Use of a Combination of Approaches to Identify and Validate Relevant Tumor-Associated Antigens and Their Corresponding Autoantibodies in Ovarian Cancer Patients

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Use of a Combination of Approaches to Identify and Validate Relevant Tumor-Associated Antigens and Their Corresponding Autoantibodies in Ovarian Cancer Patients

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Abstract

Purpose: Novel biomarkers are urgently needed to increase the sensitivity of CA125 for the early detection of ovarian cancer. Indeed, it has been shown that as much as 20% of early-stage patients do not express significant levels of this biomarker. Therefore, the possibility of using autoantibodies directed against tumor-associated antigens as putative cancer markers is being more examined. Indeed, many autoantibodies have recently been shown to correlate with cancer patient prognosis or to be suitable for detection of the disease.

Experimental Design: In this study, we have used a new approach involving the use of proteomics, immunology, and ELISA methods to identify relevant autoantibodies in the plasma of ovarian cancer patients. To do so, we developed an innovative technique called two-dimensional differential gel electrophoresis analysis of immunoprecipitated tumor antigens.

Results: This strategy allowed us to successfully identify novel circulating autoantibodies directed against the S100A7 protein in the plasma of ovarian cancer patients. Further real-time reverse transcription-PCR and immunohistochemical studies confirmed that the S100A7 mRNA and protein were highly expressed in ovarian tumors but absent in normal and benign tissues. Moreover, a preliminary study involving 138 patients confirmed that the plasma levels of anti-S100A7 antibodies are significantly elevated in early- and late-stage ovarian cancer patients compared with healthy controls and with patients with benign gynecologic diseases.

Conclusions: This shows that our approach is a valuable tool to successfully identify autoantibodies and tumor-associated antigens in cancer patients and that future research assessing their putative clinical usefulness would be worthwhile.

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Ovarian cancer is the leading cause of gynecologic cancer death worldwide. In the United States, it is the eighth most common cancer among women and the fifth most common cause of cancer death, with about 20,180 new cases and 15,310 deaths expected in 2006 (1). However, the relatively asymptomatic nature of early-stage disease and the lack of adequate screening tests have resulted in the majority of cases presenting with a late-stage disease (2). Currently, clinicians rely on a combination of serum CA125 levels and imaging to diagnose ovarian cancer in suspected patients and monitor treatment response. However, due to its low sensitivity, the currently used test to quantify the CA125 serum marker is not adequate for early detection (3). Indeed, up to 20% of ovarian cancers fail to express significant levels of the maker (4). Thus, there is an urgent need for new ovarian cancer biomarkers that could improve the sensitivity of ovarian cancer early detection.

The hunt for cancer biomarkers in the serum/plasma of patients with cancer is based on the observation that some low-molecular weight proteins that reflect the pathologic state of the patients are shed into the bloodstream (5). Therefore, because our immune system constantly surveys the body for "nonself" antigens, and generates a response against them, cancer patients often mount a humoral response to autologous tumor-associated antigens (TAAs; refs. 5, 6). Consequently, autoantibodies have recently emerged as promising biomarkers for the

detection of cancer (7). Moreover, autoantibodies directed against certain TAAs have been reported in patients with a variety of cancers, such as breast, head and neck, colon, kidney, and melanoma, but not in healthy controls (8–10). In ovarian cancer, we and others have previously reported that antibodies directed against relevant antigens, such as EpCAM, and certain heat shock proteins have a prognostic significance (11–14). Additionally, the detection of serum antibodies against the proapoptotic protein p53 was shown to predict subsequent development of lung cancer, mesotheliomas, and lymphomas (15). This suggests that circulating autoantibody levels could be useful biomarkers for cancer (16). Thus, the detection of autologous antibodies against relevant TAAs could be a powerful tool for ovarian cancer detection and/or monitoring.

Here, we report the use of a newly developed two-dimensional differential gel electrophoresis analysis of immunoprecipitated tumor antigens (2D-DITA) technique to identify relevant TAAs and their corresponding autoantibodies in patients with ovarian cancer. We successfully identified significantly elevated levels of S100A7 in ovarian cancer tissue and anti-S100A7 antibodies in the plasma of ovarian cancer patients compared with healthy controls.

Materials and Methods

Two-dimensional differential gel electrophoresis analysis of immunoprecipitated tumor antigens. Malignant ovarian tissues weighing ~800 mg were obtained from the three patients. Tissues were frozen in liquid nitrogen and grounded to a fine powder using pestle and mortar. A total of 5 μL of lysis buffer containing 1× TBS (pH 7.4), 1% Triton X-100, and protease inhibitors (Sigma-Aldrich) were added and incubated for 60 min at 4°C. The insoluble fraction was removed by centrifuging 10 min at 4,500 × *g*. The protein concentration was then determined with the bicinchoninic acid assay reagent kit (Pierce Biotechnology) according to the manufacturer's instructions.

Plasma IgGs were purified from 1.0 mL of preoperative and postoperative plasma samples obtained from the same patient using the Montage antibody purification kit and spin columns (Millipore) according to the manufacturer's instructions. The protein concentration was determined with the bicinchoninic acid protein assay reagent kit as mentioned previously. Then, 4 mg from purified IgGs (preoperative and postoperative) were desalted and concentrated to 50 μL using a Vivaspin 15 (VivaScience). A buffer exchange was done readjusting the final volume to 800 μL with 1× PBS (pH 7.4). Purified IgGs (4 mg) were incubated overnight at 4°C with 500 μL of Protein G Sepharose 4 Fast Flow (GE Healthcare). Then, the beads were washed once with 1× PBS and twice with 0.2 mol/L triethanolamine (pH 8.2; Fluka Biochemicals). Cross-linking was achieved by incubating the protein G–immobilized antibody beads with 0.2 mol/L triethanolamine/50 mmol/L dimethyl pimelimidate dihydrochloride (Sigma-Aldrich) for 45 min. Beads were then washed once with 100 mmol/L ethanolamine (pH 8.2) for 25 min followed by a 10-min wash in 1× TBS. Finally, the beads were washed once with 1× TBS containing 1% Triton X-100 before incubation with the tissue lysate prepared from the tumor tissue obtained from the same patient.

A total of 27 mg of protein lysate were added to the protein G–immobilized IgG beads prepared from the preoperative and postoperative serum samples. Both suspensions were incubated overnight at 4°C on a rocking platform. The suspensions were then washed twice with 1× TBS and once with 50 mmol/L Tris (pH 7.4). The immunoprecipitated proteins were recovered by eluting with 500 μL of two-dimensional sample buffer (Bio-Rad Laboratories) for 10 min at room temperature. Insoluble proteins were removed by a 5-min centrifugation at 7,000 × *g*. The supernatants were then concentrated to 100 μL as described previously. A further cleanup was done with the Ready Prep 2-D Cleanup kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The final pellet was resuspended in 30 μL of two-dimensional sample buffer, and the pH of the supernatants was adjusted to 8.5. CyDye3 and CyDye5 DIGE fluors (GE Healthcare) were reconstituted to 1 mmol/L stock solutions with *N,N*-dimethylformamide. Working dilutions for the fluors were prepared using 1.1 μL of stock solution and 2.4 μL of *N,N*-dimethylformamide. Cy3 and Cy5 working solutions (1.6 μL) were added to the immunoprecipitated proteins from the preoperative and postoperative samples, respectively. The labeling reactions were carried out at 4°C for 30 min in the dark. The reactions were terminated by the addition of 1 μL of 10 mmol/L lysine for 10 min. Both Cy3- and Cy5-labeled samples were combined, and the final volume was readjusted to 185 μL with two-dimensional sample buffer.

Two-dimensional gel electrophoresis on the labeled samples was done as described (17). The gels (one per patient) were scanned on a Molecular Image FX. The DeCyder software (GE Healthcare) was used to match the spots between all gels, to quantify the intensity of each spot, and to identify the ones that were significantly differentially expressed of >3-fold between preoperative and postoperative samples. Two of spots that showed highest differential signals were sent to the Harvard University/Dana-Farber Cancer Center Proteomics Core (Boston, MA) for protein identification by tandem mass spectrometry.

Cell lines and culture conditions. All cell lines and cultures were maintained at 37°C in a humidified 5% CO_2 incubator. They were grown in a 1:1 ratio of Medium 199 and MCDB 105 (both from Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin (5,000 IU/mL)/streptomycin (5,000 μg/mL; Invitrogen). The eight normal human ovarian surface epithelial cell (HOSE) cultures used in this study were established by scraping the surface of the ovary as described previously (18).

Tissue and serum samples. All tissues used in this immunohistochemical study were collected from patients treated at the Brigham and Women's Hospital between 1992 and 2000 under protocols approved by the Brigham and Women's Human Subject Committee. All tissues were collected from the primary ovarian sites from patients undergoing surgery.

Preoperative plasma samples were collected from women with ovarian cancer and benign gynecologic malignancies and from age-matched healthy controls under the Institutional Review Board approval of the parent institutions. All specimens were frozen at -80°C until needed.

Laser-based microdissection. Tissues stored in Tissue-Tek OCT medium at -80°C were sectioned at 7 μm in a cryostat (Leica). Sections were laser microdissected with the DMLMD laser-dissecting microscope (Leica). Morphologically normal ovarian epithelial cells and malignant cells were isolated.

Real-time quantitative reverse transcription-PCR. Total RNA extraction (RNeasy Mini kit, Qiagen) and cDNA synthesis (High Capacity cDNA Archive kit, Applied Biosystems) were done as described by the manufacturers. mRNA was extracted from 8 HOSE cell lines and from 37 microdissected high-grade papillary serous ovarian tumors. Real-time reverse transcription-PCR was done twice in triplicate using Taqman primer sets for S100A7 and for the housekeeping gene *cyclophilin* (both from Applied Biosystems). All the reactions were run in an ABI 7300 (Applied Biosystems). The fold change was calculated with the ΔΔCt method as previously described (19).

Immunohistochemistry. Immunostaining for S100A7 was done on 53 paraffin-embedded tissue specimens (28 papillary serous, 5 mucinous, 6 clear cell, and 14 endometrioid) using a monoclonal antibody directed against S100A7 (1:50; Abcam) and the DakoCytomation Envision kit (Dako) according to the manufacturer's protocol. The tissue sections were scored according to the following: 0 = no evidence of staining, 1 = light staining in cells, 2 = moderate staining in cells, and 3 = strong staining in most cells.

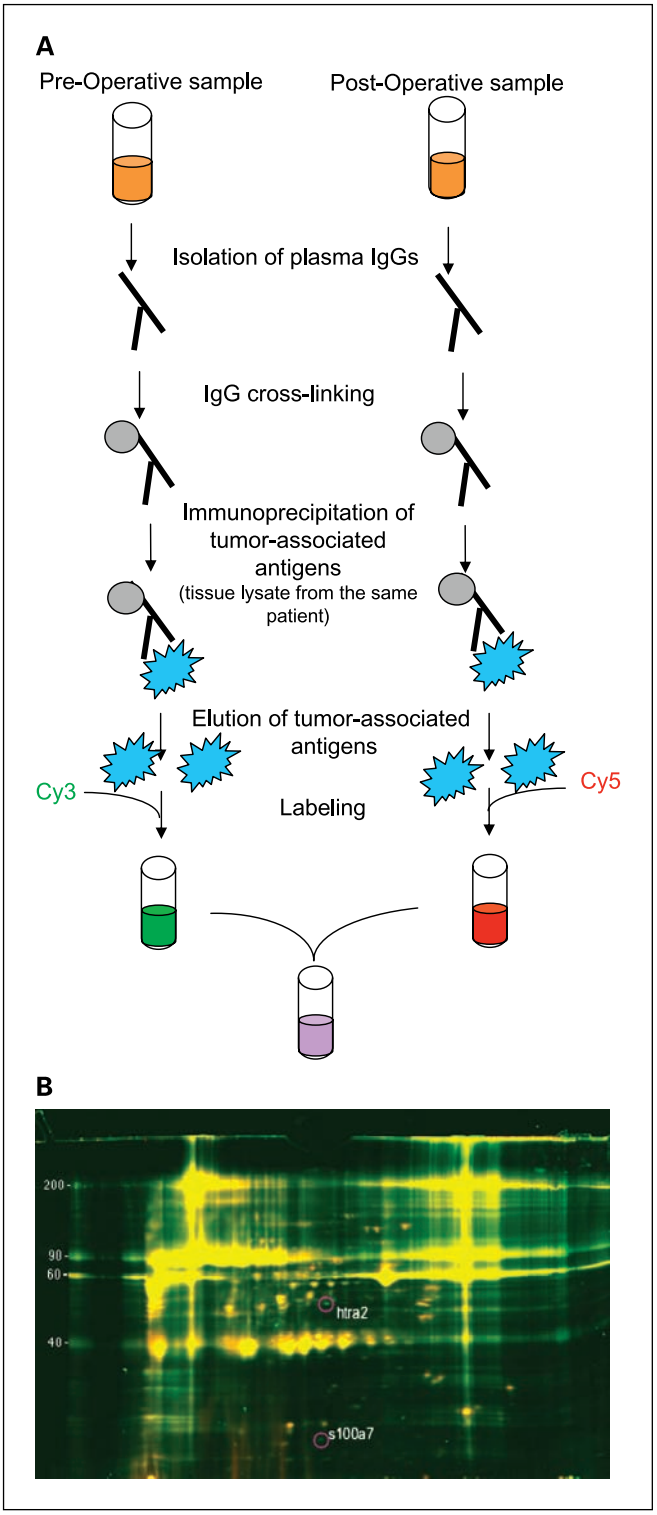

Fig. 1. *A,* schematic representation of the 2D-DITA technology. Presurgery and postsurgery plasma IgGs were isolated and cross-linked to beads. An immunoprecipitation of TAAs was then done using a tumor lysate from the same patient. Both samples were labeled either with Cy3 or Cy5 and combined. *B,* the combined presurgery and postsurgery TAAs were resolved by two-dimensional electrophoresis. Green spots, proteins down-regulated in the postoperative sample; red spots, proteins down-regulated in the preoperative sample; yellow spots, proteins that are present in both samples. One gel was run per patient. The DeCyder software was used to analyze the protein spot data in the three gels. Protein identification was done on two spots that were significantly differentially expressed in all three patients.

ELISA. Flat-bottom microtiter plates (Fisher Scientific) were coated overnight at 4°C with 0.10 μg of purified recombinant S100A7 protein (Abnova) diluted in 0.05 mol/L carbonate buffer (pH 9.7). Then, the plates were washed thrice with PBS-Tween 20 and blocked for 30 min at 37°C in PBS + 5% bovine serum albumin. The plates were washed thrice in PBS-Tween 20 and incubated 2 h with patient serum samples diluted 1:100 in blocking solution. After three washes in PBS-Tween 20, the plates were incubated for 2 h with a horseradish peroxidase–conjugated goat anti-human IgG diluted 1:5,000 in blocking solution (Chemicon). The plates were washed six times with PBS-Tween 20 and incubated 30 min in the dark with 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution (Pierce Biotechnology). The reactions were stopped by the addition of 100 μL of 0.45 mol/L H_2SO_4, and the absorbance was read at 450 nm on a plate reader (Bio-Rad Laboratories). Results are expressed as the mean absorbance of triplicate wells after subtraction of background value. The negative controls were conducted by eliminating the purified S100A7 protein, the patient serum, the secondary antibody, or the substrate for development.

Statistical analysis. A nonparametric Mann-Whitney *U* test was used to assess the statistical significance of the S100A7 mRNA expression difference between normal HOSE cells and microdissected ovarian cancer tissues and S100A7 autoantibody levels between normal individuals and different cancer groups. A Kruskal-Wallis test was used to assess the significance of the S100A7 levels in tissues between histologic types, and a Mann-Whitney test was used to compare the levels of S100A7 staining in normal versus malignant tissues.

Results

To identify autoantibodies directed against relevant ovarian TAAs, we did 2D-DITA on three pairs of plasma samples collected from the same patients before surgery and 1 month after the last cycle of chemotherapy. We hypothesized that lower levels of autoantibodies against TAAs are found in patients undergoing surgery or tumor-shrinking treatments. Lower levels of autoantibodies in the postoperative plasma samples compared with the preoperative samples should pull down smaller amount of TAAs. Subsequent Cy3-labeled TAAs pull down by the preoperative plasma samples and Cy5-labeled TAAs pull down by the postoperative samples were resolved by two-dimensional electrophoresis (Fig. 1). The two spots (TAAs) that showed the highest differential signals were cut out and sent for protein identification by tandem mass spectrometry (Fig. 2).

After mass spectrometry analysis, one spot was identified as being the HTRA2 protein, which is a 49-kDa serine protease that shows proteolytic activity against β-casein and promotes and induces cell death (20). The other spot was identified as S100A7, which is a 11-kDa protein expressed by a wide variety of cells and is involved in the regulation of cell cycle progression and differentiation (21, 22). The S100A7 protein is overexpressed in many cancers, such as breast, skin, bladder, and gastric cancer (23–26). Therefore, due to its up-regulation in many cancers, and to its chemotactic properties for immune cells, we chose to characterize its autoantibody levels in more detail.

To validate the data obtained with our 2D-DITA technology, we used quantitative real-time reverse transcription-PCR to assess whether the *S100A7* mRNA was overexpressed in ovarian cancer tissues and in normal HOSE cells. We used cDNAs from 8 HOSE cell samples and 37 microdissected high-grade

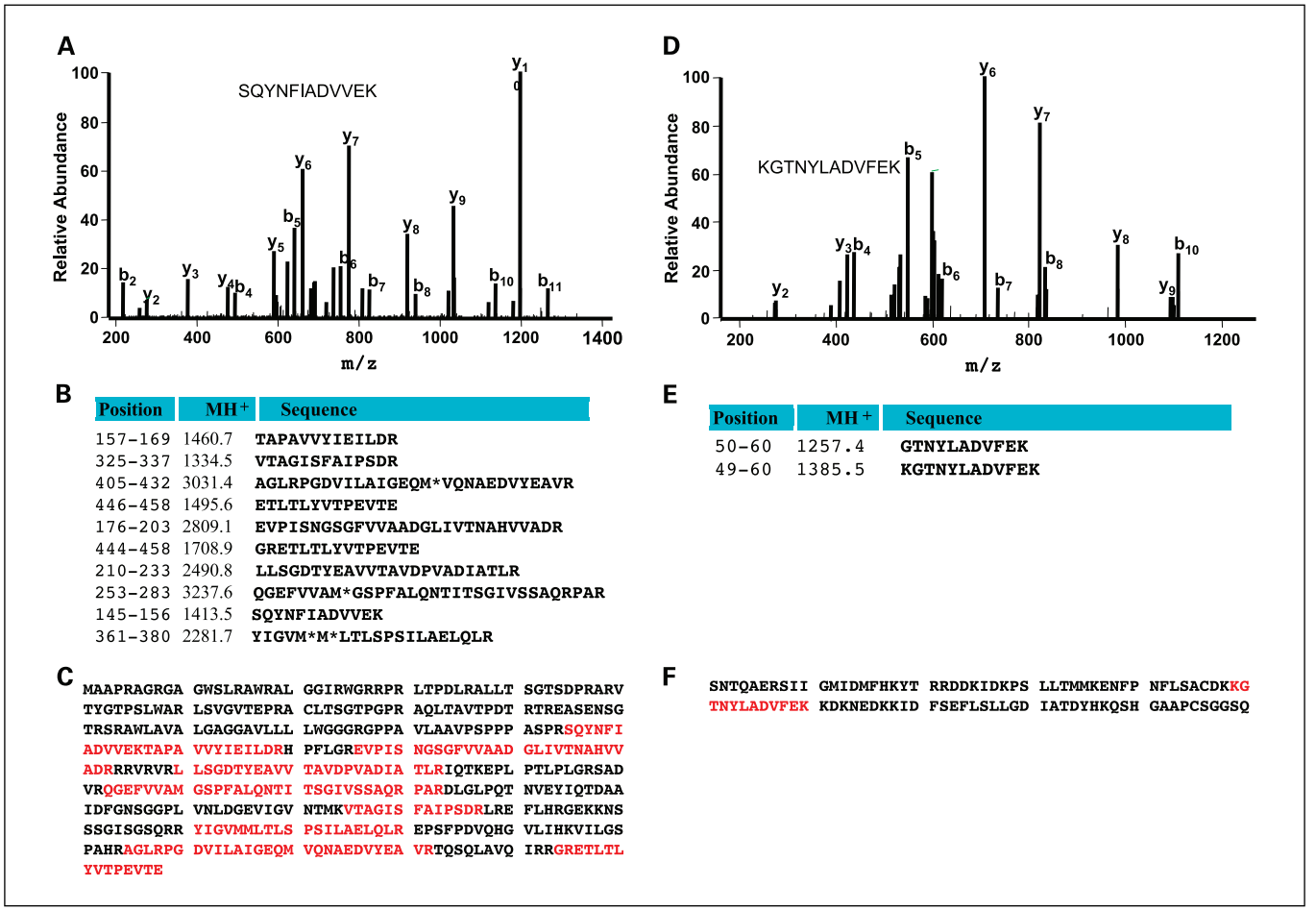

Fig. 2. Identification of peptide fragments of human HTRA2 and S100A7 by liquid chromatography-tandem mass spectrometry. *A* and *D,* mass spectrum of the SQYNFIADVVEK and KGTNYLADVFEK of human HTRA2 and S100A7, respectively. Fragmentations in the spectrum represent mainly single-event preferential cleavage of the peptide bonds resulting in the sequence recorded from both NH_2 and COOH termini (b- and y-type ions, respectively). *B* and *E,* peptides of HTRA2 and S100A7 identified from the tumor tissue lysates, respectively. *C* and *F,* sequence of human HTRA2 (Swiss-Port database no. O43464) and S100A7 (Swiss-Port database no. P31151), respectively, and the matched sequences are highlighted in red.

papillary serous ovarian carcinomas. As shown in Fig. 3, the mRNA levels of *S100A7* are significantly higher ($P < 0.001$) in most papillary serous tissues, whereas it is nearly absent in all HOSE cells examined.

Immunohistochemistry was also done on 53 ovarian cancer tissues (Fig. 4; Table 1A) and confirmed that the S100A7 protein was, indeed, overexpressed in cancerous tissues compared with normal ($P = 0.003$). Moreover, the up-regulation is not restricted to papillary serous carcinoma but is also present in mucinous, clear cell, and endometrioid ovarian cancers. Indeed, the levels of S100A7 protein are significantly elevated in nonserous ovarian cancers ($P = 0.038$). Interestingly, the protein expression levels are not significantly different between early- and late-stage disease ($P = 0.95$) or between low- and high-grade disease ($P = 0.75$). This suggests that the up-regulation of S100A7 may be an early event in the pathogenesis of ovarian cancer, and the

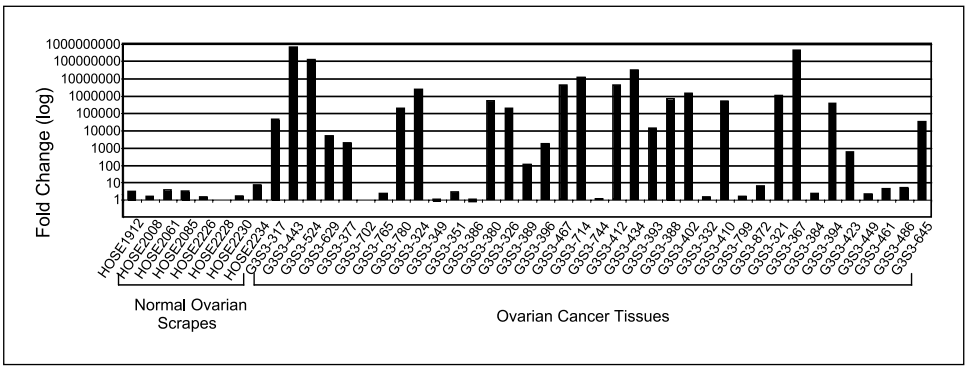

Fig. 3. Relative quantification of the *S100A7* mRNA in 8 normal ovarian surface epithelial cell samples and 37 microdissected ovarian cancer tissue samples. Each value was expressed as the mean of duplicate. The reference sample was HOSE2228, which was considered to have a value of 1. Fold change was presented in log scale.

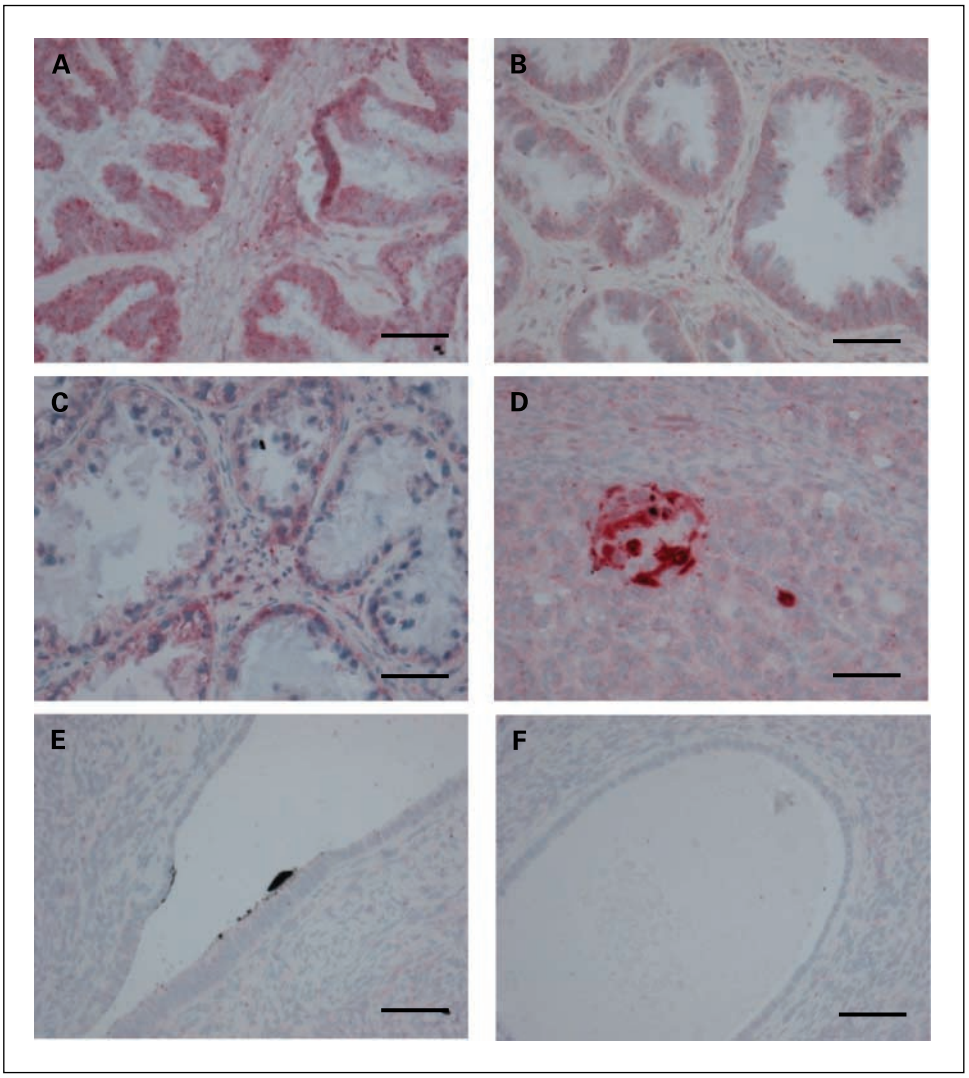

Fig. 4. Immunohistochemical staining of the S100A7 protein in a serous adenocarcinoma (*A*), an endometrioid adenocarcinoma (*B*), a clear cell adenocarcinoma (*C*), a mucinous adenocarcinoma (*D*), a normal ovary (*E*), and a benign ovarian cyst (*F*). Bar, 50 μm.

patients could mount an immune response to it very early in the progression of the disease. Furthermore, due to its secreted nature, the up-regulation of S100A7 in the tumor may trigger the development of antibodies against itself in these patients. Thus, the detection of autoantibodies directed against this protein may possibly be useful as ovarian cancer markers in all four subtypes of ovarian carcinomas.

To confirm the up-regulation of anti-S100A7 antibodies in ovarian cancer patients, we developed an ELISA assay and quantified them in the plasma of 138 ovarian cancer patients and healthy controls (11 benign gynecologic disease, 23 early-stage cancer, 69 late-stage cancer, and 35 age-matched healthy controls; Table 1B). A nonparametric Mann-Whitney *U* test shows that there is a significant difference between the levels of autoantibodies directed against S100A7 in early- and late-stage ovarian cancer patients compared with healthy controls ($P = 0.05$ and $P < 0.001$, respectively; Fig. 5). However, the levels of autoantibodies did not show significant difference among different histologic subtypes of ovarian cancer ($P = 0.627$). Notably, there is no significant difference between healthy controls and patients with benign gynecologic malignancies ($P = 0.07$). Moreover, patients with other gynecologic cancers, such as uterine and cervical, did not have elevated levels of anti-S100A7 antibodies, arguing that this biomarker is specific to ovarian cancer (data not shown).

Discussion

Recent studies showed that circulating autoantibodies recognizing relevant TAAs can be detected in cancer patients, suggesting that autoantibody-based tests may be used to differentiate cancer patient sera from normal donor sera (27–32). To identify relevant TAAs, we chose to compare the levels of antibodies between preoperative and postoperative plasma samples from the same patients. This choice was based on the hypothesis that constant stimulation of the immune system by TAAs is necessary to maintain a high level of autoantibodies. Therefore, surgically removing the tumor would prevent such stimulation. Thus, lower levels of autoantibodies are found in patients undergoing surgery or tumor-shrinking treatments (33). In fact, studies showed that anti-p53 antibodies decrease during cancer treatment. Therefore, lower levels of our autoantibodies should be found in the postoperative samples compared with the preoperative samples and they are likely to recognize TAAs (34).

Table 1A. Differential expression of S100A7 in normal and malignant ovarian tissues

	No. patients	Mean (95% confidence interval)	P
Normal/cancer			
Normal	5	0.2 (-0.14 to 0.54)	0.003*
Cancer	53	1.64 (1.35-1.91)	
Histologic types			
Serous	29	1.29 (0.91-1.68)	0.038 †
Mucinous	5	2.5 (1.11-3.89)	
Endometrioid	14	1.71 (1.19-2.24)	
Clear cell	5	2.2 (1.49-2.91)	
Grade			
Low (grade 1)	20	1.65 (1.12-2.17)	0.75
High (grade 2 and 3)	33	1.58 (1.23-1.92)	
FIGO stage			
I/II	27	1.65 (1.19-2.11)	0.95
III/IV	26	1.56 (1.2-1.92)	

Abbreviation: FIGO, International Federation of Gynecologists and Obstetricians.
*Statistically significant by Mann-Whitney test.
† Statistically significant by Kruskal-Wallis test among groups.

Using an innovative proteomic technique, which allows the simultaneous identification of autoantibodies and their corresponding TAAs, we identified elevated levels of antibodies directed against a protein called S100A7 in the plasma of ovarian cancer patients but not in healthy controls. S100A7 is a small, secreted, calcium-binding protein of the S100 protein family that was originally identified as one of the most abundant proteins in psoriatic keratinocytes (35). Further studies have shown that its expression is elevated in premalignant and squamous carcinoma of the bladder as well as in primary invasive breast tumors (25, 36–38). This suggests that the overexpression of the S100A7 protein may be an early event in tumorigenesis. Furthermore, our studies showed significantly higher levels of S100A7 protein expression in both early- and late-stage ovarian cancer compared with benign and normal ovarian tissue (Table 1). This suggests that increased S100A7 expression may be an early event in ovarian pathogenesis and that its autoantibodies may thus be a potential target for detecting the disease in its early stages.

No elevation of autoantibody levels against S100A7 has been previously described. However, due to its secreted nature, S100A7 has already been shown to act as a chemotactic factor for $CD4^+$ lymphocytes and neutrophils in the skin (39). Moreover, the S100A7 protein levels are associated with increased inflammatory cell infiltrates across all types of invasive breast tumors (38). This shows that S100A7 is an immune cell chemoattractant and that it induces an immune response in breast cancer patients. Thus, this could well be the case in ovarian cancer and may perhaps explain the presence of high levels of anti-S100A7 antibodies seen in the plasma of these patients.

Our preliminary study confirmed the presence of elevated S100A7 autoantibody levels in the plasma of ovarian cancer patients. We also showed significantly higher autoantibody levels in patients with both early- and late-stage ovarian cancer compared with patients with benign gynecologic diseases or healthy controls. Furthermore, even if the S100A7 protein levels were significantly higher in the nonserous subtypes, we did not find any significant difference in autoantibody levels among the different histologic types. This suggests that the immunogenicity of the S100A7 protein may be altered or increased by posttranslational modifications such as glycosylation in the different subtypes. Indeed, protein glycosylation is mechanistically important in the pathogenesis of autoimmune diseases (40) and this may well be the case for cancer. Therefore, the immune response generated by a patient against a specific antigen could vary considerably. This still shows that S100A7 can trigger a humoral immune response in patients with any subtype of ovarian cancer and that the autoantibody levels generated by this response are significantly higher than in healthy controls.

Moreover, because the anti-S100A7 antibody levels are significantly elevated in all subtypes of ovarian cancer, they may be useful biomarkers to improve the poor detection sensitivity of the CA125 marker for nonserous ovarian cancer. Indeed, CA125 serum levels are significantly lower in mucinous, clear cell, and endometrioid ovarian cancer patients (2).

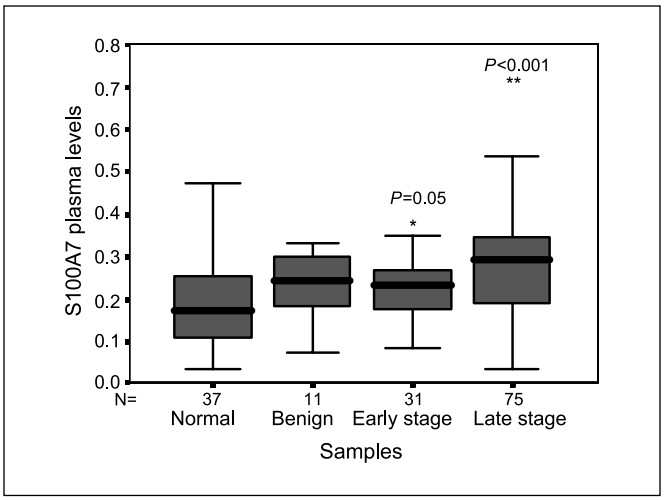

Fig. 5. Box plot analysis of S100A7 autoantibody levels in healthy controls, patients with benign gynecologic disease, and early- and late-stage ovarian cancer patients.

Table 1B. Characteristics of ovarian cancer patients used in the quantification of plasma anti-S100A7 antibodies

	No. patients	Mean antibody level (95% confidence interval)	*P*
Normal/cancer			
Normal	35	0.19 (0.154-0.226)	0.001*
Benign	11	0.221 (0.169-0.274)	
Cancer	92	0.277 (0.25-0.304)	
Histologic types			
Serous	60	0.28 (0.244-0.316)	0.627
Mucinous	10	0.211 (0.141-0.283)	
Endometrioid	10	0.272 (0.235-0.309)	
Clear cell	12	0.281 (0.197-0.0365)	
Stage			
Early	23	0.227 (0.192-0.256)	0.03*
Late	69	0.294 (0.261-0.328)	

*Statistically significant by Mann-Whitney test.

CA125 levels are available in all the cases that we screened (data not shown). However, both sensitivity and specificity estimates for ovarian cancer detection are not improved using either anti-S100A7 alone or in combination with CA125, which is likely due to the small sample size.

In conclusion, we used a novel approach to identify autoantibodies directed against relevant TAAs in the plasma of ovarian cancer patients. Our preliminary study found that anti-S100A7 antibodies are significantly elevated in the plasma of ovarian cancer patients compared with controls and with patients with benign gynecologic diseases. We have confirmed that the S100A7 protein and mRNA are overexpressed in all four histologic types of ovarian cancer tissues. Our results suggest that further studies using a larger sample size to evaluate whether anti-S100A7 antibodies alone or in combination with other autoantibodies, such as EpCAM autoantibodies (11), or other protein biomarkers can be used as diagnostic markers for ovarian cancer screening are warranted.

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