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Shared Immunoproteome for ovarian cancer diagnostics and immunotherapy: Potential theranostic approach to cancer

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Abstract

Elimination of cancer through early detection and treatment is the ultimate goal of cancer research, and is especially critical for ovarian and other forms of cancers typically diagnosed at very late stages and that have very poor response rates. Proteomics has opened new avenues for the discovery of diagnostic and therapeutic targets. Immunoproteomics, which defines the subset of proteins involved in the immune response, holds considerable promise for providing a better understanding of the early stage immune response to cancer as well as important insights into antigens that may be suitable for immunotherapy. Early administration of immunotherapeutic vaccines can potentially have profound effects on prevention of metastasis and may potentially cure through efficient and complete tumor elimination. We developed a mass-spectrometry-based method to identify novel autoantibody-based serum biomarkers for the early diagnosis of ovarian cancer that uses native tumor-associated proteins immunoprecipitated by autoantibodies from sera obtained from cancer patients and from cancer-free controls to identify autoantibody signatures that occur at high frequency only in cancer patient sera. Interestingly, we identified a subset of more than 50 autoantigens that were also processed and presented by MHC class I molecules on the surfaces of ovarian cancer cells and thus common to the two immunological processes of humoral and cell-mediated immunity. These shared autoantigens were highly representative of families of proteins with roles in key processes in carcinogenesis and metastasis, such as cell cycle regulation, cell proliferation, apoptosis, tumor suppression and cell adhesion. Autoantibodies appearing at the early stages of cancer suggest that this detectable immune response to the developing tumor can be exploited as early stage biomarkers for the development of ovarian cancer diagnostics. Correspondingly, because the T cell immune response depends on MHC class I processing and presentation of peptides, the identification of proteins that go through this pathway are potential candidates for the development of immunotherapeutics designed to activate a T cell immune response to cancer. To the best of our knowledge, this is the first comprehensive study that identifies and categorizes proteins that are involved in both humoral and cell-mediated immunity against ovarian cancer, and may have broad implications for the discovery and selection of theranostic molecular targets for cancer therapeutics and diagnostics in general.

Keywords

Immunoproteomics; auto-antigens; ovarian cancer; immunotherapy; bio-marker; early diagnosis

Introduction

Ovarian cancer is highly lethal because it is typically asymptomatic until well advanced. In the majority of cases, ovarian cancer remains undetected until survival rates are less than 25%. There is no reliable early diagnostic test for ovarian cancer that can be used to screen at early stages when the disease is treatable. The ability to diagnose at an early stage of the disease and stimulate the patient's own immune system to detect and destroy the tumor is one of the best options for the treatment and eventual cure for ovarian cancer.

In recent years, both the cell-mediated and humoral arms of the immune system have been recognized as having critical roles as probes and treatments for cancer¹. The immunoproteome, which comprises proteins involved in cellular and humoral immunity, holds considerable promise for providing a better understanding of the early stage immune response to cancer as well as important insights into antigens that may be suitable for immunotherapy.

Circulating autoantibodies against tumor-associated antigens (TAA) have been shown to be early, effective and sensitive diagnostic indicators of cancer. Autoantibodies have been observed to appear long before the manifestation of any pathology². Autoantigens represent a variety of intracellular and surface proteins, including differentiation antigens and other proteins that are overexpressed in tumors^{1, 3–6}.

The clinical application of cancer immunotherapy is based on growing evidence that cytotoxic T lymphocytes (CTL) can recognize and mount an effective attack against a wide variety of tumor cells that display specific TAA in the context of Major Histocompatibility Complex (MHC) molecules on their surfaces, including ovarian⁷ and colon tumors^{8–14}, sarcomas¹⁵, renal cell carcinomas¹⁶, pancreatic¹⁷, adenocarcinomas, and squamous tumors of the head and neck¹⁸ and the lung¹⁹. When delivered in a system designed to elicit a strong T-cell response, MHC-associated antigens have the potential to break tolerance and stimulate an immune response to prevent metastatic disease progression, and to generate a therapeutic response that can eliminate tumor cells in a cancer patient²⁰. However, the identities and distributions of these CTL epitopes and consequently their parent proteins are still largely unknown. Various methods such as CTL analysis and MHC binding motif algorithms have identified a few TAA capable of generating protective immune responses in healthy individuals at risk of developing a disease^{20, 21}. To address the question, we have optimized mass spectrometry-based methods with which we are able to conduct comprehensive surveys of native MHC-associated epitopes presented on cell surfaces. We have also developed a mass-spectrometry based differential immunoproteomics method that uses native tumor autoantigens to immunoprecipitate autoantibodies from cancer patient sera to identify autoantibody signatures that occur at high frequency in cancer patient sera and that are not found in non-cancer control sera. We chose this approach because post-translational processing can have profound effects on antibody recognition and also have important implications for the design of diagnostics and immunotherapeutics.

In the present study, for the first time, we have conducted a comprehensive screen of the tumor-reactive autoantibody profile of ovarian cancer using native tumor proteins to identify novel biomarkers, combined with a comprehensive screen of MHC-associated peptides presented on the surfaces of ovarian cancer cells. Antigens common to both screens that stimulate strong cellular (T cell) and humoral (B cell) responses against ovarian tumors are promising theranostics candidates for the development of diagnostic panels that can be used for early detection and for immunotherapy to treat early-stage disease at a time when the tumor burden is low and the tumor is most vulnerable to immune attack. The antigens that we identified perform a variety of essential tumor cell functions, including cell cycle regulation, transcriptional regulation, apoptosis, adhesion/metastasis and cell signaling.

Materials and Methods

Patient samples and cell lines

Serum samples were procured from twenty patients diagnosed with primary ovarian cancer (Tissue and Serum Repository, Kansas Masonic Cancer Research Institute, Kansas City, KS, and Duke University Comprehensive Cancer Center) and twenty age- and sex-matched control individuals (Biochemed Pharmaceuticals, Winchester, VA). The serum samples were collected from patients with histologically confirmed serous or adenocarcinoma of the ovary (the two most common forms of ovarian cancer) at the time of primary diagnosis, and with low tumor mass, regardless of the stage of the tumor prior to surgery or chemotherapy. Tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Histological classification was based on the World Health Organization (WHO) and the FIGO. Characteristics of these ovarian patients include age, stage, grade and histological type. Equal volumes of undiluted serum from each cohort were used to generate composites consisting of a random assortment of 4 individual samples from each cohort so that each serum sample would be analyzed only one time among the composites. Serum samples from all patients were collected and stored at -80°C until processing.

The human ovarian adenocarcinoma cell lines SKOV3-A2 (kindly provided by Dr. Ioannides, MD Anderson Cancer Center) (HLA type: HLA-A2, A3, A68, B18, B35) and OVCAR3 (HLA type: HLA-A2, A29, B7, B58) was obtained from the American Type Culture Collection (Rockville, MD). Cell lines were grown in RPMI 1640 media containing 10% fetal calf serum, 2mM glutamine, and 1% penicillin/streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5 % CO_2 . Cell lysates for both autoantibody and MHC peptide identification were prepared using these cell lines.

Immunoprecipitation of autoantigens

Immunoprecipitation of proteins from tumor cell lysate was carried out as follows: 2 mg of tumor cell lysate was pre-cleared by incubating with 100 μl of Protein A/G beads in microfuge tubes for 1 hour with gentle rocking at room temperature to remove any proteins bound non-specifically to Protein A/G. IgG from 400 μl of each of the serum composites was conjugated to 800 μl of Protein A/G beads with gentle rocking at room temperature for 2 hours. Beads were pelleted down and supernatant was removed. Beads were then washed thrice with PBS to remove unbound serum proteins. One half of washed beads were then incubated with pre-cleared tumor cell lysate for 2 hours at room temperature or overnight at 4°C with gentle rocking. Following incubation, supernatant was removed and saved. Beads were washed three times with 1ml PBS each. IgG-bound proteins were eluted three times consecutively using 400 μl of 0.1% trifluoroacetic acid (TFA). Saved supernatant was incubated with the remaining half of washed beads for a second round of immunoprecipitation as described above.

Fractionation and autoantigen sample preparation for mass spectrometry analysis

Immunoprecipitated samples first underwent size exclusion chromatography (SEC) in order to separate the antibodies from the antigens. SEC was performed on an ABI 140B LC system flowing at 250 $\mu\text{l}/\text{min}$ with an ABI 785A UV detector monitoring at 214nm. A Zorbax GF-250 4.6 $\times$ 250mm (Agilent) column was used with a mobile phase consisting of 1% acetic acid ($\sim\text{pH}$ 3.5) + 10% acetonitrile. Fractions were collected every minute after the elution point of IgG as determined by an IgG control injection. SEC fractions were then pooled and fractionated over an ABI 140B LC system flowing at 75 $\mu\text{l}/\text{min}$ with an ABI 785A UV detector monitoring at 214nm. Proteins were separated on a Zorbax C8 1.0 $\times$ 150mm (Agilent) column and eluted using a 0 – 60% gradient over 80 min with mobile phase A consisting of 0.1% acetic acid ($\sim\text{pH}$ 3.5) + 2% acetonitrile + 98% H_2O and mobile phase B consisting of 0.1% acetic acid ($\sim\text{pH}$ 3.5) + 98% acetonitrile + 2% H_2O . Fractions were evaporated down to $\sim 10\mu\text{l}$ using a speed-

vac. These fractions were then diluted in 25mM ammonium bicarbonate + 10% acetonitrile and 1:50 ratio (vs. total protein) of trypsin (Promega) was added and allowed to incubate at 37°C overnight. Digested fractions were then washed 3 times in mobile phase A buffer to remove any residual salts and reduced to ~20µl using a speed-vac prior to mass spectrometry analysis.

Isolation and purification of MHC associated peptides

MHC-peptide complexes from each ovarian cancer cell line were isolated by immunoaffinity purification. First, cell lysates were prepared from $1-5 \times 10^8$ cells by homogenization and freeze/thaw in buffer containing 1.0% NP40. The lysate was cleared by centrifugation at 2000 rpm for 30 minutes to remove the cell debris. MHC-peptide complexes were isolated by immunoaffinity chromatography using W632 (monoclonal antibody recognizing the pan MHC class I molecule) antibody coated protein A/G beads (UltraLink Immobilized Protein A/G, Pierce, Rockford, IL). 400µl Protein A/G beads were washed with low pH buffer followed by PBS rinses. The beads were then incubated with 0.5mg W632 antibody at room temperature for 2 hours. Labeled beads were washed three times to remove unbound antibodies. The antibody-coated beads were added to the prepared cell lysate. The mixture was incubated for two hours at room temperature with continuous rocking. The beads were separated from the lysate by centrifuging at 1000 rpm for 5 minutes. The recovered lysate was incubated for an additional 2 hours at room temperature with another set of prepared antibody-coated Protein A/G beads. The bound MHC complexes were eluted from the beads by the addition of 200µl of 0.1% TFA, pH 1.5, and the beads were centrifuged, and the supernatant removed. This process was repeated three times to give a final volume of 600µl. The eluate was then boiled for 5 minutes to denature the proteins, after which it was dried down to almost dryness using a speed-vac and reconstituted to 50µl with SEC running buffer.

Peptide fractionation

The isolated peptide mixture was first separated using size exclusion chromatography (SEC) in order to separate any proteins from the peptides. SEC was performed on an ABI 140B LC system flowing at 250ul/min in isocratic mode with an ABI 785A UV detector monitoring at 254nm. A Zorbax GF-250 4.6×250mm (Agilent, Palo Alto, CA) column was used with a mobile phase consisting of 1% acetic acid (~pH 3.5) + 10% acetonitrile. The peptide-containing fraction was collected from 14 minutes to 18 minutes as determined by a calibration mix injection. The peptide-containing fraction was further fractionated using HPLC. A 1mm × 250mm PLRP-S 100Å Polymer column was used with an ABI 140B LC system flowing at 50ul/min in gradient mode with an ABI 785A UV detector monitoring at 214nm. Solvent A was 2% ACN in H₂O + 0.1% TFA and solvent B was 80% ACN in H₂O + 0.09% TFA using a gradient of 2% B to 60% B in 60 minutes. Fractions were collected at one-minute intervals and further processed for mass spectrometry analysis.

LC/MS/MS analysis of tryptic and MHC peptides

Tryptic peptide fractions and MHC peptide fractions were identified by the technique of Collision Activated Dissociation (CAD) on an ion trap mass spectrometer (Qtrap, Applied Biosystems and LCQ, Thermo Finnigan) equipped with on-line microcapillary HPLC (Eldex) and a custom-made microspray ionization source. An aliquot of the fraction was loaded onto the microcapillary column, picofrit 360×75µm, 15µm tip (New Objective) packed with C18 resin (Reliasil) using a pressure bomb. The column was then washed for several minutes with buffer A (2% acetonitrile + 1% acetic acid) to wash away salts. A 3hr gradient (5%–65% buffer B (90% acetonitrile + 1% acetic acid)) was used to elute the peptides directly into the mass spectrometer. Mass data were acquired from 500 – 1800 amu. The spectra were processed in real-time to determine the abundant ion species. The ion species were immediately isolated

within the ion trap and fragmented to produce CAD spectra representing the selected ion. This ~12 second process was repeated for the entire duration of the 3 hr peptide fractionation.

Data analysis and interpretation

Mass spectrometry data (spectra) were compared to a non-redundant human protein database using Sequest (Thermo Finnigan) and Mascot (Matrix Science) software. Results were returned as a confidence score and peptides that scored above a threshold value were manually verified. In addition, we synthesized selected peptides and confirmed the sequences by co-elution experiments in the mass spectrometer. If multiple peptides (tryptic fragments) mapped to the same protein with adequate scores, the protein was considered positively identified. Using the identified proteins, we generated two separate non-redundant autoantigen databases; the first representing the proteins found in the cancer serum group; the second representing the proteins found in normal serum samples. Using the identified MHC peptides and parent proteins, we generated two separate non-redundant databases; the first, representing the peptides found in the SKOV3-A2 cell line; the second, representing the peptides found in OVCAR3 cell line. Data comparison was made using an Excel spread sheet format to identify common peptides.

Results

Immunoproteomics analysis of ovarian cancer

We applied immunoaffinity purification and mass spectrometry methodologies to identify targets involved in humoral and cell mediated immunity in ovarian cancer. As shown in figure 1, we developed two independent processes. One process was the identification of serum autoantibody-reactive autoantigens identified by immunoaffinity isolation of tumor proteins using serum samples collected from patients diagnosed with ovarian cancer. In a separate procedure, we identified MHC-peptide complexes from ovarian cancer cell lines by immunoaffinity purification with antibodies to pan MHC Class I molecules. The cell lines used in these studies were adenocarcinomas. HLA-A2-positive tumors were selected because of the technical ease of identifying and characterizing HLA-A2 MHC-associated peptides, and relevant because more than 40% of the U.S. population is A2-positive. Following ongoing preclinical validation of these epitopes, these antigens may be candidates for formulation into a multiantigen immunotherapeutic vaccine and clinically tested in cancer patients.

Autoantibody screening and identification

Serum samples from 20 patients (age range 42–76) with either adenocarcinoma or serous carcinoma, collected at the time of primary diagnosis, were obtained for autoantibody identification. Ovarian cancer is often diagnosed at advanced stages; therefore the criteria for sample inclusion in this discovery-stage screening study were collection at the time of primary diagnosis and prior to any intervention such as surgery and chemotherapy. Many ovarian cancer patients present with low tumor mass even at these later stages, and although it would be ideal to use samples from patients diagnosed at the earliest stages of disease (I/II) we relied on samples for which there was a confirmed diagnosis of ovarian cancer to generate a broad panel of targets. When we have identified and validated a panel of diagnostic targets, it will be straightforward and necessary to screen various forms of ovarian cancer and high-risk individuals (e.g. stage I, stage II, patients with elevated CA125 or familial history of ovarian cancer) to assess the time of appearance of these markers.

Autoantibody profiles were identified by using native tumor proteins immunoprecipitated by autoantibodies in sera from ovarian cancer patients and non-cancer controls. Five different cancer composites were prepared by pooling sera from four different patients. Each of two control composites consisted of pooled sera from four different non-cancer samples. Each composite was independently processed. Immunoaffinity purified (IAP) samples were first

fractionated by size exclusion chromatography (SEC) and then by reverse phase HPLC. The HPLC fractions were individually digested with trypsin and subjected to LC/MS/MS analysis followed by database searches to identify proteins immunoprecipitated by the autoantibodies. The data from all of the cancer and control composites were combined in a spreadsheet to identify autoantigens that were only found in cancer patient sera. Of particular interest were high frequency antigens that appeared in the majority (3, 4, or 5) of the cancer composites, which are considered to be highly immunologically significant.

MHC peptide screening and identification

MHC-peptide complexes were isolated from the ovarian cancer cell lines SKOV3-A2 and OVCAR3. Both lines share the HLA-A2 haplotype but also express other different haplotypes. MHC-peptide complexes were isolated by immunoaffinity purification using antibodies to pan MHC class I molecules (W632), which recognize all HLA-A, B and C haplotypes. We chose this antibody because we were interested in identifying all proteins processed through the MHC class I pathway, regardless of haplotype, because peptides associated with any haplotype are potentially capable of activating haplotypes-matched T cells. By using pan MHC class I antibodies, we expected that the mass spectrometry analysis would identify some HLA-A2 specific peptides common to both cell lines, as well as additional peptides processed and presented by the additional HLA types represented among the two cell lines. We reasoned that proteins that go through class I processing will present different peptides depending upon the HLA composition of the cells. For any peptides identified in the database, we were able to identify the parent protein that was processed and presented in association with MHC, regardless of the HLA haplotype through which the protein was originally processed.

The autoantibody and MHC peptide datasets were merged into a single spreadsheet to identify common autoantigens (Figure 1).

From several thousand hits in the composite datasets, we identified approximately 100 autoantibody targets that were common to a majority of the five cancer composites and not present in control sera (manuscript in preparation). Eight proteins present in all five composites and not found in control sera were also found to be processed and presented by MHC class I molecules on the tumor cells (Table 1). Seven out of the eleven peptides derived from these eight proteins were consistent with a predicted HLA-A2 motif²² and were found in both the OVCAR3 and SK-OV3-A2 cell lines. Other peptides were most likely processed and presented by other HLA types not shared by the cell lines. Interestingly, identifying multiple peptides for some of these antigens indicated that these proteins were efficiently processed and capable of generating multiple T cell ligands, all of which are potential immunotherapeutic targets.

Similarly, nine autoantibody-reactive TAAs common to four out of five (80%) of the cancer composites and not found in control sera were also found in the MHC peptide dataset (Table 2). Six out of the nine peptides fit the HLA-A2 (shared HLA locus) motif and were present in both cell lines. Autoantibody-reactive TAAs common to sixty percent (3 out of 5) of the composite samples and not found in control sera shared sixteen different MHC peptides (Table 3). Among these peptides, nine peptides showed HLA-A2 motif and were present in both cell lines. Two proteins generated more than one MHC-associated peptide, indicating efficient processing for presentation in the MHC class I pathway. Some of the same HLA-A2 peptides were also found to be MHC processed and presented in breast tumor cells, for example vimentin; and some of the same parent proteins were found to be common to ovarian and breast cells, for example vinculin, KIF13B, timeless homolog, and breast cancer type 2 susceptibility protein (data not shown).

Functional characterization and categorization of autoantigens common to the autoantibody and MHC-associated protein datasets

Our goal was to identify a subset of targets that potentially have both diagnostic and therapeutic properties. A comprehensive analysis of antigens involved in both arms of the immune system enabled us to elucidate interesting clues about antigen recognition and antigen processing in ovarian tumor cells. Antigens common to both humoral and cellular immunity are involved in numerous different tumor transformations pathways and represent critical processes in transcription/replication (11%), kinases (8%), cell signaling and growth (40%), apoptosis (3%), tumor suppression (9%) cell cycle regulation (9%) and cell adhesion (20%) (Figure 2). The fact that many different and critical tumor pathways are represented in the antigen panel is encouraging for the development of multiantigen immunotherapeutic vaccines, which could be designed to elicit immune responses against many different facets of tumor cell function and maximize the probability of extensive CTL activation and tumor eradication.

Discussion

Identification of autoantigens that are reliable early-stage cancer diagnostic biomarkers or that have clinical immunotherapeutic application has been particularly challenging due to the lack of focused use of sensitive analytical methods to characterize the compositions of functionally relevant cellular compartments such as the immunoproteome. Few biological applications have succeeded in utilizing the large body of available proteomic and genomic information to assess suitable candidates for the development of reliable early-stage diagnostics or clinically effective immunotherapeutics for cancer, and in particular, ovarian cancer. Even less progress has been made in the characterization of novel targets that activate both arms of the immune system. During the past twenty years, the immune response to cancer has been clearly shown to be an important aspect of cancer biology because the proteins involved in the humoral and cellular immune responses also participate in many critical oncogenesis processes including signal transduction, cell cycle regulation, cell proliferation and apoptosis. Autoantibody and T cell responses involved in tumor rejection have been particularly well-characterized in melanoma^{23–25}. Furthermore, evidence supports a correlation between the presence of certain markers and the stage of cancer development^{26–31}. These markers reflect a diversity of processes characteristic of carcinogenesis, including oncogenic point mutations^{32, 33}, altered and atypical gene transcripts, alternate reading frames, pseudogenes³⁴, and antisense strands of DNA³⁵. Examples of autoantigens that have been well-studied in melanoma include the cancer-testis antigen families MAGE³⁶, GAGE, and BAGE, for which a number of class I MHC-restricted epitopes have been identified³⁷. The antigen NY-ESO-1 has also been shown to be recognized by both CD4⁺ and CD8⁺ T cells in melanoma^{38–40}, and anti-NY-ESO-1 titer was found to directly correlate with tumor load⁴¹. Differentiation antigens, which are expressed by both cancer cells and their normal cell counterparts⁴², have been studied in patients with breast, colon, and pancreatic cancer, and are best characterized for melanoma. They include tyrosinase, tyrosinase-related protein (TYRP)-1/gp75, TYRP-2/DCT, gp100/pmel17 and Melan-A/MART-1^{33, 36–50}. Anti-tyrosinase in various stages of melanoma may be used as a tumor marker for disease recurrence or progression⁵¹ and tyrosinase is one of several peptide components in immunotherapeutic vaccines under evaluation for treatment of melanoma⁵². *Her-2/neu* is a target of cytotoxic T-cell lines in patients with breast and ovarian tumors^{53, 54} and in colorectal cancer cell lines^{55, 56}.

Characterization of serologic responses to TAA have important implications for cancer detection and prognosis because autoantibodies appear in the serum long before tumors are detectable by conventional tests, such as imaging^{2, 20, 57}. Our approach of using native autoantigens for screening parallels recent work in prostate cancer that suggests that serum profiling using native autoantigens is a sensitive technique that distinguishes cancer from benign hyperplasia, and therefore has important implications for improving the reliability and specificity of diagnostic testing for prostate disease⁵⁸. Many studies on cancer autoantibodies have used serum probing of recombinant proteins (cDNA libraries^{1, 3–6} or phage display

59, 60 or denatured proteins resolved by 2-DE. These methodologies have significant limitations because of the absence of native antigen conformation and post-translational modifications^{9, 61–63} or generation of ‘mis-sense’ or ‘nonsense’ products that do not occur in vivo and that cannot be identified⁵⁹. Therefore, these approaches may potentially overlook proteins relevant to cancer specificity that possess features that can profoundly influence antigen specificity^{64–66} and highly relevant to the design of diagnostics and immunotherapeutics. To overcome these limitations, we used native tumor proteins generated from tumor cells to immunoprecipitate autoantibodies from ovarian cancer patient sera (Tables 1–3). While we identified several antigens in our database that were common to a phage display study reported earlier⁶⁰, most of the overlap was observed to be low-frequency autoantibodies identified in only one or two of our composite samples. The panels represented in Tables 1, 2, and 3 differ in two ways from earlier studies: first, we selected autoantibodies that were present in a majority of our composite samples (minimum 3 of 5), and second, we only report autoantibody biomarkers for which there a corresponding MHC peptide was identified, which significantly reduced the overall biomarker panel size of interest to this work.

Cell-mediated immunity elicited by MHC-associated antigens expressed on human tumors and recognized by cytotoxic T lymphocytes has been demonstrated for ovarian⁷ and a number of other cancers^{8–19}. To characterize the repertoire of T cell-inducing antigens in ovarian cancer, we assessed MHC Class I-restricted peptide epitopes obtained directly from the surfaces of ovarian cancer cells and analyzed by mass spectrometry. The data indicate that a number of autoantigens that induce autoantibody response also go through the MHC class I pathway and thus are potential T cell targets (Tables 1–3). This is significant for tumor-rejection therapy because cytotoxic T cells only recognize antigens that are displayed in the context of MHC molecules, therefore in order to elicit a T cell response, a protein must be processed (i.e., fragmented) through the MHC pathway and one or more of its peptides presented in the context of MHC molecules associated with one or more of the A, B, or C haplotypes. A few T-cell epitopes associated with ovarian tumors have been identified using MHC binding algorithm motif predictions^{54, 67, 68}, including peptides derived from the HER-2/neu proto-oncogene, epithelial mucin, MUC1 and p53^{20, 69}. However, endogenous T cell epitopes associated with ovarian cancer are still largely unknown and our data represent the first analysis of endogenous processing and presentation of the largely untapped reservoir of potential candidates for ovarian cancer immunotherapy.

The genesis of autoantibody production in cancer is not well understood, however the presence of an antibody response to any antigen is important because this may suggest that there is already present a population of specific CD4+ T cells at a relatively early stage of disease that could be expanded and used for the immunotherapy. One of the limitations of cancer immunotherapy has been tolerance to self proteins as antigens, one well-studied example being the Her-2/neu protein⁷⁰. The presence of a repertoire of autoantibodies against tumor-associated proteins indicates that the tolerance to self antigens has been broken or may indicate a type 2 cellular response depending on the subclass of IgG specific for the antigen. The presence of autoantibodies can also be an excellent surrogate marker for the presence of a CD4 + helper T cell response, which will help to prime, expand, and most importantly to sustain CD8+ T cell responses as demonstrated by several groups for NY-ESO-1 antigen^{71–75}. This study indicates that there are natural breaks in tolerance to some self proteins in patients with cancer (as shown by antibody production), and that these antigens may be more amenable to generating a cell mediated immune response than others. At an early stage of tumor development, it is possible that the immune system can recognize changes in normal self-antigens and mount an immune response to eliminate those cells. At the same time, by virtue of the oncogenic changes occurring in cancer cells, these proteins are also abnormally produced, making them susceptible to proteasome degradation and MHC class I pathway

processing⁷⁶, so that they are presented by MHC molecules on the cancer cell surface in the form that is critical for T cell recognition and activation.

The data presented in our study clearly demonstrate that autoantigens capable of eliciting both T and B cell responses are present in ovarian cancer. Consistent with earlier findings, we identified targets with roles in key processes in carcinogenesis and metastasis, such as cell cycle regulation, cell proliferation, apoptosis, tumor suppression, and cell adhesion (Figure 2). Our work has also shown that by using strategies designed for biomarker discovery it is possible to identify functional targets for diagnostics, anticancer drugs and vaccination protocols. Currently, we are evaluating a panel of autoantibody biomarkers for the development of an array-based test that is capable of detecting different types of ovarian cancer at its earliest stages. In addition, we are characterizing panels of CTL epitopes for functional anti-tumor activity against selected HLA-A2 specific peptides, which may lead to clinical applications through the development of a cancer vaccine. As part of the preclinical characterization we will evaluate peptide-specific CTL activity using various types of carcinomas as targets to determine the broad applicability of these antigens to multiple tumor types. Once the immune effectiveness of these antigens is confirmed, they will also be evaluated for use as theranostic targets, consistent with the concept that ovarian cancer treatment could be individually tailored to the patient's immunological profile, based on composition of the autoantibody panel identified by diagnostic serum screening. More broadly, similar types of analyses could be applied to other cancers for the discovery of diagnostic and therapeutic autoantigens.

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Figure 1. Illustration of the immunoproteomics processes used to identify antigens common to autoantibody profiles in cancer patient serum and MHC-associated peptides and parent proteins from ovarian tumor cell lines.

Figure 2.
Autoantigens common to humoral and cell-mediated immunity grouped according to cellular function.

Table 1

Serum autoantibody-reactive autoantigens present in five of five cancer composites and autoantigens that are also MHC-processed and presented on cell lines OVCAR3 and SKOV3-A2.

Parent Protein Identity	SwissProt No.	Functional category	Peptide
A-kinase anchor protein 9	/:spt Q99996	Signal transduction	VDSVVITESD
Eukaryotic translation initiation factor 4 gamma	/:spt Q04637	regulation of translational initiation	AIIEEYLHL [*] VLMTEDIKL [*]
Midasin (MIDAS-containing protein)	/:spt Q9NU22	Putative nuclear chaperone	SLADDSVLRL [*]
RAD50	/:trm Q92878	DNA repair/recombination; telomere repair	EYVEKFYRI
Talin 1	/:spt Q9Y490	Cytoskeletal structure; motility; binds to vinculin	ALNELLQHV [*]
Vinculin	/:spt P18206	Cell adhesion; motility	ESILEPVAQQ
Vimentin	/:spt P18206	Cell adhesion; motility; interacts with vinculin	NLAEDIMRL [*] SLQEEIAFL [*] KLEGEESRISL [*]
Centrosome-associated protein 350 (CAP350)	/:trm Q8WY20	Centrosome-microtubule anchoring	ILSKKDLPL [*]

^{*} Peptides with HLA-A2 motif

Table 2

Serum autoantibody-reactive autoantigens present in four of five cancer composites and autoantigens that are MHC-processed and presented on cell lines OVCAR3 and/or SKOV3-A2

Parent Protein Identity	SwissProt No.	Functional category	Peptide
60S ribosomal protein L34, mitochondrial precursor (L34mt)	/:spt Q9BQ48	Structural component of ribosome	IKRKNKHGW
Cullin homolog 7 (CUL-7)	/:spt Q14999	endothelial proliferation and/or differentiation	SLHTELSV [*] YMADRLLG [*]
Hypothetical protein KIAA0090	/:trm Q14700	membrane protein of unknown function	LLIDDEYKV [*]
Glycogen debranching enzyme (Glycogen debrancher)	/:spt P35573	Glycogen degradation	TIAEVGKWLQA
Probable RNA-dependent helicase p68 (DEAD-box protein p68)	/:spt P17844	RNA-dependent ATPase	YLLPAIVHI [*]
Serine/threonine-protein kinase tousled-like 1	/:spt Q9UK18	Intracellular protein transport; signaling	MDELASLDP
TBP-associated factor 172 (TAF-172) (TAF(II)170)	/:spt O14981	Regulation of transcription	SLADVHIEV [*]
ZID, zinc finger protein with interaction domain	/:trm Q15916	Regulation of transcription	ALKKHLTSV [*]

* Peptides with HLA-A2 motif

Table 3

Serum autoantibody-reactive autoantigens present in three of five cancer composites and autoantigens that are MHC-processed and presented on cell lines Ovar3 and/or SKOV3-A2

Parent Protein Identity	SwissProt No.	Functional category	Peptide
Inositol 1,4,5-trisphosphate receptor type 3	/:spt Q14573	Signal transduction	TLFNVIKSV*
Microtubule-associated protein 1A (MAP 1A) Proliferation-related protein p80	/:spt P78559	Cell structure	ETELTYPTN
Golgi autoantigen, golgin subfamily A member 3 (Golgin-160)	/:spt Q08378	Golgi transport	RLDSELKEL*
ATP-binding cassette, sub-family A, member 3	/:spt Q99758	Nucleotide binding and membrane transport	EQVFLSFAH
DNA-dependent protein kinase catalytic subunit (EC 2.7.1.37)	/:spt P78527	molecular sensor for DNA damage	LLQDFNRFL*
ADP-ribosylation factor GTPase-activating protein 3	/:spt Q9NP61	Intracellular protein transport and secretion	FASHVSPEV
Inner centromere protein	/:spt Q9NOS7	Cytokinesis; mitosis	EFSKEPELM
C-jun-amino-terminal kinase interacting protein 2	/:spt Q13387	regulation of JNK cascade, anti-apoptosis	FDVOFLGSVE
Death associated transcription factor 1	/:spt Q9BTC0	Tumor suppressor	SPLEDLSPCPA
Ribosome-binding protein 1 (Ribosome receptor protein)	/:spt Q9P2E9	Protein targeting and transport	ALQKRLDEV*
FKBP-rapamycin associated protein (FRAP)	/:spt P42345	Cell cycle regulation	AIASLIGVEG
Ras-related protein Rab-27A	/:spt P51159	GTPase	LDLIMKRME
Plakophilin 2	/:spt Q99959	Beta catenin family Cell adhesion	GLIDSLVHYV
DRIM protein	/:trm O75691	negative regulation of cell proliferation	ALMELFPKL* SLLDLHTKV*
Nuclear protein NP220	/:trm Q14966	RNA/DNA binding; splicing	EEDEDSLAD
Transcriptional repressor CTCF (CCCTC-binding factor)	/:spt P49711	Putative tumor suppressor	NMEEQPINI*
Timeless homolog	/:trm Q9UNS1	epithelial cell morphogenesis	AILAHLNTV*
Centriole associated protein CEP110	/:trm Q9Y489	RNA Pol II transcription factor	NLNQFLPEL*
Colon cancer-associated protein Mic1	/:trm Q9Y5M0	Marker for p53 activation	LHELVIKTL

* Peptides with HLA-A2 motif