanced-stage cases versus healthy volunteers for which an AUC of 0.86 (95% CI, 0.78-0.90) was derived. However, we obtained an AUC of 0.71 (95% CI, 0.67-0.76) for discrimination between advanced-stage ovarian cancer and women with nonmalignant gynecologic conditions suggesting that these markers, like CA125, are affected by nonmalignant gynecologic conditions.

Many other biomarkers, including CA125, are significantly less predictive for early-stage ovarian cancer patients. It is therefore interesting that the autoantibody-based test may be similarly effective for discrimination of sera of both early-stage and late-stage ovarian cancer patients from healthy women. Although no information is available on the relationship of NY-CO-8 and ovarian carcinogenesis, both p53 mutation (14, 16, 43) and HOXB7 overexpression have been described in early-stage ovarian cancer (11, 21).

Differences in specimen processing can confound biomarker studies. The similar AUCs obtained using ovarian cancer cases from different institutions (ULSM and GOG) suggests that this autoantibody-based test is likely resistant to the confounding effects of collection at different sites.

Pattern recognition analysis of proteomic profiles has been used to discriminate sera of ovarian cancer patients from those of healthy women. However, the biological underpinning of these patterns is yet unclear (44). By contrast to proteomic profiles, the significance of the TAAs p53 and HOXB7 in cancer biology has been studied extensively for ovarian and other cancers (9, 43, 45–47). A partial cDNA sequence NY-CO-8 was initially identified as a colon cancer antigen. However, subsequent analysis of the serologic reactivity to NY-CO-8 suggests that it is a naturally occurring autoantigen (42). The presence of NY-CO-8-reactive autoantibody in healthy controls that are suppressed in cancer patients may account for its negative association with ovarian cancer in our study. However, we observed no generalized suppression of antibody levels in ovarian cancer patients (Fig. 1); thus, the possibility of a protective effect warrants further investigation. Indeed, a similar phenomenon was recently described for MUC1-specific antibody (48). The full-length NY-CO-8 gene was recently cloned, and its product, CCCAP, was shown to associate with centrosomes (49). Intriguingly, disruption of the centrosomes is an early event in the genesis of ovarian cancer. Furthermore, the breast and ovarian cancer hereditary susceptibility genes BRCA1 and BRCA2 contribute to a centrosome function that is believed to help maintain the integrity of the chromosome segregation process (50, 51).

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AUTHOR QUERIES

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Q1: Running head: Antibody to Ovarian Tumor Antigens for Detection. Short title OK?

Q2: Provide city and state (country, if foreign) of Qiagen.

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INTRODUCTION - Project 3

Metastatic ovarian cancer is almost an incurable disease and is responsible for the highest mortality rate (20%) among patients with gynecologic malignancies. Current efforts to reduce this mortality rate, including improvement of early detection and treatment, have been relatively unsuccessful. Existing therapies for advanced disease, such as chemotherapy and radiation therapy, rarely result in long-term benefits in patients with locally advanced and metastatic disease [1-3]. Immunotherapy provides a promising alternative approach for the control of ovarian cancer.

The ideal cancer therapy should have the potency to eradicate systemic tumors and control metastases, as well as the specificity to discriminate between malignant and normal cells. In both of these respects, the immune system is an attractive therapeutic pathway. The immune system has multiple complementary effector mechanisms capable of killing target cells, including T cells, macrophages, granulocytes, and natural killer cells. T cells can generate tumor-specific immune responses via a vast array of clonally distributed antigen receptors which can recognize tumor–specific antigens. It is well established that T cells recognize peptide fragments of cellular proteins bound to major histocompatibility complex (MHC) molecules on the surfaces of cells, and any cellular protein (including those from which tumor antigens derive) can be presented to T cells in this way. Thus, identification of tumor-associated antigens uniquely expressed in ovarian cancer is important for the development of antigen-specific cancer immunotherapy. Mesothelin represents an ideal target antigen for the control of ovarian cancers since it is expressed in over 95% of ovarian cancers and is absent or expressed at low levels in normal tissues [4].

DNA vaccines targeting human mesothelin provide a potentially effective approach to the control of mesothelin-expressing ovarian cancer. DNA vaccines are considered to be a potentially promising form of vaccine for the control of infectious diseases and cancers since they offer many advantages over other conventional vaccines such as peptide or attenuated live pathogens. For instance, DNA vaccines are relatively safe and can be administered repeatedly without adverse effects. In addition, DNA vaccines are relatively easy to produce on a large scale and are able to yield products with high purity and stability. Most importantly, effective DNA vaccine delivery systems, such as the direct intradermal administration of DNA vaccines via gene gun to professional antigen presenting cells (APCs), have been well established. Using this delivery method, we have previously developed several innovative strategies to enhance DNA vaccine potency by modifying the properties of DNA-transfected APCs (for reviews, see [5,6]).

One of the strategies that we have recently developed to enhance DNA vaccine potency is the employment of single-chain trimer (SCT) technology to bypass antigen processing and ensure stable MHC class I presentation of the antigenic peptide by transfected APCs. We have previously constructed a DNA vaccine encoding an SCT composed of an immunodominant CTL epitope of human papillomavirus type 16 (HPV-16) E6 antigen, β2m, and H-2K^b MHC class I heavy chain (pIRES-E6-β2m-K^b). Transfection of human embryonic kidney cells (293 cells) with pIRES-E6-β2m-K^b DNA has been shown to bypass antigen processing and lead to stable presentation of E6 peptide. Furthermore, C57BL/6 mice vaccinated with pIRES-E6-β2m-K^b exhibited significantly increased E6 peptide-specific CD8$^+$ T cell immune responses and more potent anti-tumor effects against E6-expressing tumors compared to mice vaccinated with DNA encoding wild-type E6 [7]. These findings indicate that a DNA vaccine encoding an SCT may potentially improve DNA vaccine potency and elicit antigen-specific immune responses capable of controlling tumors or infectious diseases.

In the current study, we evaluated the efficacy of a DNA vaccine employing an SCT targeting a human mesothelin-specific CTL epitope (aa540-549) using HLA-A2 transgenic mice. HLA-A2 transgenic mice have extensively been used to identify functional, human CTL epitopes and to evaluate the efficacy of candidate vaccines, since these transgenic mice may have CTL repertoires similar to those of humans. We demonstrated that HLA-A2 transgenic mice vaccinated with DNA employing an SCT of HLA-A2 linked to the human mesothelin epitope aa540-549 (pcDNA3-Hmeso540-β2m-A2) were capable of generating strong human mesothelin peptide (aa540-549)-specific CD8$^+$ T cell immune responses. Furthermore, mice vaccinated with pcDNA3-Hmeso540-β2m-A2 were able to more effectively prevent the growth of HLA-A2 positive, human mesothelin-expressing tumor cell lines compared to vaccination with control DNA. The implications of this approach for clinical application were discussed.

Body:

Cells transfected with DNA encoding human mesothelin (pcDNA3-Hmeso) or infected with vaccinia encoding human mesothelin (Vac-Hmeso) express human mesothelin.To characterize human mesothelin expression of cells transfected with DNA or infected with vaccinia, TK- cells were either transfected with pcDNA3-Hmeso or infected with Vac-Hmeso vaccinia. Transfected or infected cells were characterized by staining with human mesothelin-specific antibodies followed by flow cytometry analysis. TK- cells transfected with pcDNA3-Hmeso expressed human mesothelin, as shown in Figure 1A. In constrast, TK- cells transfected with pcDNA3

vector did not express human mesothelin. Similarly, TK- cells infected with Vac-Hmeso expressed human mesothelin, while TK- cells infected with wild type vaccinia did not express human mesothelin (Figure 1B). These results confirm that we have successfully generated a human mesothelin-expressing DNA construct and vaccinia virus.

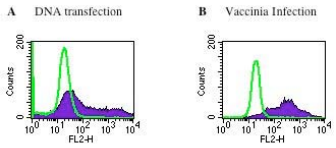


Figure 1. Flow cytometry analysis to characterize human mesothelin expression of cells transfected with DNA or infected with vaccinia. (A) TK- cells transfected with pcDNA3-Hmeso (filled histogram) or pcDNA3 vector (clear histogram). **(B)** TK- cells infected with Vac-Hmeso (filled histogram) or wild-type vaccinia (clear histogram). Trasfected or infected TK- cells were stained with human mesothelin-specific antibodies and characterized by flow cytometry analysis .

Identification of human mesothelin epitopes aa116-125 and aa540-549 as HLA-A2-restricted human mesothelin-specific CTL epitopes

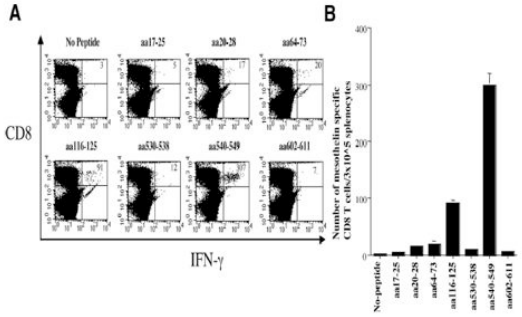
The availability of a human mesothelin-expressing DNA construct and vaccinia virus allows us to design prime and boost vaccination regimens in order to generate high numbers of human mesothelin-specific $CD8^+$ T cells in vaccinated mice. Using splenocytes derived from HLA-A2 transgenic mice vaccinated with pcDNA3-Hmeso and boosted with Vac-Hmeso, we were able to develop quantitative human mesothelin-specific $CD8^+$ T cell immunological assays. In order to identify candidate HLA-A2-restricted human mesothelin-specific CTL epitopes, we used the BioInformatics & Molecular Analysis Section (BIMAS) database for HLA-A2 peptide binding predictions. We identified several candidate 9- or 10-residue peptides for human mesothelin. Their positions, MHC restrictions, sequences and scores are presented in **Table 1**.

Table 1. Candidate HLA-A2 restricted human mesothelin-specific CTL epitopes predicted by BIMAS

Peptide name Start position	MHC class I	Peptide sequence	BIMAS score
Peptide-20	HLA2	SLLFLLFSL	1054
Peptide-17	HLA2	ALGSLLFLL	284
Peptide-64	HLA2	QLLGFPCAEV	257
Peptide-116	HLA2	ALPLDLLLFL	270
Peptide-530	HLA2	VLPLTVAEV	271
Peptide-540	HLA2	KLLGPHVEGL	311
Peptide-602	HLA2	LLGPGPVLTV	271

Bioinformatics & Molecular Analysis Section (BIMAS) was used to analyze various 9-residue peptides from mesothelin for H-2 binding predictions. The sequences, positions, MHC restrictions, and scores (according to the numbers of mesothelin-specific, IFN-γ-expressing CTLs generated by the candidate peptide) of the potential human mesothelin epitopes were determined.

To determine which candidate peptides represent true HLA-A2-restricted human mesothelin-specific CTL epitopes, we used the candidate peptides identified by BIMAS to stimulate splenocytes from vaccinated HLA-A2 transgenic mice. As shown in **Figure 2**, splenocytes from vaccinated mice pulsed with human mesothelin peptides aa116-125 or aa540-549 generated significantly greater numbers of human mesothelin-specific, IFN-γ-expressing CTLs than splenocytes pulsed with the other human mesothelin peptides ($p<0.001$). These data indicate that the human mesothelin peptides aa116-125 and aa540-549 can be recognized by $CD8^+$ T cells generated by HLA-A2 transgenic mice primed with pcDNA3-Hmeso and boosted with Vac-Hmeso. Furthermore, there are no CD8-specific T cell responses to these epitopes when using the same vaccines to vaccinate C57BL/6 mice (data not shown). Thus, these data confirm that aa116-125 and aa540-549 human mesothelin epitopes are HLA-A2-restricted human mesothelin-specific CTL epitopes but not H-2 D^b, K^b-restricted CTL epitopes. Furthermore, between these two identified peptides, aa540-549 generated greater numbers of IFN-γ-expressing CTLs than aa116-125.



Vaccination with pcDNA3-Hmeso540-β2m-A2 enhances the human mesothelin-specific $CD8^+$ T cell responses in vaccinated HLA-A2 transgenic mice

The identification of aa540-549 as a strong HLA-A2-restricted human mesothelin-specific CTL epitope allowed us to generate a DNA vaccine encoding a SCT of HLA-A2 linked to this epitope (pcDNA3-Hmeso540-β2m-A2) (Figure 3**). We also generated a DNA vaccine encoding an SCT of HLA-A2 linked to OVA (pcDNA3-OVA-β2m-A2) as a negative control.**

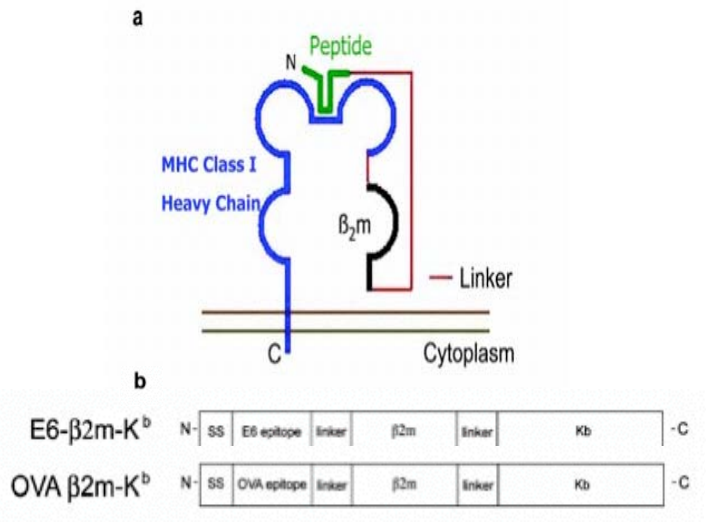


Figure 3. Diagrams depicting structure of our SCT and structures of chimeric DNA constructs. (A) Diagram of a peptide:β2m:MHC SCT on cell surface. **(B)** Diagram of an SCT encoding human mesothelin aa540-549 linked to β2m and an HLA-A2-restricted MHC Class I molecule, and a SCT encoding OVA linked to β2m and an HLA-A2-restricted MHC Class I molecule. Each was cloned into the pcDNA3 vector to make the DNA vaccines used in the study.

To assess the immunogenicity of our DNA vaccine, we vaccinated mice with pcDNA3-Hmeso540-β2m-A2 or pcDNA3-OVA-β2m-A2 and then performed intracellular cytokine staining with flow cytometry analysis to characterize human mesothelin-specific $CD8^+$ T cell precursors using human

mesothelin aa540-549 peptide as a stimulant. HLA-A2 transgenic mice vaccinated with pcDNA3-Hmeso540-β2m-A2 generated a significantly higher frequency of human mesothelin-specific IFN-γ-secreting CD8$^+$ T cell precursors compared to mice immunized with pcDNA3-OVA-β2m-A2 (Figure 4**). These data indicate that vaccination with DNA encoding an SCT of HLA-A2 linked to a human mesothelin-specific CTL epitope is capable of generating strong human mesothelin-specific CD8$^+$ T cell immune responses in HLA-A2 transgenic mice.**

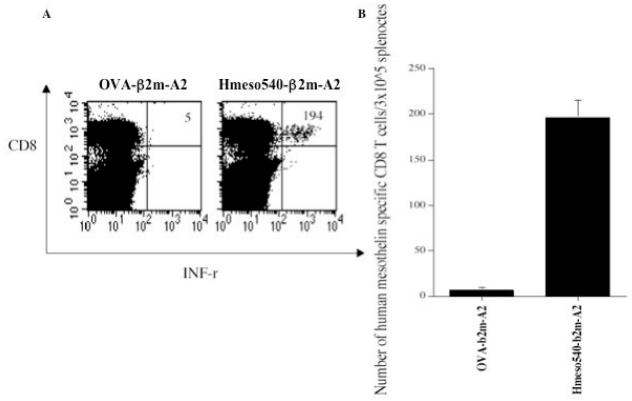


Figure 4. Characterization of human mesothelin-specific CD8$^+$ T cells in vaccinated HLA-A2 transgenic mice. Mice (5 per group) were immunized with pcDNA3-OVA-β2m-A2 or pcDNA3-Hmeso540-β2m-A2. Splenocytes from vaccinated mice were collected and stimulated with human mesothelin-specific peptides (aa540-549). Intracellular cytokine staining followed by flow cytometry analysis was performed. **(A)** Representative figure of the flow cytometry data. **(B)** Bar graph depicting the number of human mesothelin peptide (aa540-549)-specific IFN-γ-secreting CD8$^+$ T cell precursors/3 × 10^5 splenocytes (mean±SD). The data presented in this figure are from one representative experiment of two performed.

A Syngeneic tumor cell line expresses human mesothelin and HLA-A2

In order to generate a tumor cell line expressing human mesothelin and HLA-A2 for use in tumor prevention experiments in HLA-A2 transgenic mice, we have transduced TC-1/A2 cell lines with a retrovirus containing human mesothelin to generate TC-1/A2/Hmeso. We have previously generated an HLA-A2-expressing murine tumor model, TC-1/A2, that is capable of growing consistently in HLA-A2 transgenic mice through subcutaneous injection [8]. We have further characterized the expression of HLA-A2 and human mesothelin in TC-1/A2/Hmeso cells using flow cytometry analyses. TC-1 cells were used as negative controls. As shown in **Figure 5,** TC-1/A2/Hmeso cells (filled histogram) showed significantly higher expression of both HLA-A2 and human mesothelin compared to TC-1 cell lines (clear histogram). These data indicate that the TC-1/A2/Hmeso tumor cell line is successfully engineered to express significant levels of both HLA-A2 and human mesothelin proteins. Furthermore, TC-1/A2/Hmeso was able to successfully grow in HLA-A2 transgenic mice when subcutaneously challenged after DNA vaccination.

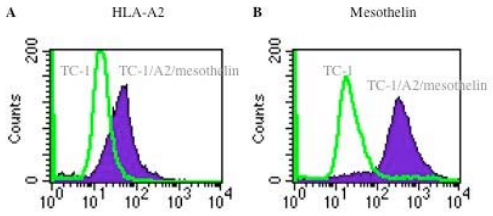


Figure 5. Flow cytometry analysis to demonstrate the expression of HLA-A2 and human mesothelin in TC-1/A2/Hmeso cell line. TC-1/A2/Hmeso was generated by transducing TC-1/A2 with a retrovirus containing human mesothelin as described in the Materials and Methods. **(A)** Characterization of HLA-A2 expression in TC-1/A2/Hmeso (filled histogram) and TC-1 (clear histogram). The cell lines were stained with HLA-A2-specific monoclonal antibody followed by flow cytometry analysis. **(B)** Characterization of human mesothelin expression in TC-1 TC-1/A2/Hmeso (filled histogram) and TC-1 (clear histogram). The cell lines were stained with human mesothelin-specific antibody followed by flow cytometry analysis.

pcDNA3-Hmeso540-β2m-A2 vaccination prevents the growth of human mesothelin-expressing TC-1/A2/Hmeso tumors in mice

Given the immunogenicity of pcDNA3-Hmeso540-β2m-A2 (see **Figure 4**), we next explored whether it could elicit effective protective anti-tumor effects against the HLA-A2 positive, human mesothelin-expressing tumor cell line, TC-1/A2/Hmeso. Tumor cells were injected subcutaneously one week after DNA vaccination. During a 6-week follow-up period, 60% of mice receiving pcDNA3-Hmeso540-β2m-A2 remained tumor free. All of the pcDNA3-OVA-β2m-A2-immunized mice exhibited tumor growth by the second week **(Figure 6)**. This suggests that vaccination with pcDNA3-Hmeso540-β2m-A2 can generate significant protective effects against human mesothelin-expressing tumors. Thus, our data indicate that a DNA vaccine encoding an SCT of a human MHC class I molecule linked to a CTL epitope from human mesothelin antigen is capable of generating a strong antigen-specific $CD8^+$ T cell immune response and antitumor effects in vaccinated HLA-A2 transgenic mice.

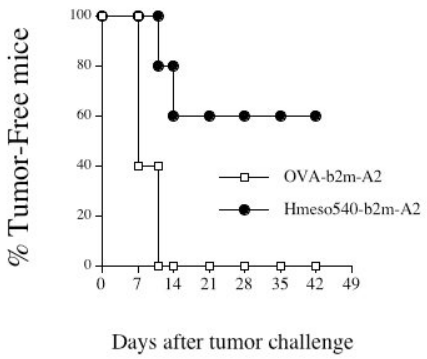


Figure 6. *In vivo* tumor protection experiment to demonstrate the antitumor effect generated by pcDNA3-Hmeso540-β2m-A2 against human mesothelin-expressing TC-1/A2/Hmeso tumors in HLA-A2 transgenic mice. Mice (5 per group) were immunized with OVA-β2m-A2 or Hmeso540-β2m-A2 four times at one week interval. At 1 week after the last vaccination, mice were challenged subcutaneously with $5x10^4$ TC-1/A2/Hmeso cells/mouse and monitored for evidence of tumor growth by palpation and inspection thrice a week.

KEY RESEARCH ACCOMPLISHMENTS:

♦ We have identified human mesothelin epitopes aa116-125 and aa540-549 as HLA-A2-restricted human mesothelin-specific CTL epitopes.

♦ We have shown that vaccination with pcDNA3-Hmeso540-β2m-A2 DNA enhances the human mesothelin-specific $CD8^+$ T cell responses in vaccinated HLA-A2 transgenic mice.

♦ We have generated a syngeneic tumor cell line expresses human mesothelin and HLA-A2.

♦ We have shown that pcDNA3-Hmeso540-β2m-A2 vaccination prevents the growth of human mesothelin-expressing TC-1/A2/Hmeso tumors in HLA-A2 transgenic mice.

REPORTABLE OUTCOMES:

C.-F. Hung, R. Calizo, Y.-C. Tsai, L. He, and T.-C. Wu (2006) A DNA vaccine encoding a single-chain trimer of HLA-A2 linked to human mesothelin peptide generates anti-tumor effects against human mesothelin-expressing tumors. Human Gene Therapy (submitted).

CONCLUSIONS:

The employment of single-chain trimers (SCT) of HLA-A2 linked to the human mesothelin epitope aa540-549 represents a potential opportunity for the clinical translation of DNA vaccines against human mesothelin-expressing tumors, particularly ovarian cancer cells.

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