Autoantibody Profiling for Cancer Detection

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- Antibody • Tumor antigen • Cancer detection • Proteomics
- Protein microarray

It is widely appreciated that making major inroads in the fight against cancer is going to depend, in part, on early detection. Detecting cancers based on serum profiling is a particularly attractive concept because a single draw of blood would allow screening for different cancers. There is increasing evidence for a humoral immune response to cancer in humans, as demonstrated by the identification of antibodies against a number of tumor antigens in patients with various tumor types.[1–7] Proteins not present in normal cells may elicit a host immune response that affords a dramatic amplification of signal in the form of antibodies relative to the amount of the corresponding antigen. The immune response occurs early during tumor development and as a result the presence of autoantibodies against tumor antigens in serum might provide an effective means for cancer screening and early diagnosis.

One of the most investigated humoral immune response targets in cancer is the tumor suppressor p53. The p53 gene plays a critical role in maintaining genomic integrity and the p53 pathway is frequently inactivated in almost all common cancer types.[8–10] In 1982, Crawford and colleagues[11] first demonstrated the presence of antibodies against human p53 proteins in 9% of breast patient sera. Subsequently, anti-p53 autoantibodies were detected in patients with a variety of cancers including ovarian, liver, colon, lung, pancreas, and prostate.[12–14] Poor prognosis was shown to be associated with the occurrence of antibodies against p53 among colon cancer patients.[15] Although there is a strong correlation between p53 missense mutations and p53 autoantibodies, some patients have antibodies in the absence of detectable p53 mutations, suggesting that overexpression of wild-type p53 may be sufficient to induce a humoral response. The prevalence of anti-p53 antibodies ranges from 3% to 30% depending on the cancer type. Although it is unlikely that cancer or precancerous conditions can be screened for or diagnosed based only on the detection of p53 antibodies, it is likely that the identification of panels of antigens that induce a humoral response in a cancer type will allow detection of cancer at an early stage.

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CLASSIFICATION OF TUMOR ANTIGENS

Tumor antigens can be loosely categorized into four groups: (1) tumor antigens encoded by genes that are linked to tumorigenesis (eg, L-*myc*, c-*myb*, p21 *ras*, HER2/neu, cyclin B1, survivin, livin);[16–22] (2) tumor antigens encoded by genes referred to as "cancer/testis antigens" whose expression is restricted to tumors and germ cells;[23] (3) tumor antigens encoded by genes that are mutated in tumors (eg, p53);[24,25] and (4) tumor antigens encoded by genes whose protein products exhibit aberrant posttranslational modifications, such as glycosylation or proteolytic cleavage (eg, annexin I, calreticulin).[26,27]

It is not clear why certain proteins can elicit an autoantibody response with tumor development. The mechanisms responsible for induction of immune reactivity against tumor antigens are ill defined.[28] The diversity of tumor antigens may indicate different underlying mechanisms for the autoantibody response directed against them. Although tumor antigens expressed on the cell surface are readily accessible for an immune response, intracellular tumor antigens may become immunogenic as a result of distinct processes associated with tumor development and progression including apoptosis and inflammation, which affect antigen presentation and promote a humoral immune response.

For most antigens identified to date, only a subset of patients with a tumor type develops a humoral response to a particular antigen. Tumors exhibit substantial heterogeneity in protein expression, which may partially explain why heterogeneity in the immune response to particular antigens may occur. Other factors, such as major histocompatibility complex (MHC) polymorphisms, may also influence the immune response against a particular antigen.

STRATEGIES FOR IDENTIFICATION OF TUMOR ANTIGENS

Ever since the early observations of cancer immunity, efforts have been made to identify antigens that can trigger a humoral immune response. Early attempts[29–32] relied on an approach referred to as "autologous typing" with a focus on cell-surface antigens, whereby cancer patient sera were reacted with autologous cancer cells. Although several antigens profiled by this approach were defined later,[33,34] it was difficult to purify these antigens for further characterization partially because of the low titers of cancer autoantibodies. Advances in biochemical and molecular biologic techniques have been applied to the discovery of novel tumor antigens resulting in substantial expansion of the tumor antigen repertoire. The potential clinical use of individual antigens has been limited, however, because of poor predictive value. There is a substantial need to develop novel technologies to discover tumor antigen marker panels with the specificity and sensitivity necessary for early screening and diagnosis of cancer. A brief discussion on current strategies for the detection of tumor antigens that induce a humoral immune response follows.

A Candidate-Based Approach

A targeted strategy with predefined candidates is a trial and error approach. A candidate antigen can be "guessed" and "tested" by ELISA or Western blot analysis. For example, because cytoplasmic tyrosine kinase (cSrc) was found to be overexpressed, activated, and in some cases mutated in carcinoma, it was investigated as a tumor antigen. Autoantibodies against three proteins (cSrc; Fyn, another member of the Src family not found to be activated in cancer; and Csk, a COOH-terminal Src tyrosine kinase that downregulates cSrc activity) were assessed by ELISA. Although no autoantibodies were detected against cSrc or Fyn, up to 20% of cancer patients with carcinoma had high-affinity autoantibodies against Csk.[35] A requirement for this approach is the availability of antigens, which can be either purified from cancer cell cultures or produced from a recombinant expression source, such as bacteria, yeast, or insect cells. The purity of recombinant proteins could affect assay results.[36]

Affinity-Based Enrichment

A variation on absorption-based assays as an example of affinity-based approaches is a column-based strategy in which two separate affinity columns are prepared from immobilized immunoglobulins (IgG in most cases) isolated from pooled sera from cancer patients or healthy controls. Proteins from cancer cell lysates are first passed through the affinity column coupled with IgG from healthy controls, then through the column coupled with IgG from cancer patients. Proteins bound to the second column are eluted, characterized, and served as candidate tumor antigens for further validation.[37]

Major Histocompatibility Complex II Bound Peptides

The presentation of peptides derived from tumor antigens by MHC class II molecules is a necessary step in the process of humoral immune response. Tumor antigens can be deduced from the sequence of peptides bound to MHC class II molecules. In one study, peptides eluted from HLA-DR molecules on human monocyte-derived dendritic cells that were pulsed with necrotic melanoma cells were sequenced by a combination of two-dimensional capillary liquid chromatography and electrospray ionization tandem mass spectrometry. Peptides originating from potential tumor antigens were identified using this approach including one epitope derived from the melanoma-associated protein melanotransferrin.[38]

Recombinant Escherichia coli*/Phage/Combinatorial Libraries*

SEREX based on expression library screening has proved to be a useful strategy for tumor antigen identification and has been relied on for studies of many different cancer types.[39] A vast number of entries have been deposited into the Cancer Immunome Database (https://www2.licr.org/CancerImmunomeDB/), which was developed to share tumor antigens discovered using SEREX. In this approach, cancer patient and healthy control sera are used to probe cDNA expression libraries constructed from tumor tissues, cancer cell lines, or testes. Clones that exhibit differential reactivity between the two sera groups[40–43] are isolated and identified. A high-throughput approach has been developed using phage display of cDNA libraries from cancer sources. In one study, a T7 phage display cDNA library was constructed from an ovarian cancer cell line.[44] Four rounds of differential biopanning were performed with negative and positive selection using pooled sera from healthy women and a late-stage ovarian cancer patient. Individual plaques from the selected phage library were arrayed on nitrocellulose membranes and screened with cancer and normal control sera for differential reactivity. A variation on the cDNA library approach is to use random peptide library phage display to select peptides recognized by circulating antibodies in cancer patient sera. This strategy was applied to prostate cancer and resulted in the identification of a member of the heat shock protein family, GRP78, as a tumor antigen.[4]

Proteomic Approaches

Proteomic approaches for tumor antigen identification have been increasingly recognized for canvassing individual proteins in the whole proteome, in their modification states as they occur in cells, for their antigenicity in a high-throughput multiplexed manner. Given that proteins are subject to post-translational modifications, proteomic approaches hold the promise to discover antigens with epitopes that result from post-translational modifications.

The authors initially implemented a proteomic approach for the identification of tumor antigens that elicit a humoral response using two-dimensional polyacrylamide gel electrophoresis (PAGE) to separate cellular proteins from tumor tissue or tumor cell lines and then screening sera from cancer patients for antibodies that react against the separated proteins by Western blotting. Proteins that specifically react with sera from patients with the same tumor type are identified by mass spectrometry. This strategy has been applied to several tumor types leading to the identification of multiple antigens that have potential as cancer markers for early diagnosis through their detection in serum or through serum profiling for corresponding autoantibodies. Some of the identified antigens have moved to an independent validation phase.[7,27,45–48]

In one study, proteins from a pancreatic tumor cell line (Panc-1) were separated by two-dimensional PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes.[48] Sera obtained from 36 newly diagnosed patients with pancreatic cancer, 18 patients with chronic pancreatitis, 33 patients with other types of cancers, and 15 healthy donors were screened individually for the presence of antibodies to Panc-1 pancreatic tumor cell line proteins. Autoantibodies were detected against either one or two calreticulin isoforms identified by mass spectrometry in sera from 21 of 36 patients with pancreatic cancer. One of 18 chronic pancreatitis patients and 1 of 15 healthy controls demonstrated autoantibodies to calreticulin isoform 1; none demonstrated autoantibodies to isoform 2. None of the sera from patients with colon cancer exhibited reactivity against either of the two isoforms. One of 14 sera from lung adenocarcinoma patients demonstrated autoantibodies to calreticulin isoform 1;2 of 14 demonstrated autoantibodies to isoform 2. Calreticulin antibodies were also identified in sera from patients with liver cancer.[26] Remarkably, the isoform that elicits antibodies in liver cancer is different from the isoforms that elicit antibodies detectable in sera from subjects with pancreatic cancer, providing a clear illustration of the merits of a proteomic approach for identification of diagnostic cancer antigens based on the analysis of natural proteins, as opposed to synthetic peptides or recombinant proteins that do not have representation of the various isoforms present in tumor cells that result from post-translational modifications and processing of proteins.

The major bottleneck for two-dimensional Western blotting has been the laborious nature of preparing two-dimensional gels from lysates followed by transfer of proteins onto membranes for screening with individual sera. This has limited both the number of sera that could be screened and the number of cell lines and tumors for which this screening approach could be applied. The authors have explored a complementary approach for comprehensive analysis of proteins in their modified forms, which is to array protein fractions following liquid-based separation of lysates isolated from tumor cells and tissues for high throughput screening for tumor antigens that react with antibodies in patient sera. This type of array is referred to as “natural protein microarray.” Proteins in reactive fractions are identified by mass spectrometry.

Although the concept of a “multianalyte microspot immunoassay” system was first proposed back in the late 1980s,[49] it was not until the development and success of DNA microarrays that protein microarrays gained attention, with substantial progress in recent years. Protein microarrays have emerged as a promising approach to meet the pressing need for systematic analysis of thousands of proteins in parallel. Protein microarrays that contain natural proteins derived from tumor cells have the potential to substantially accelerate the pace of discovery of tumor antigens to yield a molecular signature for immune responses directed against protein targets in different types of cancer. In a study of colon cancer, microarrays printed with 1760 distinct protein fractions, prepared from the LoVo colon adenocarcinoma cell line, were hybridized with individual sera.[50] A fraction that exhibited IgG-based reactivity with 9 of 15 colon cancer sera was found to contain ubiquitin C-terminal hydrolase L3 by tandem mass spectrometry (ESI-Q-TOF). The highest levels of ubiquitin C-terminal hydrolase L3 mRNA among the 329 tumors of different types analyzed by DNA microarrays were found in colon tumors. Independent validation by Western blotting demonstrated that ubiquitin C-terminal hydrolase L3 antibodies existed in 19 of 43 sera from patients with colon cancer and in 0 of 54 sera from subjects with lung cancer, colon adenoma, or otherwise healthy. These preliminary findings led to the conclusion that a natural protein microarray approach has the necessary sensitivity and throughput for the identification of tumor antigens that have induced an antibody response in patients with specific cancers. By using protein extracts from a cancer cell line or from one or more tumor tissues, limitations of other approaches based on recombinant proteins or peptides that lack epitopes resulting from post-translational modifications are overcome.

The application of protein microarrays also encompasses the use of recombinant proteins and peptides with the advantage of requirements of minimal volumes of serum for clinical samples, in addition to their high throughput.[51–53]

AUTOANTIBODIES IN LUNG CANCER

Lung cancer is the leading cause of cancer deaths among men and women in the United States. Lung cancer caused an estimated 160,390 deaths in 2007, accounting for 28.7% of all cancer deaths. The chance for a successful treatment is much higher when cancer is diagnosed at an early stage. Lung cancer has been associated with a paraneoplastic cerebellar degeneration syndrome with occurrence of antineuronal antibodies, such as anti-Hu, anti–P/Q-type voltage-gated calcium, and anti–N-type voltage-gated calcium.[54–61] In one study, using recombinant recoverin, a neuronal calcium-binding protein, as an antigen, autoantibodies against recoverin (anti-Rc) were detected by Western blotting in sera from 15% of patients with small cell lung cancer and from 20% of patients with non–small cell lung cancer. Only two anti–Rc-positive cases were observed among 86 patients with nonmalignant pulmonary disorders and none among 50 healthy individuals.[62]

Targeted studies resulted in the discovery of several antibodies against oncogenes, tumor suppressor genes, and genes that are important in cancer development. For example, anti–c-Myc and anti–L-Myc antibodies were reported to occur in 13.2% and 10% lung cancer patients compared with 3.3% and 0% in healthy controls, respectively.[22,63] Several studies of p53 in lung cancer have been reported.[12,64] Although the frequencies of serum p53 antibodies were statistically higher in lung cancer patients than in the control subjects in most studies, the association of antibodies with clinical parameters, such as survival, is still not well-defined.

Survivin and livin are members of the inhibitor of apoptosis protein family and are highly expressed in cancer cells, but show little or no expression in normal tissues. Rohayem and colleagues[65] reported that 21.6% of lung cancer patient sera (N = 51) reacted with purified recombinant survivin in an ELISA. The presence of antibodies against p53 was also tested for the same collection of sera, and four sera from lung cancer patients contained anti-p53 antibodies (7.8%). Yagihashi and colleagues[20] examined the prevalence of anti-livin and anti-survivin antibodies in lung cancer patients with ELISA using recombinant proteins. Nineteen (51.3%) of 37 lung cancer patients were positive for anti-livin antibodies. Thirty-one samples from the same lung cancer patients were also assayed for anti-survivin antibodies. Eighteen patients (58.1%) were positive for anti-survivin antibodies and 21 patients (71%) were positive for antibodies to survivin, livin, or both. Intensity of anti-livin antibody responses did not correlate with intensity of anti-survivin responses.

In another targeted study, Chang and colleagues[66] examined whether serum from non–small cell lung cancer patients exhibited immunoreactivity against the antioxidant enzyme peroxiredoxin-I using Western blotting after their discovery of Prx-I overexpression in non–small cell lung cancer tissue. They found that 25 (47%) of 53 non–small cell lung cancer patient sera had autoantibodies against Prx-I, whereas only four (8%) sera from 50 healthy subjects showed reactivity to Prx-I. Prx-I itself was detected in the sera from 18 (34%) of 53 non–small cell lung cancer patients but in only one serum from 50 controls. Moreover, 17% of non–small cell lung cancer sera were positive for both Prx-I antibody and antigen but none of control sera. It is interesting to note that Prx-I was found to be secreted by lung adenocarcinoma cells (A549 cell line) but not by noncancerous lung cells (BEAS 2B) or breast cancer cells (MCF7).

Tan and coworkers[67] examined antibody frequencies for a panel of seven tumor-associated anitgens (TAAs) (c-*myc*, cyclin B1, IMP1, Koc, p53, p62, and survivin) in 527 cancer patients (64 breast cancers, 45 colorectal cancers, 91 gastric cancers, 65 hepatocellular carcinomas, 56 lung cancers, and 206 prostate cancers) and 346 healthy controls. Recursive partitioning was used to assess whether each subject could be accurately classified based on his or her antibody reactivity profile to the seven TAAs. In the case of lung cancers, the classification tree had a sensitivity of 0.8 at a specificity of 0.90 when normal means $\pm$ 2 SDs were used as standard cutoffs for immunoassay positivity. Antibody to cyclin B1 was the initial discriminating node for lung cancers.[68]

In 1998, Gure and colleagues[3] first applied SEREX to lung cancer. Serologic analysis of a recombinant cDNA expression library constructed from a lung adenocarcinoma with the autologous patient serum led to the isolation of 20 clones representing 12 different genes. Embryonic neural proteins were identified as tumor antigens by screening cDNA libraries derived from small cell lung cancer cell lines using pooled sera of small cell lung cancer patients.[69] Brass and colleagues[70] screened a cDNA library generated from a lung squamous carcinoma with autologous patient serum and 35 clones representing 19 genes were isolated. Diesinger and colleagues[71] generated two cDNA libraries from squamous cell lung carcinoma and isolated 15 immunogenic antigens (including *eIF-4 G*) using autologous sera. In a follow-up study, antibodies against recombinant *eIF-4 G* were detected in five (15%) of 33 heterologous sera from lung squamous carcinoma patients, but not in 17 control sera from individuals without tumors and 17 sera from patients with squamous cell carcinoma of the head and neck.

Zhong and colleagues[72] introduced the application of T7 phage display libraries to the identification of circulation antibodies to non–small cell lung cancer antigens. Sera from healthy individuals were used for negative selection. Forty-five immunoreactive phage clones were identified as having significant sequence identity with cDNA from known or suspected tumor-associated proteins. Antibodies against five phage-expressed proteins (HSP70, HSP90, p130, GAGE, and BMI-1) were measured using ELISA in patient (N = 40) and control sera (N = 49). HSP70 had the best performance with an area under the curve of 0.73. The combined area under the curve was 0.84. Zhong and colleagues[73] later implemented a protein microarray approach to T7 phage display analysis and identified five genes (GAGE7, BAC clone RP11-499F19, SEC15L2, PMS2L15, and EEF1A) that have a combined diagnostic accuracy of 88.9%.

Proteomic approaches have led to the identification of tumor antigens in lung cancer. In one study, sera obtained at the time of diagnosis from 64 patients with lung cancer were investigated for the presence of IgG antibodies to A549 adenocarcinoma cell line proteins and to autologous tumor tissue proteins that were separated by two-dimensional gel electrophoresis. Serum from nine of 64 patients with lung cancer (**Table 1**), consisting of six patients with adenocarcinoma, two with squamous cell carcinoma, and one with SCLC, exhibited IgG-based reactivity against a group of three proteins with an estimated molecular weight of 25 kd and with a pI between 5 and 5.6 (**Fig. 1**). Reactivity was specific to IgG1 among the IgG subtypes examined (IgG1–4). The identity of this set of proteins was determined by mass spectrometry after trypsin digestion and corresponded to protein gene product 9.5 (PGP 9.5). The lung cancer specificity of PGP 9.5 autoantibodies was determined by screening sera from 99 patients with other types of cancer and 71 sera from noncancer controls (see **Table 1**). Only one serum in the cancer control group, from a patient with hepatocellular carcinoma, exhibited immunoreactivity against PGP 9.5 proteins. The noncancer control group consisted of sera from 61 healthy subjects, including 15 chronic smokers, and from 10 patients with chronic lung disease. Only one serum (from a healthy postpartum, nonsmoker female subject) exhibited immunoreactivity against PGP 9.5 proteins.

Increased levels of PGP 9.5 mRNA and protein have been previously reported in non–small cell lung cancer tissue based on serial analysis of gene expression and immunohistochemistry.[74,75] To determine the cellular distribution of PGP 9.5 and its possible occurrence as a secreted protein, subcellular compartments from the A549 adenocarcinoma cell line were investigated by Western blotting. PGP 9.5 was readily detected in the membrane and the secreted protein fractions. Interestingly, two sera from lung cancer patients that did not contain autoantibodies against PGP 9.5 exhibited circulating PGP 9.5 protein. Circulating PGP 9.5 protein was not detected in

Table 1
Anti–PGP 9.5 autoantibodies in subject sera

	Number of Subjects	PGP 9.5 Auto AB Positive
Lung cancer	**64**	**9**
Adenocarcinoma	40	6
Squamous cell carcinoma	18	2
Small cell carcinoma	4	1
Large cell carcinoma	2	0
Other types of cancer	**99**	**1**
Brain cancer	14	0
Neuroblastoma	23	0
Breast cancer	11	0
Melanoma	7	0
Liver cancer	44	1
Other controls	**71**	**1**
Healthy nonsmokers	46	1
Chronic smokers	15	0
Chronic lung disease	10	0

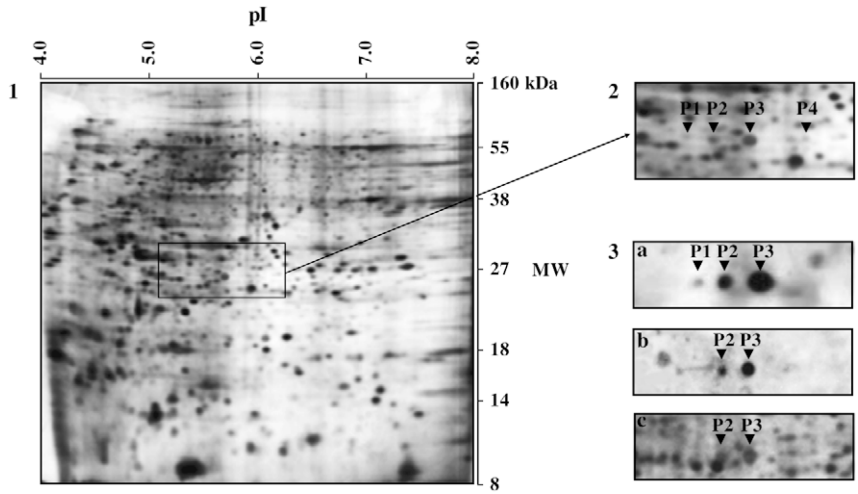

Fig. 1. Two-dimensional PAGE and Western blot analysis of A549 lung adenocarcinoma cell proteins. Panel 1 shows the A549 two-dimensional protein pattern after silver staining. The boxed area is shown on panel 2, in which arrows point to the location of PGP 9.5 forms (spots P1–P3) recognized by sera from patients with lung cancer and the position of the form P4, which is recognized by a polyclonal rabbit anti–PGP 9.5 antiserum that also recognizes P1 to P3. Panel 3 shows close-ups of Western blots from A549 cell proteins (a) and tumor tissue proteins from a patient with lung cancer (b) hybridized with his autologous serum showing reactivity against PGP 9.5 proteins. A close-up of the autologous tumor tissue two-dimensional protein pattern stained with silver nitrate (c) shows P2 and P3 PGP 9.5 variants.

any of the control sera. In all, PGP 9.5 was found to elicit autoantibodies or to occur in circulation among newly diagnosed patients with lung cancer.

In another study following a similar approach as for PGP9.5, a set of sera from 54 newly diagnosed patients with lung cancer and 60 patients with other cancers and from 61 non-cancer controls were investigated. Sera from 60% of patients with lung adenocarcinoma, 33% of patients with squamous cell lung carcinoma, but none of the non-cancer controls had IgG-based reactivity against proteins identified as glycosylated annexins one and II in A549 two-dimensional Western blots (**Table 2**). Positive sera were generally reactive against annexins I and II at the highest serum dilution tested, which was one per 1000. Reactivity was not limited to patients with advanced-stage disease. Sera showed similar reactivity against annexins I and II in autologous tumor protein blots and in blots prepared from normal lung tissue, as in A549-derived blots. Sera from lung cancer patients that exhibited IgG-based reactivity against annexins I or II exhibited reactivity that was specific to IgG1 among the IgG subtypes examined, and also exhibited IgM-based reactivity. None of the sera from other cancer types or from noncancer controls exhibited autoantibodies against annexin II. Sera from six of 60 patients with other types of cancers, namely four of 17 with esophageal cancer, one of 14 with brain tumor, and one of 11 with breast cancer, exhibited annexin reactivity.

Annexin expression in lung tumors was assessed by immunohistochemistry, using monoclonal antiannexin I and II antibodies. Annexin I was abundantly expressed in a diffuse manner in most adenocarcinomas and squamous cell carcinomas. Intense annexin II immunoreactivity was also detected in most tumors in a predominantly

Table 2
Antiannexins I and II autoantibodies in subject sera

	Number of Subjects	Annexin I Auto Ab Positive	Annexin II Auto Ab Positive
Lung cancer	**54**	**16**	**18**
Adenocarcinoma	30	12	11
Squamous cell carcinoma	18	3	4
Small cell carcinoma	4	1	2
Large cell carcinoma	2	0	1
Other types of cancer	**60**	**6**	**0**
Brain cancer	14	1	0
Breast cancer	11	1	0
Melanoma	7	0	0
Liver cancer	11	0	0
Esophageal cancer	17	4	0
Other controls	**61**	**0**	**0**
Healthy subjects	51	0	0
Chronic lung disease	10	0	0

membranous pattern. There were no appreciable differences in annexin I and II expression, by immunohistochemical analysis, between autoantibody positive and negative lung cancer patients. Interestingly, annexin I was subjected to N- or O-deglycosylation. The resulting products were separated by SDS electrophoresis and analyzed by Western blotting. N-deglycosylation by endoglycosidase F induced a basic shift of the protein, whereas no change was observed after O-glycosidase treatment, compared with untreated annexin I. Two sera were tested that exhibited IgG-based immunoreactivity against annexin I. These sera did not react against endoglycosidase F–treated annexin but exhibited IgG-based immunoreactivity against annexin I after O-glycosidase treatment.

A recent study by Pereira-Faca and colleagues[76] screened two sets of newly diagnosed lung cancer sera for their reactivity by Western blotting with chromatographically fractionated protein extracts from three lung cancer cell lines (A549, H23, and H522). One set consisted of sera from 19 newly diagnosed subjects with lung adenocarcinoma and 19 matched controls. A second independent set consisted of sera from 26 newly diagnosed subjects with lung adenocarcinoma and 24 controls matched for age, gender, and smoking history. One protein that exhibited significant reactivity with both sets of cancer sera ($P = .0008$) was confidently identified by mass spectrometry as 14-3-3 theta. Remarkably, significant autoantibody reactivity against 14-3-3 theta was also observed in an analysis of a third set consisting of 18 prediagnostic lung cancer sera collected as part of the Beta-Carotene and Retinol Efficacy Trial (CARET) cohort study, relative to 19 matched controls ($P = .0042$). A receiver operating characteristic curve constructed with a panel of three proteins consisting of 14-3-3 theta identified in this study, plus annexin 1 and PGP 9.5 proteins previously identified as associated with autoantibodies in lung cancer as described previously, gave a sensitivity of 55% at 95% specificity (area under the curve = 0.838) in discriminating lung cancer at the preclinical stage from matched controls.

Natural protein microarrays[77] were also applied to the discovery of tumor antigens in lung cancer. In one study,[78] the lung adenocarcinoma cell line A549 was relied on to determine reactivity of its arrayed proteins with sera from lung cancer patients and controls. A total of 150 mg of A549 proteins was separated into 20 fractions by isoelectric focusing. Each fraction was subjected to reverse-phase high-performance liquid chromatography and 92 fractions were collected. A total of 1840 fractions were printed in duplicate onto nitrocellulose slides. Slides were hybridized each with a serum sample and autoantibody reactivity was detected by an indirect immunofluorescence method.[79] Two typical images of antigen slides are shown in **Fig. 2**. A distinct reactivity pattern in each slide is clearly observed in a comparison of the two images. A total of 19 lung cancer and 14 control seras were investigated. For each fraction, the number of cancer sera that gave higher fluorescent intensity than the second highest normal serum was determined. The number of fractions expected to have "N" or more patient samples bigger than the second biggest normal was estimated by simulation. **Table 3** provides a summary of the statistical results. The probability that nine or more cancer sera has values for a spot higher than the second largest control is less than 0.008. At this significance level, the number of fractions expected by chance is 15. The number of fractions that fit the criteria was 63, however, which was 4.1 times more than expected by chance alone.

An important promise of autoantibodies to tumor antigens is their potential for early detection. In a recent study, proteins from human lung adenocarcinoma cell line A549 lysates were subjected to extensive fractionation.[80] The resulting 1824 fractions were spotted in duplicate on nitrocellulose-coated slides. The microarrays produced were used in a blinded validation study to determine whether annexin I, PGP 9.5, and 14-3-3 theta antigens previously found to be targets of autoantibodies in newly diagnosed subjects with lung cancer are associated with autoantibodies in sera collected at the presymptomatic stage and to determine whether additional antigens may be identified in prediagnostic sera. Individual sera collected from 85 subjects within a year before a diagnosis of lung cancer and 85 matched controls from the CARET cohort were hybridized to individual microarrays. The evidence obtained indicated the occurrence in lung cancer sera of autoantibodies to annexin I, 14-3-3 theta, and a novel lung cancer antigen, LAMR1, which precede onset of symptoms and diagnosis. The findings do provide supportive evidence for the potential use of autoantibodies for the diagnosis of lung cancer before onset of symptoms.

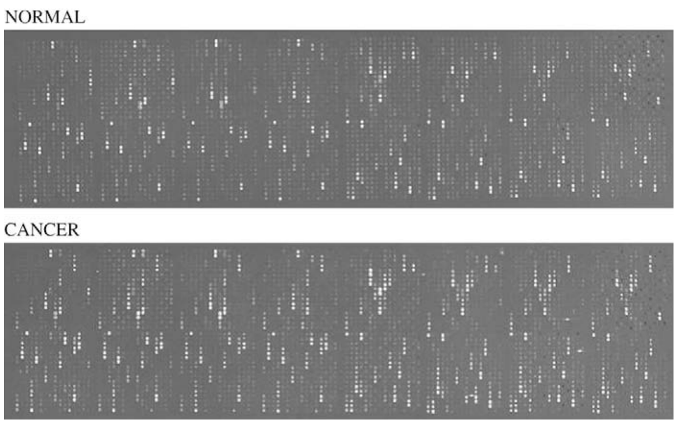

Fig. 2. Two typical slide images. The top one was hybridized with a normal serum sample and the bottom a lung cancer patient serum sample.

Table 3
Statistical analysis results (M2-statistic)

	M2-Statistic		
N	Numbers Obtained Experimentally	Numbers Expected by Chance	Obtained/Expected
0	1840	1840	1
1	1655	1474	1.12
2	1262	1050	1.20
3	879	688	1.28
4	607	422	1.44
5	406	244	1.66
6	255	133	1.91
7	174	69	2.53
8	106	34	3.16
9	63	15	4.11
10	34	6	5.21
11	13	3	5.02
12	8	1	8.67
13	4	0	NA
14	0	0	NA
15	0	0	NA
16	0	0	NA
17	0	0	NA

Column 2 shows the number of fractions with "N" or more cancer samples that gave higher intensity than the second largest normal sample obtained from the protein microarray experiment; column 3 shows the number expected by chance; column 4 shows the ratio of the number actually obtained to the number expected by chance. A total of 18 cancer and 15 normal samples were studied.

SUMMARY

The identification of tumor antigens that elicit an antibody-based immune response may be useful in cancer screening and immunotherapy. Clearly, tumors may develop in the presence of this antibody-mediated immune response. It has been increasingly realized that each individual autoantibody marker might have limited use because its classification performance by itself may not meet the requirement of a clinical assay. Identification of panels of tumor antigens that have restricted expression to particular cancer types and that induce autoantibodies, in combination with other clinical modalities, has the potential to yield a molecular signature for blood-based screening and diagnosis. The identification of tumor antigens that have elicited humoral immune responses also has direct relevance to cancer therapeutics because many of these antigens are also involved in cell-mediated immune reactions, which is the principal mechanism for tumor immunity.[81–85]

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