Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays

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Communicated by Sherman M. Weissman, Yale University School of Medicine, New Haven, CT, September 10, 2007 (received for review March 15, 2007)

Ovarian cancer is a leading cause of deaths, yet many aspects of the biology of the disease and a routine means of its detection are lacking. We have used protein microarrays and autoantibodies from cancer patients to identify proteins that are aberrantly expressed in ovarian tissue. Sera from 30 cancer patients and 30 healthy individuals were used to probe microarrays containing 5,005 human proteins. Ninety-four antigens were identified that exhibited enhanced reactivity from sera in cancer patients relative to control sera. The differential reactivity of four antigens was tested by using immunoblot analysis and tissue microarrays. Lamin A/C, SSRP1, and RALBP1 were found to exhibit increased expression in the cancer tissue relative to controls. The combined signals from multiple antigens proved to be a robust test to identify cancerous ovarian tissue. These antigens were also reactive with tissue from other types of cancer and thus are not specific to ovarian cancer. Overall our studies identified candidate tissue marker proteins for ovarian cancer and demonstrate that protein microarrays provide a powerful approach to identify proteins aberrantly expressed in disease states.

autoantibodies | tissue microarray | tissue marker | differential expression | cancer antigen

Epithelial ovarian cancer is the fifth leading cause of cancer deaths in women in the United States. In 2007, ≈22,400 women will be diagnosed with ovarian cancer and 15,300 women will die from the disease (1). Identifying proteins that reveal differences in the stages of neoplastic differentiation will be informative in understanding the disease. They may be useful for diagnostics and may also suggest useful targets for therapeutic intervention.

Thus far relatively few differentially expressed proteins have been identified, particularly for early disease stages, during which there are few symptoms and early detection is most important. Detection during stage I or II results in a 60–90% 5-year survival; diagnosis at later stages (III and IV) results in a survival rate of <20% (2). Presently, the most common marker for detecting ovarian cancer is CA-125, which corresponds to a group of cell surface glycoproteins of unknown function. Although CA-125 is elevated in the serum of 80% of patients with advanced ovarian cancer, it identifies <10% of patients with early-stage (I and II) disease (3, 4). Thus, identification of other candidate proteins may ultimately prove valuable for early diagnosis and for predicting cancer prognosis and treatment outcomes.

Several strategies have been used to identify components aberrantly expressed in cancer tissue. Serum profiling using mass spectrometry (5, 6) and gene expression profiling (7) have been used to identify differentially expressed proteins and RNAs, respectively. However, proteins identified by mass spectrometry have low reproducibility, and it remains unclear whether preferential expression of genes is reflected at the protein level.

Another strategy for protein discovery is to use serum autoantibodies, in which antibodies produced by the immune response to the cancer are screened for, which may be indicative of disease (8, 9). Many proteins affected before or during tumor formation elicit an immune response (e.g., p53) (10). Probing of fractionated tumor lysates spotted on membranes has been used to search for autoantibodies (11). Although this technique can probe many proteins, the subsequent identification of the reactive proteins is difficult. Another method involves the serological analysis of proteins expressed in *Escherichia coli* from cDNA libraries. Several hundred autoantigens have been discovered by this method, including tumor-associated autoantigens found in ovarian cancer (12, 13); however, it is generally limited to the interrogation of linear epitopes, and the expression of some epitopes in bacteria is difficult.

We have developed a proteomic approach to search for autoantigens using protein microarrays. Protein microarrays have been successfully used to identify autoantigens involved in autoimmune disease (14), but not cancer. Sera from women with various stages of ovarian cancer were used to probe a human protein microarray containing 5,005 proteins. Tumor-associated autoantibodies present more commonly in the diseased population were identified. Four candidate markers for ovarian cancer were further analyzed for their ability to differentiate normal versus cancerous ovarian tissue.

Results

Identification of Autoantibodies Associated with Ovarian Cancer. To identify tumor-associated autoantibodies present in the sera of individuals with ovarian cancer and their reactive antigens, sera from 30 diseased patients and 30 age-matched healthy individuals were incubated with proteome microarrays containing 5,005 human proteins purified from insect cells and spotted in duplicate (Fig. 1*A*). The presence of autoantibodies bound to each protein spot was detected by using a fluorescently labeled secondary antibody; a control microarray was probed with only the fluorescently labeled anti-human IgG antibody. Proteins that displayed signal intensities greater than or equal to 2 standard deviations above background were identified by using ProCAT software (15). Of 5,005 proteins present on the microarray, 1,845 were bound by autoantibodies from cancer patients, whereas 1,441 were bound by autoantibodies from healthy individuals. Of these, 730 reacted solely with sera from cancer patients, whereas 326 reacted solely with sera from healthy patients (Fig. 1*C*).

Identification of Tumor-Associated Autoantigens. Our initial studies did not reveal any antigens that either solely or in combination were recognized by antibodies from either all diseased or all healthy patients, even when the results were subdivided into the different disease stages, I, II, III, and IV. We therefore used three statistical methods to identify antigens that displayed either a greater intensity and/or a greater frequency of positive signals with the sera of either diseased or healthy individuals. The analyses included pairwise *t*

Author contributions: M.E.H. and M.S. designed research; M.E.H. and I.P. performed research; G.M. contributed new reagents/analytic tools; M.E.H., I.P., and K.H. analyzed data; and M.E.H., I.P., and M.S. wrote the paper.

Conflict of interest statement: M.S. consults for Invitrogen Corporation and Affomix Corporation. M.H. currently works for Microbia Precision Engineering.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0708572104/DC1.

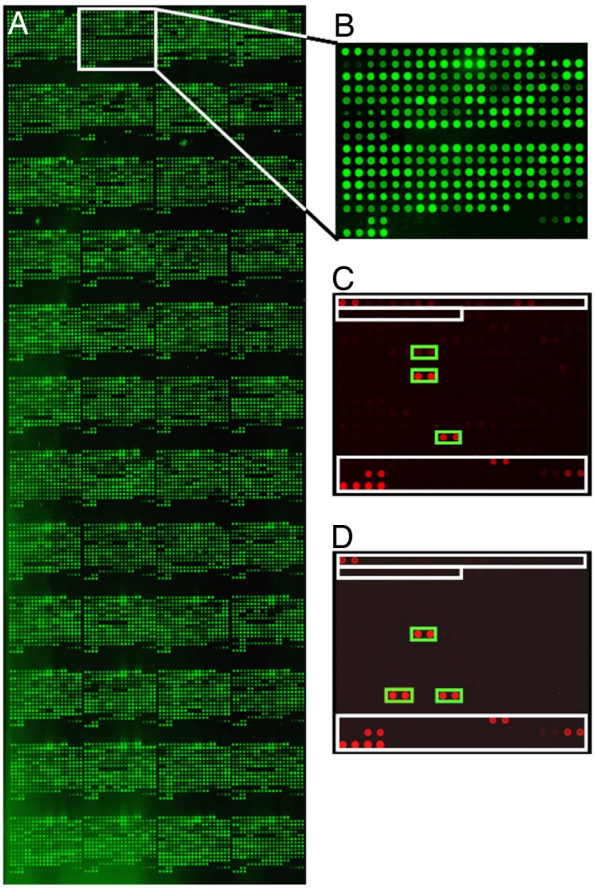

Fig. 1. Identification of tumor-associated autoantibodies and targeted protein antigens. (*A*) A protein microarray comprising 5,005 GST fusion proteins probed with anti-GST antibody. (*B* and *C*) Proteins visualized with anti-GST antibody (*B*) or with serum from ovarian cancer patients and healthy women (*C*). White boxes, positive and negative controls; green boxes, reactive proteins.

testing, ReliefF (a machine learning method of classifier identification), and the Proteome Prospector informatic program. Forty-five autoantigens present in at least two of the three lists were grouped into a list of tumor-associated autoantigens. In addition, the top 45 autoantigens that were in only one list were also included. In total 90 protein antigens targeted by tumor-associated autoantibodies were identified, and two antigens appeared more often in the samples from non-disease individuals. A subset of these proteins is listed in Table 1, and the full listing of antigens, including their source of identification, is present in supporting information (SI) Table 2.

Initial Validation Using Immunoblot Analyses. Because a number of antigens exhibited stronger serum antibody reactivity with the disease patients relative to healthy individuals we reasoned that these antigens might be overrepresented in the tissues of cancer patients. We therefore selected four antigens, lamin A/C, structure-specific recognition protein 1 (SSRP1), Ral binding protein 1 (RALBP1), and ZNF265 from an initial group of ≈10 proteins that exhibited the strongest reactivity in the cancer patients relative to the control patients for further study. The lamin A/C proteins are products of two alternative spliced RNAs from the LMNA gene and are structural components of the nuclear envelope (16). Their association with ovarian cancer was suggested by pairwise *t* tests. SSRP1 has been implicated in the mechanism of cell death caused by cisplatin (17), and RALBP1 is involved in xenobiotic transport, including the transport of antitumor drugs such as doxorubicin (18, 19). SSRP1 and RALBP1 were identified as potential tumor-associated autoantigens by both pairwise *t* testing and Proteome Prospector. SSRP1 was also identified by the ReliefF analysis. Finally, ZNF265 is a component of the mRNA splicing machinery and is potentially involved in the regulation of alternative splicing (20). ZNF265 was identified as a potential tumor-associated antigen by Proteome Prospector. Antibodies to each of these proteins were obtained from commercial sources and used to examine protein expression in cell lysates by using immunoblot analysis and in tissue by using immunohistochemistry staining.

Protein lysate arrays containing tissue extracts from ovarian and other cancer tissues along with matched controls were probed in dot blot assays. Each lysate contained samples from two or more individuals that were pooled before spotting on the array. Antibodies to lamin A/C and SSRP1 exhibited a stronger signal in the samples from ovarian cancer patients relative to matched controls of healthy individuals. For SSRP1, staining was also evident in the other cancerous tissues samples including breast, brain, and liver tissues, and it was more evident in healthy tissues of the colon and esophagus. For lamins, staining was elevated only in the ovarian samples and not in other samples. RALBP1 and ZNF265 signals were not evident in the dot blot assays, indicating that they are low-abundance proteins or do not react well with their target proteins in these assays (data not shown).

To further confirm the dot blot assay, protein samples of cancer and normal cells were also analyzed by immunoblot analysis using antibodies specific for lamin A/C and SSRP1. The lamin A/C proteins, which migrate as two bands of 70 and 60 kDa, are greatly elevated in the cancer samples relative to controls (Fig. 2*B*). SSRP1 was also elevated in the cancer sample relative to healthy controls (data not shown). As a control the same samples were probed with p53; this protein was reduced in the cancer tissue relative to normal

Table 1. Select list of differentially expressed proteins as identified through protein microarray analysis of serum autoantibodies

Accession no.	Protein	GO process	Function
NM_005572	Lamin A	Nuclear membrane organization	Part of nuclear lamina
NM_003146	SSRP1	Regulation of transcription	Involved in response to drug therapy
NM_006788	RALBP1	Transport	May contribute to multidrug resistance
NM_006147	IRF6	Regulation of transcription	Transcription factor
BC032852	MAGEB4	Unknown	Cancer marker
NM_004645	COIL	Unknown	Found in nuclear coiled bodies
NM_014062	NOB1P	Unknown	Related to adenocarcinoma antigen
NM_054016	FUSIP1	RNA splicing	Repressor of mRNA splicing
BC032851	CBLB	Signal transduction	Homolog to the oncogene v-cbl
NM_133480	TADA3L	Regulation of cell cycle	Coactivator for p53 transcription

GO, Gene Ontology.

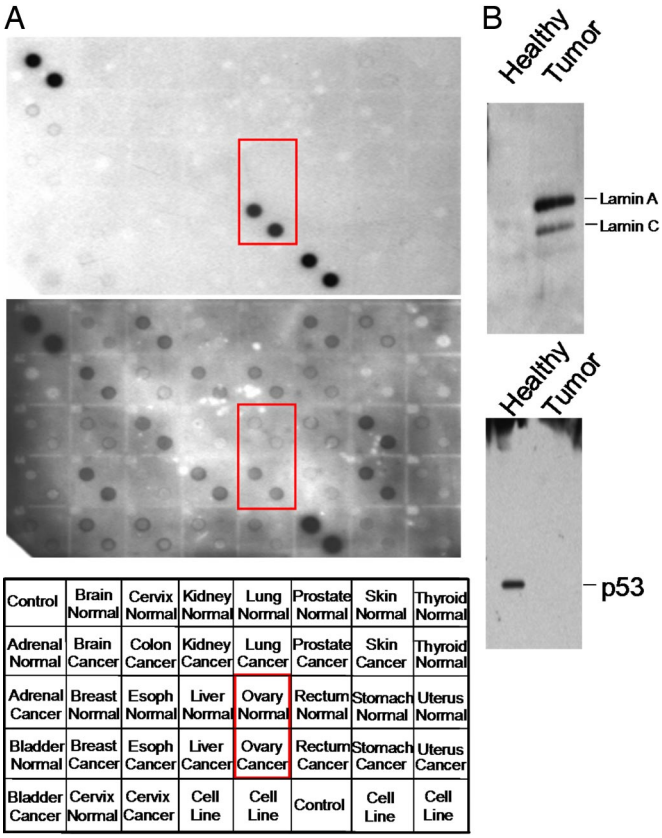

Fig. 2. Immunoblot analysis of candidate tumor markers. (*A*) Filters spotted with protein lysates were probed with anti-lamin A/C (*Top*) and SSRPI (*Middle*) antibodies. The tissue origin of each sample is shown in the key (*Bottom*). (*B*) Western blot analysis of healthy and diseased ovarian tissue using antibodies specific for proteins lamin A/C (*Upper*) and p53 (*Lower*).

cells (Fig. 2*B*). Thus, lamin A/C and SSRP1 are elevated in cancer tissue relative to healthy tissue.

Differences in Protein Patterns in Cancer Tissue Using Tissue Microarrays. We next examined the expression of protein antigens in healthy and ovarian cancer tissues using tissue microarrays. Tissue microarrays allowed us to investigate the *in situ* tissue expression of the protein of a large number of samples from different cancer patients. Lamin A/C, SSRP1, RALBP1, ZNF265, and CA-125 antibodies were used to stain 30 samples from ovarian cancer patients (median age 49.5 years, range 22–68 years) and 30 control samples from unmatched healthy individuals (median age 41.5 years, range 17–69 years). For each antibody samples from the same patients were used thereby allowing a direct comparison of results for each protein. Enhanced staining was observed in most cancer patients relative to those of control patients for lamin A/C, SSRP1, and RALBP1 (SI Fig. 7). Increased staining of tissue above background levels was not detected for ZNF265 in cancer patients (data not shown). Lamin A/C and SSRP1 exhibited the greatest differential staining between cancer and control patients; RALBP1 also showed differential staining, but less than that of lamin A/C and SSRP1.

We also compared the staining of lamin A/C, SSRP1, RALBP1, and ZNF265 in 10 additional patients in which ovarian cancer tissue and noncancerous material were isolated from the same individual. Positive immunoreactivity of lamin A/C, SSRP1, and RALBP1 is evident in the cancer cells relative to the non-cancer tissue isolated from the same patient (Fig. 3). In each case, one exception was observed in which a sample labeled as noncancerous exhibited strong staining for each protein; the staining pattern suggested that cancerous tissue had infiltrated the normal tissue for that particular sample. ZNF265 did not display staining above background in cancer cells (data not shown). Thus, these tissue staining results demonstrate that increased lamin A/C, SSRP1, and RALBP1 staining is observed in ovarian cancer tissue relative to non-cancer material. Because of the ineffectiveness of ZNF265 staining we discontinued the analysis of this antigen.

One concern with immunostaining is that we might detect proteins with common epitopes rather than the protein of interest. For lamin A/C, additional antibodies to a different part of the protein (the N terminus versus the C terminus for the initial antibody) were available. The N-terminal antibody was used to stain the tissues from the 40 cancer tissues and 40 normal patients, including the 10 samples from matched diseased–healthy tissues from the same patients. The second lamin A/C antibody showed a staining pattern very similar to that shown for the first antibody (data not shown). Thus, these experiments with two different antibodies indicate that lamin A/C and not a cross-reactive protein is elevated in cancer tissue relative to normal tissue.

Our immunostaining results of lamin A/C, SSRP1, and RALBP1

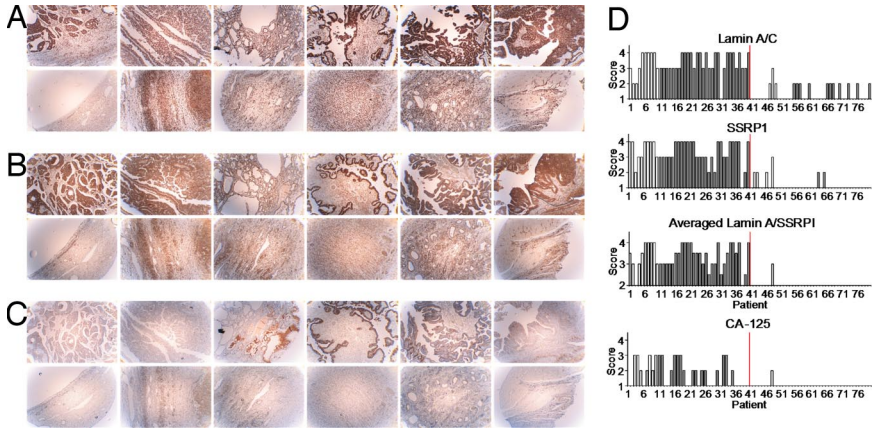

Fig. 3. Identification of differentially expressed proteins in ovarian cancer. (*A–C*) Representative examples of staining of tissue microarrays containing tumor tissue and healthy tissue taken 1.5 cm apart were probed with antibodies specific for lamin A/C (*A*), SSRP1 (*B*), or CA-125 (*C*). In each staining, diseased tissue (upper row in pair) is compared with healthy tissue (lower row in pair). A full set of tissue staining images can be found in SI Figs. 7–10. (*D*) Staining scores for patients from both matched and unmatched tissue microarray cores. Open bars represent scoring for matched samples, and filled bars represent unmatched samples. Cancerous tissue samples are left of the red medial vertical line, and healthy tissue samples are on the right.

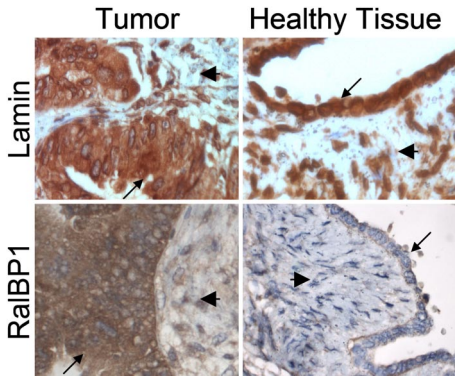

Fig. 4. Staining for lamin A/C and RALBP1 in ovarian tissue sections. (Magnification: ×40.) Note intense staining of epithelial cells (long arrows) for RALBP1 and low staining of stromal cells (short arrows). Lamin A/C is found in epithelial as well as in stromal cells.

were compared with that of the ovarian cancer serum marker CA-125 using tissue sections from the same patients. CA-125 staining is weaker in samples from both cancer patients and normal individuals; however, the differential staining pattern appeared dramatically weaker than that of lamin A/C and SSRP1 and slightly weaker than that of RALBP1. Thus, lamin A/C and SSRP1 appear to be qualitatively stronger markers for the cancer tissue.

Integration of Multiple Staining Results Provides Maximum Sensitivity and Specificity. To quantify our tissue staining results we used a manual scoring system in which two individuals blindly scored a randomized mixture of images from the 80 matched and unmatched cancer and normal tissue samples. A scale of 1–4 was used for each sample with 1 equal to low-level staining similar to that observed with that of most normal tissue samples and 2–4 signifying increasing levels of staining signal. The results from each scorer were averaged and are summarized in Fig. 3*D*. Upon un-blinding of the scores, high scores were observed for lamin A/C and SSRP1 in the cancer samples relative to the normal tissues. Weaker scores were observed for RALBP1 (data not shown) and CA-125. The sensitivity of staining for lamin A/C, SSRP1, RALBP1, and CA-125 was found to be 97.5%, 97.5%, 85.0%, and 55.0%, respectively; the specificity was 65.0%, 85.0%, 87.5%, and 97.5%, respectively. From these results, no one antigen alone was found to be capable of identifying the disease with both high sensitivity and specificity.

We also examined the effects of combining the results from the different makers (SI Table 3 and Fig. 3*D*). When the results of lamin A/C and SSRP1 are averaged maximum accuracy is achieved; 38 of 40 cancer patients were correctly scored, and one healthy tissue was scored as cancerous. This latter sample was the one isolated as healthy tissue from a cancer patient and mostly likely was cancerous material. Thus, our sensitivity is 95%, and specificity is 97.5%. Inclusion of the results from RALBP1 or CA-125 reduced the sensitivity and specificity. Although these studies have been performed on only one set of samples because of the limited number of samples available, robust tissue diagnosis is readily achieved by using a combination of anti-lamin A/C and SSRP1 antibodies; this diagnosis is superior to that for the CA-125 antigen in tissue sections.

Expression of Autoantigens in Epithelial and Stromal Cells. In addition to analyzing tissue sections at a gross morphological level, the different antigens were examined in detail in epithelial and stromal cells. As shown in Fig. 4 each of the lamin A/C and RALBP antigens are abundantly expressed ovarian cancer epithelial cells, and there appears to be many more of these cells relative to healthy tissue; for lamin A/C the protein is present in the nuclear periphery. For lamin

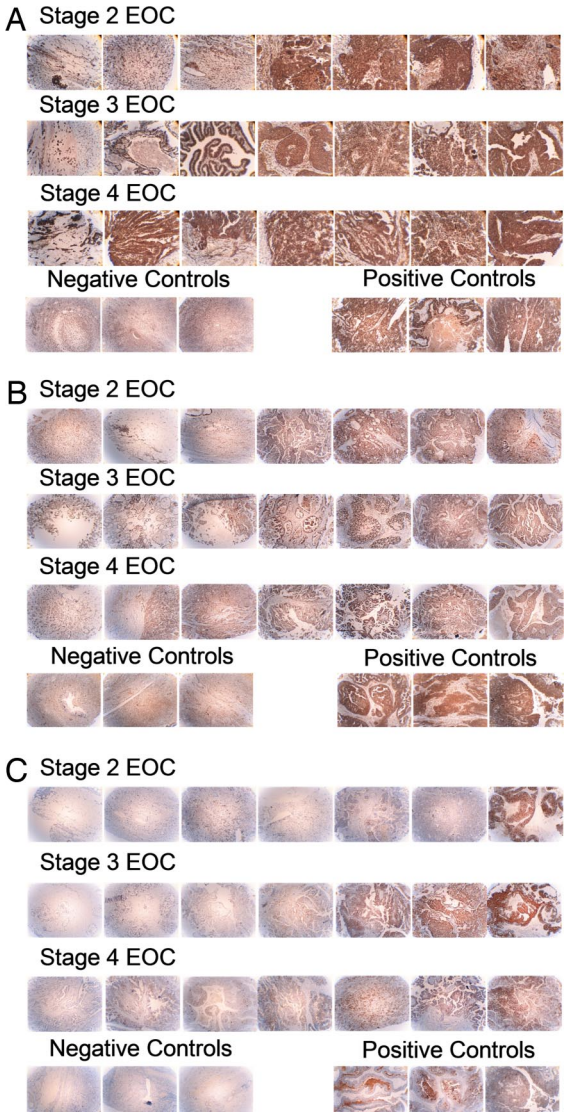

Fig. 5. Tissue microarray analysis of different-stage ovarian tumor tissue. Microarrays containing representative tissue from stage II, stage III, and stage IV tumors were probed for lamin A/C (*A*), SSRP1 (*B*), and CA-125 (*C*). Staining of positive and negative control samples was performed in a separate parallel experiment. Representative images are shown. Total tissue samples for each stage: stage II, 7; stage III, 24; stage IV, 13. Complete sets of tissue staining images can be found in SI Figs. 7–10.

A/C expression is also evident in normal epithelial cells. Similar results were obtained for SSRP1. For RALBP the expression in normal cells was heterogeneous: in two samples epithelial cells stained whereas in another they did not (Fig. 4). Thus, RALBP is specific in some tissues.

In addition to their presence in epithelial cells, the lamin A/C and SSRP1 antigens were also detected in stromal cells of both cancer and normal tissue. RALBP was readily detected in all cancer samples but only a subset of normal tissues. Thus, the lamin A/C, SSRP1, and RALBP staining cells are more prevalent in ovarian cancer tissues; for RALBP protein is present only in some healthy tissues.

Many Early-Stage Samples Are Detected by Using Anti-Lamin A/C and SSRP1 Antibodies. In addition to staining matched samples we also examined lamin A/C and SSRP1 distribution in a different set of samples that were typed according to stage (Fig. 5). Robust signals

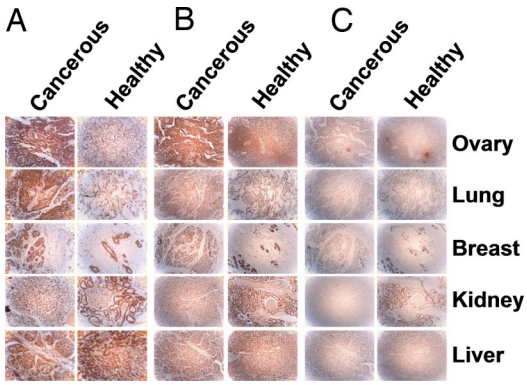

Fig. 6. Candidate protein antigen distribution in other healthy and diseased tissues, probing for lamin A/C (*A*), SSRP1 (*B*), and RALBP1 (*C*). Three samples were analyzed for each tissue and disease state. Representative images are shown. Full sets of images are in SI Figs. 7–10.

were observed on all stage III and IV samples with each antibody. In addition, four of seven stage II samples reacted strongly with the anti-lamin A/C and anti-SSRP1 antibodies, indicating that many early-stage disease tissues can be detected. Similar sections from the sample individuals stained with anti-CA-125 antibodies exhibited reduced signals in many stage III and IV patients and fewer (one of seven) stage II patients. Thus, both lamin A/C and SSRP1 staining outperform that of CA-125 for detecting ovarian cancer tissues, including early-stage samples.

Elevated Staining of Antigens in Other Cancer Types. We also examined lamin A/C, SSRP1, and RALBP1 distribution in other types of cancer and normal tissues, including those of kidney, liver, breast, esophagus, and uterus. Cancer and normal tissues isolated from matched individuals were stained with anti-lamin A/C, SSRP1, RALBP1, and CA-125 antibodies. Increased staining of lamin A/C, SSRP1, and RALBP1 was evident in cancer tissues of the ovaries, breast, and lungs relative to healthy tissue (Fig. 6). Interestingly, strong staining was observed in noncancerous kidney tissue relative to cancer tissues. Strong staining was observed in both cancer and normal liver tissues. These different results indicate that these antigens are prevalent not only in ovarian cancer tissues but also in tissues of many types of cancers and a subset of healthy tissues.

Discussion

In this study we used sera and protein microarrays to identify proteins aberrantly expressed in ovarian cancer. From a list of candidate tumor autoantigens we identified three with enhanced expression in ovarian cancer tissue relative to healthy tissue. Only a subset of patients produced antibodies to these proteins, and the autoantibody reaction in these patients exhibited a poor correlation with disease even when cancer stages were taken into consideration. A lack of tight correlation between cancer state and autoantibodies to specific antigens may reflect heterogeneity in the disease state, the timing of the disease, or most likely the immune response to the antigens in different individuals. Nevertheless, the protein microarray screening was able to identify candidate proteins that, when examined, further identified three antigens that exhibited strong signatures in ovarian cancer tissue relative to normal tissue.

Other groups have performed autoantibody screens to identify ovarian cancer antigens. Nelson and colleagues (21) screened cDNA libraries expressed in *E. coli* with sera from 50 cancer patients and 20 healthy individuals and found several reactive antigens (21). Tainsky and colleagues (22) used serum from one patient to identify several candidate proteins; their method showed moderate sensitivity and specificity (55% and 98%, respectively). The use of only one serum in the initial screen may have limited the diversity of the resulting antigens. The proteins identified by using our approach were distinct from those of the other studies. We presume that unbiased screening of a large number of proteins using protein microarrays increases the frequency of finding a large number of candidate antigens, including low-abundance proteins.

RNA expression studies to identify differentially expressed protein have been performed by other groups and have identified many candidates, including lamin A/C, which was overexpressed in our study. However, many of our markers, including SSRP1, did not display increased RNA expression in ovarian or other cancers. Thus, for many genes RNA and protein levels do not correlate, and approaches that examine protein levels are required to identify aberrantly expressed antigens.

The nuclear envelope and lamin proteins have been implicated in a variety of cancers and diseases. The nuclear envelope has been reported to exhibit a distinct morphology in cancer cells relative to normal tissues (23). RNA expression of the lamin A/C gene is reduced in hematological malignancies, including lymphomas (24), and the protein has been previously reported to be at lower levels in malignant ovarian cancer cells than in benign tumor cells (25). We find that these proteins are elevated in ovarian cancer tissue relative to normal tissue. This difference likely reflects differences in tissue types investigated in these studies, the type of diseases, and/or differences between mRNA and protein levels. Regardless, the role of lamins in human disease is becoming increasingly evident (26, 27).

Many of the antigens that we detected were not specifically related to cancer but are prevalent in normal cells. We presume that these represent an aspect of the disease state that is not evident in normal tissues. Immunofluorescent subcellular localization of the lamin A/C proteins in ovarian cancer tissue reveals that these proteins are in their normal distribution around the nuclear periphery (data not shown). We hypothesize that lamins and many other proteins identified in our screen might be overexpressed or that there is an increase in a specific cell type in cancer tissues. It is believed that many ovarian cancers form through growth of epithelial cells that invaginate or remain inside the ovary. Many of our markers are highly expressed in these rapidly proliferating cells, as well as in those of other cancers (e.g., breast and lung) and in several normal tissues (e.g., kidney). However, not all epithelial cells of normal and cancer tissues readily stain with anti-lamin A/C, SSRP1, and RALBP1 proteins. The staining cells may represent either a specialized type of epithelial cells or precursor stem cells that are overproliferating in the tissues. Further characterization of the antigens is likely to provide clues as to the nature of the cancerous cells.

Two differentially expressed proteins, SSRP1 and RALBP1, have been implicated in drug resistance or the mechanism by which the drug exerts its effects. RALBP1 was found to be overexpressed in cancer cell lines resistant to the chemotherapeutic agent gemcitabine (28). In our study, the serum samples were drawn before any application of chemotherapy. Perhaps SSRP1- and/or RALBP1-expressing tissue is poised to confer drug resistance and/or the presence of serum antibodies correlates with the development of resistance.

Our results revealed three of four candidate tissue markers that when immunostained produced a robust signature of cancer in tissue sections. As such they are useful for biopsies, but not routine screening, which requires analysis of fluids. However, at least some material may be present in the blood, and thus they may be useful candidates for a serum test. Regardless of whether this is the case, these markers should be useful for tissue diagnostics and further characterization of the disease state. They may also be useful targets for therapeutic intervention.

Materials and Methods

Patient Sample Information. Details of the serum collection have been published (6). Samples from 30 individuals with ovarian

cancer and 30 healthy patients were analyzed. For the diseased population, the median age was 70.5 years, with the oldest and youngest patients at 99 and 40 years, respectively. On a per-stage basis, patients with stage I disease ($n = 3$) had a median age of 61 years; stage II ($n = 11$), 66 years; stage III ($n = 12$), 72 years; and stage IV ($n = 4$), 81 years. The healthy population comprised age-matched subjects. None of the patients with ovarian cancer had undergone any treatment for the disease at the time the samples were taken.

Detection of Tumor-Associated Autoantibodies. Serum samples were diluted 1:150 in Tris-buffered saline solution with 0.1% Tween 20 detergent (TBS-T). A total of 300 μl of the diluted serum sample was overlaid onto a ProtoArray human protein microarray, V3.0 (Invitrogen, Carlsbad, CA). A Lifterslip (Erie Microarray, Portsmouth, NH) was positioned over the array surface. Primary serum incubations were performed at 4°C for 2 h. The arrays were washed with TBS-T, and bound serum antibodies were detected by incubation with Alexa Fluor 594 goat anti-human IgG (H+L) (Invitrogen) diluted 1:2,000 in TBS-T at 4°C for 1 h. Arrays were washed with TBS-T and dried at room temperature, and slide fluorescence was measured with GenePix Pro 6.0 Software (Molecular Devices, Sunnyvale, CA).

Statistical Analysis. Three approaches were used to identify antigens showing significant difference between the healthy and cancerous samples: (*i*) paired *t* tests. (*ii*) ReliefF (a supervised method was used). ReliefF ranks the features according to their importance for the classification task, thereby providing a quality estimate of each feature according to how well feature values discriminate between instances close to each other. This process is then repeated several times. (*iii*) The array manufacturer's software. This analysis relies on a statistical test based on Chebyshev's inequality principle, which determines whether a fluorescent signal on the microarray is significant from built-in negative controls.

To compile a list of ovarian cancer-specific antigens, proteins that appeared in at least two of the three lists were compiled into a master list (SI Table 2). In addition, those markers that appear on only one list, but with a high degree of statistical confidence, were selected. Markers selected for further study were chosen based on their rank order and the availability of commercial antibodies.

Dot Blot and Western Analysis. Dot blot analysis of antigen prevalence in healthy and cancerous tissues was performed by using DiscoverLight Human Tissue Arrays (Pierce, Rockford, IL). Western analysis was performed by using 5 μg of total protein from the ovarian Human Tissue Lysate Set (Pierce) and standard techniques.

Immunohistochemistry. Immunohistochemical detection of antigen content in healthy and cancerous tissues was performed by using tissue microarrays (US Biomax, Rockville, MD). Deparaffinization and antigen retrieval were accomplished by using Trilogy solution (Cell Marque, Rocklin, CA) and heating/pressure supplied by a conventional pressure cooker. Endogenous peroxidase activity was inhibited by using 0.3% hydrogen peroxide. Nonspecific interactions were blocked by using normal horse serum. Primary antibodies were diluted in TBS-T and incubated for 1 h. Supplier and other antibody information can be found in SI Table 4. Bound antibodies were detected by using biotin-linked anti-mouse/rabbit secondary antibody and streptavidin-conjugated HRP enzyme in conjunction with DAB chromagen. Tissue was counterstained with hematoxylin.

We thank L. Kung and J. Mok for critical review of the manuscript, X. Zhu and A. Sboner for assistance with statistical analysis, and D. Rimm for advice and help with photographs. This research was supported by grants from the National Institutes of Health.

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