Occurrence of Autoantibodies to Annexin I, 14-3-3 Theta and LAMR1 in Prediagnostic Lung Cancer Sera

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ABSTRACT

Purpose

We have implemented a high throughput platform for quantitative analysis of serum autoantibodies, which we have applied to lung cancer for discovery of novel antigens and for validation in prediagnostic sera of autoantibodies to antigens previously defined based on analysis of sera collected at the time of diagnosis.

Materials and Methods

Proteins from human lung adenocarcinoma cell line A549 lysates were subjected to extensive fractionation. The resulting 1,824 fractions were spotted in duplicate on nitrocellulose-coated slides. The microarrays produced were used in a blinded validation study to determine whether annexin I, PGP9.5, and 14-3-3 theta antigens previously found to be targets of autoantibodies in newly diagnosed patients 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 patients within 1 year before a diagnosis of lung cancer and 85 matched controls from the Carotene and Retinol Efficacy Trial (CARET) cohort were hybridized to individual microarrays.

Results

We present evidence for 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.

Conclusion

Our findings suggest potential utility of an approach to diagnosis of lung cancer before onset of symptoms that includes screening for autoantibodies to defined antigens.

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INTRODUCTION

There is increasing evidence for a humoral immune response to cancer in humans, as demonstrated by the identification of autoantibodies against a number of intracellular and surface antigens in patients with various tumor types.¹⁻¹⁰ Interest in the humoral response against tumor antigens relates in part to the potential screening and diagnostic utility of autoantibodies and their corresponding antigens.

A number of circulating autoantibodies in lung cancer have been identified by screening expression libraries with patient sera.^{5,6,11-14} However, there is a need to identify additional autoantibody targets to increase specificity and sensitivity. Several proteomics methods are emerging as useful means to discover autoantibody biomarkers.^{15,16} The merit of a proteomic approach is that it allows proteins, in their post-translational modification states as

they occur in cells, to be analyzed for their antigenicity. Our previous studies using two-dimensional gels of lung tumor cell lysates and Western blotting uncovered autoantibodies in the sera of patients with lung cancer against annexin I, PGP9.5, and 14-3-3 theta proteins.¹⁷⁻¹⁹ More recently, we have implemented a method that uses liquid-based procedures to separate intact proteins in tissue and tumor cell lysates.^{20,21} Several hundreds of distinct protein-containing fractions are spotted onto microarrays, interrogated using various sources of sera, and quantitatively analyzed for bound antibodies. We successfully identified anti-PGP9.5 antibodies in lung cancer sera, anti-UCHL3 antibodies in colon cancer sera, as well as autoantibodies in prostate cancer sera, collected at the time of diagnosis, using this microarray approach²²⁻²⁴; thus, this established the potential of natural protein microarrays to uncover

antigens that induce an antibody response in cancer in a relatively high throughput approach.

In the present study, prediagnostic sera were used to determine whether a set of antigens consisting of annexin I, PGP9.5, and 14-3-3 theta, previously found to be associated with autoantibodies at the time of diagnosis, discriminate between cases and control before onset of symptoms and for the discovery of additional antigens. We present evidence for the occurrence of autoantibodies against a novel antigen, LAMR1, in lung cancer and provide evidence for occurrence of autoantibodies against annexin I, 14-3-3 theta, and LAMR1 in prediagnostic sera.

MATERIALS AND METHODS

Materials

Nitrocellulose-coated FAST slides were purchased from Whatman (Sanford, ME). Alexa 647-labeled antihuman immunoglobulin G (IgG) and recombinant protein arrays were purchased from Invitrogen (Carlsbad, CA).

Serum Samples

Serum samples and controls used in this study were obtained after patients and controls gave informed consent. Sera from newly diagnosed patients with lung cancer and matched controls were collected through the Community Clinical Oncology Program at the University of Michigan, Ann Arbor, MI. Prediagnostic blood samples from patients with lung cancer and matched controls were randomly chosen in pairs from the Carotene and Retinol Efficacy Trial (CARET) serum bank.^{25,26} The distribution of histology and time from blood draw to diagnosis for the 85 prediagnostic lung cancer samples are shown in Appendix Tables A1 and A2 (online only).

Natural Protein Microarray Production

A total of 50 mg of proteins from the human lung adenocarcinoma cell line A549 lysates were first separated by anion exchange high-performance liquid chromatography, followed by reverse-phase chromatography as described previously.²⁰ A total of 1,824 fractions were collected from the two-dimensional separation. Fr_XX_YY denotes the YYth fraction from the reverse-phase high-performance liquid chromatography of the XXth fraction from the anion exchange chromatography. Fractions were lyophilized and resuspended in 25 μ L of printing buffer (250 mmol/L of Tris-HCl, pH 6.8, 0.5% sodium dodecyl sulfate, 25% glycerol, 0.05% TritonX-100, 62.5 mmol/L of dithiothreitol). All 1,824 fractions, together with printing buffer as negative controls and purified human IgG as positive controls, were printed in duplicate onto nitrocellulose-coated slides using a contact printer. These slides were designated as full A549 natural protein microarrays. An A549 natural protein microarray containing selected fractions (targeted array) was produced in a similar way, whereby only selected fractions of interest (Fr_00_84, Fr_09_38, Fr_15_39 and Fr_15_46) were printed in duplicate on 16-pad FAST slides.

Detection of Autoantibodies in Serum Specimens

Serum samples were hybridized with protein microarrays using an indirect immunofluorescence protocol, and local background-subtracted median spot intensities for downstream statistical analysis were generated as described previously.²⁷

Mass Spectrometry Analysis

Samples were trypsin-digested and subjected to mass spectrometry analysis on an LTQ-Orbitrap (Thermo Fisher Scientific Inc, Waltham, MA) as described previously.²¹

Statistical Analysis

Intensity data were linear normalized to make the 25th and 75th percentiles of the distribution of the intensities for each sample agree exactly with the average of the 25th and 75th percentiles of all samples by

linear transformations. Linear normalized data were then standardized fraction by fraction. Each sample/fraction intensity was subtracted off the mean of the same fraction for all samples in the same printing batch and divided by the standard deviation of the fraction for all samples in the corresponding printing batch. A two-sample *t* test was applied to each single fraction to compare the difference in mean intensity between cancers and controls.

The discriminatory capacity of selected fractions was also evaluated by the receiver operating characteristic curve. The area under the curve (AUC) was calculated, which corresponds to the Mann-Whitney test statistic. Parallel analyses using a generalized version of the real boosting algorithm with 10-fold cross-validation was also performed on the 1,824 fractions in order to select the best combination of fraction(s) that can discriminate between cases and controls.^{28,29} The results were treated as part of a separate biomarker discovery process. Even if a promising fraction was identified as a previously identified antigen associated with lung cancer, it was treated as an independent confirmation of the previous findings rather than as part of the validation study, because that antigen has not been established for validation at the design stage of the study.

For combined analysis, the markers were integrated by a summation of the dichotomized markers, whereby each marker was dichotomized by its optimal cutoff point, which corresponds to the minimum classification error rate. The 95% CI band of the receiver operating characteristic curve was estimated from 500 bootstrap procedures. The combination rule should be treated as a discovered biomarker and requires further validation; however, it does provide information on the complementarities of informative antigens.

Fig 1. Reproducibility of natural protein microarrays. Quantitative reproducibility was assessed by hybridization of the same pooled sample on six different microarrays. Pearson correlations between replicate spots on each of the six different microarrays were all 0.99, and correlations between replicate microarrays were greater than 0.96. Representative scatter plots were presented. (A) Correlation of duplicate spots on the same array; (B) correlation of two different arrays hybridized with the same serum sample. RFU, relative fluorescent units.

RESULTS

Validation Study of Autoantibodies to Annexin I, PGP9.5, and 14-3-3 Theta in Prediagnostic Sera

Annexin I, PGP9.5, and 14-3-3 theta were previously identified as inducing an autoantibody response in lung cancer, based on two-dimensional Western blot analysis of sera from newly diagnosed patients with lung cancer.¹⁷⁻¹⁹ We have developed natural protein microarrays to screen tumor-derived proteins for antigens that induce autoantibodies, based on extensive protein fractionation followed by spotting of aliquots from individual fractions.³⁰ Natural protein-containing microarrays were used to investigate the occurrence of autoantibodies to annexin I, PGP9.5, and 14-3-3 theta reactivity in prediagnostic sera. Serum specimens from the CARET cohort, which consists of subjects at increased risk for lung cancer observed longitudinally,²⁶ were relied on to investigate the occurrence of autoantibodies to lung cancer antigens within a year before diagnosis. Each case and control pair was matched for age at enrollment (5-year intervals), sex, intervention arm (active vitamins or placebo), exposure population (asbestos or heavy smoker), baseline smoking status (active or former), year of enrollment, and year of blood draw. Eighty-five prediagnostic lung cancer specimens and an equal number of matched controls were used for this study (Appendix Tables A1 and A2).

A549 natural protein microarrays were prepared from two-dimensional separations of a batch of A549 cell lysates. Quantitative reproducibility of microarrays was assessed by replicate analysis. Reproducibility across microarrays was assessed by hybridization of the same sample on different microarrays. Reproducibility within slides was assessed by replicate spots on the same microarray. Correlation of replicate spot intensity measures in the same microarray was 0.99. Correlation of spot intensity measures between different microarrays hybridized with the same sera was 0.96 (Fig 1). The median IgG reactivity for cancer samples, across the entire spotted array, was similar to that for normal controls (data not shown). However, the number of fractions with significant *P* values less than .05 with measures for cancer greater than for control was higher than the number of fractions for which control was greater than cancer. Of 1,824 arrayed fractions, 68 fractions gave a *P* value less than .05 with mean intensity for cancer greater than control, whereas there were only 16 fractions with a *P* value less than .05 with mean intensity for controls greater than for cancer.

Annexin I was localized on the A549 natural protein microarrays in Fr_00_84. PGP9.5 was localized in Fr_09_38, and 14-3-3 theta was localized in Fr_15_46 based on reactivity with corresponding monoclonal antibodies. After blinded analysis of all sera, the prediagnostic cases had significantly higher mean annexin I autoantibody levels than

Fig 2. Reactivity of (A) annexin I, (B) 14-3-3 theta, (C) PGP9.5, and (D) LAMR1 containing spotted fractions with all 85 + 85 Carotene and Retinol Efficacy Trial sera.

those of controls (t test $P = .001$; Fig 2). The P value for 14-3-3 theta was also significant at .01 (Fig 2). On the other hand, the average PGP9.5 autoantibody reactivity of prediagnostic cases was not significantly different from that of matched high-risk controls from CARET ($P = .2$; Fig 2). These results were in concordance with prior results based on two-dimensional Western blot analysis of 18 of the 85 CARET prediagnostic sera used in the present study.¹⁹

Differential Reactivity to LAMR1 in Prediagnostic Lung Cancer Sera

A boosting logistic regression method with leave-ten-percent-out for cross-validation was initially used to determine whether additional arrayed proteins exhibited differential reactivity with prediagnostic cases relative to control sera. A spotted fraction (Fr_15_39), which was among the most informative in each of 10 iterations of the model-building procedure, yielded LAMR1 protein identification with high confidence by mass spectrometry based on mass spectral matching to 21 unique peptides in the LAMR1 sequence (65% coverage). The mean level of autoantibodies against LAMR1 in prediagnostic lung cancer sera was significantly higher than that in matched controls, with a P value of .017 (Fig 2). The combined AUC for all three antigens, annexin I, 14-3-3 theta, and LAMR1, in CARET prediagnostic sera versus matched controls was 0.73 (Fig 3). The sensitivity and specificity at the optimal cutoff point was 51% and 82%, respectively.

CARET lung cancer cases represented three groups: adenocarcinoma, squamous cell carcinoma, and other non-small-cell lung cancer (Appendix Table A1). An analysis of variance test was performed to determine correlation between autoantibody levels against annexin I, PGP9.5, 14-3-3 theta, LAMR1, and lung cancer subtypes. Annexin I, PGP9.5, and LAMR1 did not show significant reactivity difference among the three subtypes. A significant P value of .007 was obtained for 14-3-3 theta, with the adenocarcinoma and the squamous cell

Table 1. Reactivity Differences Between Cases in the 0 to 6 Months Group and the 7 to 12 Months Group

Antigen	Blood Draw to Diagnosis (months)	No.	Mean Reactivity	P^*
Annexin I	0-6	44	0.20	.72
	7-12	41	0.29	
PGP9.5	0-6	44	0.28	.05
	7-12	41	-0.11	
LAMR1	0-6	44	0.40	.07
	7-12	41	-0.05	
14-3-3 theta	0-6	44	0.40	.06
	7-12	41	-0.03	

*Two-sample t test.

carcinoma groups exhibiting lesser reactivity than other non-small-cell lung cancer. CARET cancer sera analyzed were collected within 1 year before diagnosis (Appendix Table A2). We looked for a relationship between autoantibody levels and the time from blood draw to diagnosis by stratifying the samples into two groups, one group with cases collected between 0 and 6 months (inclusive) before diagnosis and the other group between 7 and 12 months (inclusive) before diagnosis. The mean reactivity for cases in the 0 to 6 months group was higher than that for cases in the 7 to 12 months group, with t test P values of .05, .06, and .07 for PGP9.5, 14-3-3 theta, and LAMR1, respectively. There was equivalent reactivity between the two groups for annexin I (Table 1). When comparing cases in each group against matched controls, the differences were also more significant in the 0 to 6 months group than in the 7 to 12 months group for PGP9.5, 14-3-3 theta, and LAMR1 (Table 2).

Assessment of Autoantibody Reactivity to LAMR1 in Sera From Newly Diagnosed Patients With Lung Cancer

The occurrence of autoantibodies to LAMR1 in sera from newly diagnosed patients with lung cancer was determined using spotted microarrays containing purified recombinant LAMR1 that were reacted with sera from 45 newly diagnosed patients with lung cancer and from an equal number of healthy controls that were matched for age, sex, and time of blood collection. Increased LAMR1 reactivity among lung cancer sera relative to controls was observed based on two-sample t test ($P = .024$). Most of the patients with lung cancer (32 of 45 patients) had adenocarcinoma. A significant P value of .03 was also obtained for the 32 patients with adenocarcinoma relative to matched controls.

Fig 3. Combined receiver operating characteristic analysis of the LAMR1, annexin I, and 14-3-3 theta fractions based on reactivity with all 85 + 85 Carotene and Retinol Efficacy Trial sera. The yellow 95% CI band in the plot was estimated from 500 bootstraps. AUC, area under the curve.

DISCUSSION

Our findings indicate that autoantibodies against LAMR1, annexin I, and 14-3-3 theta were significantly elevated in preclinical sera of patients with lung cancer compared with matched high-risk controls who did not develop lung cancer during the period of follow-up. A combination of LAMR1, annexin I, and 14-3-3 theta autoantibodies yielded an AUC of 0.73 in preclinical lung cancer sera. Although autoantibodies to various cancer antigens have been reported in the sera of newly diagnosed patients with lung

Table 2. Reactivity Differences Between Cases and Controls in Different Groups Stratified by Blood Draw Time to Diagnosis

Antigen	Blood Draw to Diagnosis (months)	Cancer		Normal		P*
		Mean	SD	Mean	SD	
Annexin I	0-6	0.20	1.16	-0.197	0.80	.067
	7-12	0.29	1.25	-0.293	0.49	.006
	All	0.24	1.20	-0.244	0.67	.001
PGP9.5	0-6	0.28	1.11	-0.111	1.06	.093
	7-12	-0.11	0.64	-0.078	1.07	.886
	All	0.10	0.93	-0.095	1.06	.214
LAMR1	0-6	0.40	1.41	-0.158	0.49	.017
	7-12	-0.05	0.65	-0.207	1.06	.414
	All	0.18	1.13	-0.182	0.81	.017
14-3-3 theta	0-6	0.40	1.13	-0.254	0.60	.001
	7-12	-0.028	0.95	-0.132	1.13	.654
	All	0.20	1.06	-0.195	0.89	.010

Abbreviation: SD, standard deviation.

*Two-sample *t* test.

cancer,^{1,5,6,11-14,17,18,31-37} an important aspect of this study is testing for occurrence of autoantibodies in prediagnostic sera and demonstrating significant autoantibody reactivity against LAMR1, annexin I, and 14-3-3 theta. The sample size, 85 patients and an equal number of controls, and the characteristics of controls (heavy smokers or subjects who have been exposed to asbestos) are important features of this study.

We previously reported the occurrence of autoantibodies against annexin I, PGP9.5, and 14-3-3 theta in newly diagnosed patients with lung cancer.^{17,18} In this study, we have validated the occurrence of autoantibodies to annexin I and 14-3-3 theta in prediagnostic lung cancer sera using natural protein arrays, yielding significant *P* values of .001 and .01, respectively, for differences with matched controls. The occurrence of autoantibodies to LAMR1 in lung cancer reported here is a novel finding. The full-length *lamr1* gene encodes a 33-kDa precursor protein with 295 amino acids.³⁸ Its precursor and post-translationally modified forms serve diverse biologic functions in vivo. The 33-kDa precursor protein dimerizes after acylation to form the mature 67LR,³⁹ which was initially purified using affinity chromatography on Sepharose columns conjugated with laminin and designated as the 67-kDa laminin receptor (67LR).⁴⁰⁻⁴² The 33-kDa precursor is an evolutionarily conserved ribosomal protein associated with the 40S subunit of the translational machinery.^{43,44} A 44-kDa protein originally identified as an oncofetal antigen by Coggin et al was subsequently found to be encoded by the same gene as 67LR.^{45,46} The precursor also serves as the receptor for the prion protein in eukaryotic cells.⁴⁷ 67LR plays a role in cancer invasion and metastasis, related to its high affinity to laminin, an important component of basement membrane.⁴⁸ Overexpression of 67LR has been observed in melanomas, lymphomas, and epithelial tumors.⁴⁹⁻⁵¹ Expression of 67LR is correlated with poor prognosis in non-small-cell lung cancer.⁵² There is evidence that the monomeric membrane-associated 44-kDa oncofetal antigen/immature laminin receptor protein (OFA/iLRP), but not 67LR, is immunogenic,⁴⁵ providing support for our finding of autoantibodies in lung cancer. OFAs are expressed in fetal cells and a variety of cancers but are not present in normal neonatal or adult tissues.⁴⁶ Immunization of adult hamsters with irradiated fetal hamster or mouse cells provides strong immu-

nity to SV40-induced tumorigenesis.⁵³⁻⁵⁵ OFA/iLRP was later identified as the protective antigen on the membrane of rodent and human fetal and tumor cells.^{45,56-58} Studies of OFA/iLRP have largely focused on cellular immunity and its potential utility in T-cell based immunotherapy.⁵⁹⁻⁶⁵ Although humoral immune response could be induced in mice immunized with recombinant OFA/iLRP,⁶⁶ the occurrence of autoantibodies against OFA/iLRP in human patients with cancer has not previously been reported. Although OFA/iLRP is a glycosylated protein,⁶⁷ our data suggest that autoantibodies are not restricted to a glycan-containing epitope in OFA/iLRP, given the reactivity observed with recombinant LAMR1 in the sera of patients with lung cancer. This is consistent with previous findings that bacterially expressed recombinant OFA/iLRP was competent in inducing cytotoxic T lymphocyte-mediated target cell lysis.⁶⁴ It is interesting that OFA/iLRP expression was found to precede clear histologic evidence of malignant T cells or clinical lymphoma in irradiated mice that went on to develop T-cell lymphomas,⁶⁸ which is consistent with an early immune response during tumorigenesis and our demonstration of autoantibodies in preclinical sera.

In the present study, no significant difference in PGP9.5 reactivity was observed between prediagnostic cases as a group and controls, in contrast with prior findings based on analysis of sera collected at the time of diagnosis of lung cancer. This may be related to differences in patient and tumor characteristics or to the temporal pattern of PGP9.5 expression and/or immune response to PGP9.5 in lung cancer. In support of the latter is the finding of increased reactivity among patients within 6 months from diagnosis compared with patients whose blood was collected 6 to 12 months before diagnosis. Nevertheless, autoantibodies to PGP9.5 may have diagnostic utility in symptomatic patients in conjunction with an imaging modality.

We have acquired for the first time data related to temporal changes of humoral immune response to a set of tumor antigens in lung cancer for patients whose blood was collected over a period ranging from the time of diagnosis to within a year before diagnosis as part of the CARET high-risk cohort. PGP9.5, 14-3-3 theta, and LAMR1 showed increase in reactivity in prediagnostic sera, with higher reactivity closer to diagnosis (0 to 6 months) relative to farther from diagnosis (7 to 12 months). Interestingly, however, reactivity to

annexin I did not show a relationship to time from diagnosis within the 1-year time frame. This may indicate that an immune response to annexin I occurred earlier during the course of lung cancer development compared with PGP9.5, 14-3-3 theta, and LAMR1. It has been demonstrated before that the sugar moiety on annexin I was critical for its antigenicity.¹⁸ We speculate that this post-translational modification may occur early during tumor development. Alternatively, a sugar-containing epitope may be more immunogenic than a peptide backbone yielding a detectable immune response early. Tumor antigens with different temporal reactive patterns may have different clinical utility for screening and diagnosis. These findings point to the value of prediagnostic sera in assessing the significance of autoreactivity to particular antigens.

Although significant reactivity with prediagnostic sera was observed in this study for a small panel of antigenic proteins, consideration of a screening modality for lung cancer that includes testing for autoantibodies would depend on the availability of additional antigenic targets to augment the sensitivity and specificity achieved in this study. An initial application of such a panel may be in conjunction with an imaging screening modality for patients at an increased risk for lung cancer. Studies aimed at identifying and validating novel

antigens are currently being undertaken through the National Cancer Institute Early Detection Research Network (<http://edrn.nci.nih.gov>) and through other efforts.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

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