Identification of 14-3-3θ as an Antigen that Induces a Humoral Response in Lung Cancer

Sandra R. Pereira-Faca,[1] Rork Kuick,[2,3] Eric Puravs,[2] Qing Zhang,[1] Alexei L. Krasnoselsky,[1] Douglas Phanstiel,[1] Ji Qiu,[1] David E. Misek,[2] Robert Hinderer,[2] Martin Tammemagi,[4] Maria Teresa Landi,[5] Neil Caporaso,[5] Ruth Pfeiffer,[5] Cim Edelstein,[1] Gary Goodman,[1] Matt Barnett,[1] Mark Thornquist,[1] Dean Brenner,[3] and Samir M. Hanash[1]

[1]Fred Hutchinson Cancer Research Center, Seattle, Washington; [2]Department of Pediatrics, University of Michigan; [3]University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan; [4]Brock University, St. Catharines, Ontario, Canada; and [5]Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland

Abstract

We have implemented a strategy to identify tumor antigens that induce a humoral immune response in lung cancer based on the analysis of tumor cell proteins. Chromatographically fractionated protein extracts from three lung cancer cell lines were subjected to Western blotting and hybridization with individual sera to determine serum antibody binding. Two sets of sera were initially investigated. One set consisted of sera from 19 newly diagnosed subjects with lung adeno-carcinoma 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 ($P = 0.0008$) was confidently identified by mass spectrometry as 14-3-3θ. Remarkably, significant autoantibody reactivity against 14-3-3θ 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 cohort study, relative to 19 matched controls ($P = 0.0042$). A receiver operating characteristic curve constructed with a panel of three proteins consisting of 14-3-3θ identified in this study, plus annexin 1 and protein gene product 9.5 proteins previously identified as associated with autoantibodies in lung cancer, 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. [Cancer Res 2007;67(24):12000–6]

Introduction

Lung cancer is the leading cause of cancer mortality in the United States and in other industrialized countries (1). The relative proportion of lung cancer represented by adenocarcinoma continues to increase especially in women and nonsmokers (2). Because treatment is efficacious largely for patients diagnosed at early stages of disease, the discovery of effective biomarkers for early detection, applicable to individuals at increased risk, is relevant to reducing lung cancer mortality. A promising approach for the identification of cancer markers that may be useful for early detection is the analysis of serum for autoantibodies against tumor proteins. There is increasing evidence for an immune response to

cancer in humans, shown in part by the identification of autoantibodies against a number of intracellular and surface antigens detectable in sera from patients with different cancer types (3–5). Circulating autoantibodies in lung cancer have been reported to have potential for either diagnosis or prognosis (6). Several proteomics methods are emerging as useful means to discover autoantibody biomarkers (4, 7). Our previous studies uncovered autoantibodies against annexin and protein gene product (PGP) 9.5 proteins in lung cancer patient sera collected at the time of diagnosis (8, 9). There is a need to identify additional autoantibody targets to increase specificity and sensitivity and to define a panel for potential use for early detection or screening. Here, we report the identification of the 14-3-3θ protein as a target for autoantibodies in lung cancer. We also provide evidence for the occurrence of autoantibodies against a panel consisting of 14-3-3θ, annexin 1, and PGP 9.5 proteins in sera collected at the preclinical stage of lung cancer.

Materials and Methods

Cancer cell lines. Three lung adenocarcinoma cell lines (A549, H23, and H522) were separately cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere in 5% CO_2. Culture plates containing adherent cells were washed four times with 20 mmol/L of Tris-HCl (pH 6.8) containing 150 mmol/L of NaCl and cells were harvested by adding 1.5 mL/plate of lysis buffer consisting of Tris-HCl (pH 6.8), 0.5% *n*-octyl β-D-glucopyranoside, 150 mmol/L of NaCl, 5 mmol/L of $MgCl_2$, 5 mmol/L of $MgCl_2$, 5 mmol/L of $CaCl_2$, and a cocktail of protease inhibitors (Roche). Cell lysis was done with four cycles of freeze-thawing and the whole cell lysate from each cell line was obtained after centrifugation at $2,000 \times g$, 4°C for 1 h.

Protein fractionation and one-dimensional SDS-PAGE. For initial analysis, whole cell lysates from the three lung cancer cell lines were combined and subjected to reversed-phase chromatography fractionation. A Resource RPC 3 mL column (GE Healthcare) was used. Buffer A consisted of 0.1% trifluoroacetic acid and buffer B was 90% acetonitrile/0.095% trifluoroacetic acid. Chromatography was carried out at a flow rate of 1.5 mL/min. The gradient consisted of 0% to 15% solvent B for 25 min, 25% to 100% solvent B for 60 min. A total of 11 fractions were collected based on chromatographic features. Fractions were resuspended in electrophoresis buffer [0.125 mol/L Tris (pH 6.8), 4% SDS, 20% glycerol, 2% DTT] after lyophilization and were loaded in 12% acrylamide gels (8.5 × 13.5 cm; Bio-Rad) and run at 30 mA/gel. One gel was stained with SYPRO Ruby (Molecular Probes) and others were transferred for 2 h (100 V/gel) to nitrocellulose membranes (Bio-Rad) for Western blotting.

Two-dimensional PAGE. In subsequent analyses, proteins in lysates from the cultured A549 lung adenocarcinoma cell line were separated using two-dimensional PAGE as described previously (10). Briefly, cultured A459 cells were lysed in solubilization buffer [8 mol/L urea (Bio-Rad), 2% NP40,
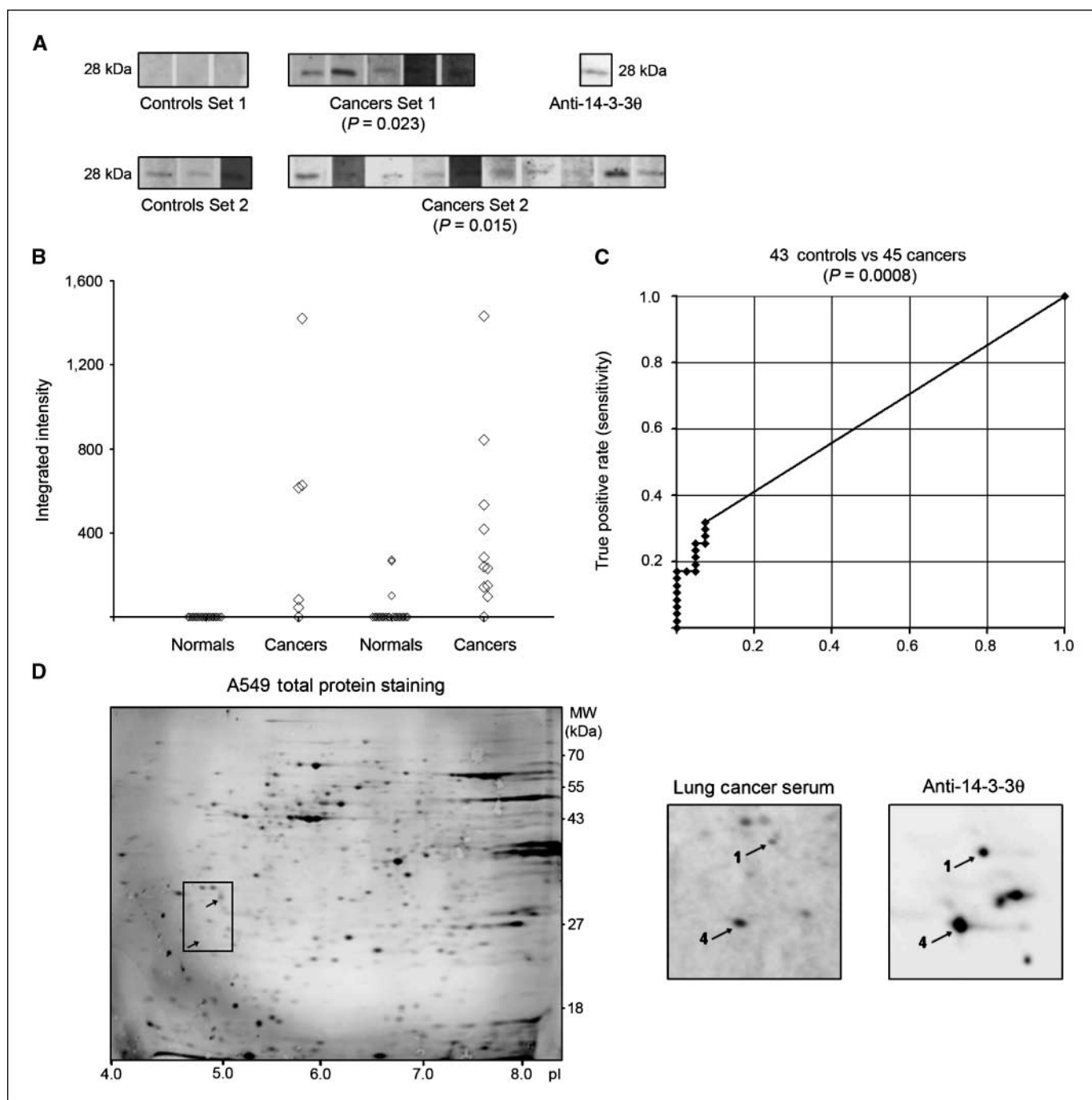
Figure 1. Reactivity of sera from newly diagnosed lung cancer subjects with 14-3-3θ. Proteins purified from three lung cancer cell lines were fractionated by reversed-phase chromatography and each fraction was loaded onto one-dimensional SDS gels and subjected to Western blot analysis using serum as primary antibody and antihuman IgG as the secondary antibody. *A,* 14-3-3θ band reactive with five lung cancer sera and none of the controls in the first set of sera analyzed, and with 10 cancer sera plus three controls (smokers) in a second independent set. Western blot analysis using monoclonal antibody to the 14-3-3θ isoform is also shown. *B,* integrated intensity is plotted for this band for all sera analyzed in both sets, demonstrating differential reactivity with cancer sera compared with controls. *C,* ROC curve for cancer sera versus controls, combining data from both sets of sera ($P = 0.0008$ one-sided rank-sum test). The AUC was 0.635. *D,* identification of 14-3-3θ isoforms. Protein profile of the A549 lung adenocarcinoma cell line by two-dimensional PAGE and total protein staining with SyproRuby. *Arrows,* 14-3-3θ isoforms of interest. *Insets,* Western blots performed with lysates from A549 cells and an antihuman 14-3-3θ monoclonal antibody and with one reactive lung cancer serum as primary antibodies.

2% carrier ampholytes (pH 4–8; Gallard/Schlessinger), 2% β-mercaptoethanol, and 10 mmol/L phenylmethylsulfonyl fluoride]. Two hundred micrograms of solubilized protein were applied onto individual isoelectric focusing gels. Isoelectric focusing was done using (pH 4–8) carrier ampholytes at 700 V for 16 h, followed by 1,000 V for an additional 2 h.

The first-dimension gel was loaded onto the second-dimension gel, after equilibration in 125 mmol/L of Tris (pH 6.8), 10% glycerol, 2% SDS, 1% DTT, and bromophenol blue. For the second-dimension separation, a gradient of 11% to 14% acrylamide (Crescent Chemical) was used. The resolved proteins were transferred onto an Immobilon-P polyvinylidene difluoride

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Table 1. Subject characteristics of lung cancer sera from set 2

Patient*	Age (y)	Cigarettes (packs/y)	Cancer stage	14-3-3 Reactivity [†]
1	62	57.5	IA	0.0
2	73	42.0	IA	98.4
3	59	44.0	IA	0.0
4	59	53.8	IA	0.0
5	66	67.2	IA	0.0
6	65	47.0	IA	0.0
7	68	43.0	IB	844.4
8	77	60.0	IB	0.0
9	75	57.0	IB	534.4
10	66	60.0	IB	238.9
11	54	39.0	IB	148.4
12	74	57.0	IB	0.0
13	63	34.5	IB	0.0
14	59	121.0	IIA	0.0
15	73	59.0	IIB	0.0
16	65	49.0	IIB	229.6
17	58	21.0	IIB	0.0
18	51	46.5	IIB	140.1
19	57	31.5	IIB	417.5
20	68	56.0	IV	0.0
21	49	33.0	IV	0.0
22	61	94.0	IV	0.0
23	52	31.0	IV	1,433.7
24	70	56.0	IV	0.0
25	56	30.8	IV	0.0
26	71	30.6	IV	282.9

*All cancer subjects were male smokers.

[†]Reactivity for 14-3-3θ protein was measured by integrated intensity on one-dimensional Western blots.

membrane (Millipore). Protein patterns in some blots were visualized directly by SYPRO Ruby staining.

Western blotting. Nitrocellulose or polyvinylidene difluoride membranes were blocked overnight with 5% nonfat dry milk (Bio-Rad) in PBS, then incubated for 2 h with normal or cancer serum at a dilution of 1:500 in PBS containing 0.1% Tween 20, at room temperature and under gentle agitation. After 1 h of washing with PBS/0.1% Tween 20 (6 × 10 min) membranes were incubated with 1:1,000 dilution of horseradish peroxidase–conjugated sheep anti-human IgG (GE Healthcare) in PBS/0.1% Tween 20 for 1 h at room temperature. Membranes were then washed for 1 h and the chemiluminescence immunodetection was done with enhanced chemiluminescence reagents (GE Healthcare). Hyperfilm films (GE Healthcare) were exposed for 30 s for optimal image visualization. For protein localization, Western blotting was done using an antibody for 14-3-3θ isoform (Sigma) at a 1:5,000 dilution. As a secondary antibody, an anti-mouse IgG (GE Healthcare) was used at 1:2,000 dilution. Additional monoclonal antibodies used were 14-3-3γ (1:2,500; Abcam), 14-3-3β (1:2,500; Abcam), 14-3-3β + ε + ζ (1:2,500; GeneTex).

Imaging and statistical analysis. Western blot films were scanned in transmittance mode in an Epson Expression 1680 scanner using 300 dpi. For one-dimensional blot analysis TotalLabTL100 v2006 (NonLinear) software was used to obtain background-corrected band-integrated intensities. For two-dimensional blot analysis, reactive protein spots were detected and quantified using BioImage 2-D Analyzer software, followed by matching and review using previously developed software (11, 12). Measurements in cases and controls were compared using one-sided Wilcoxon rank-sum tests for which P values were computed based on one million random permutations of the sample labels.

Protein identification by mass spectrometry. One-dimensional bands or two-dimensional protein spots were excised directly from gels or nitrocellulose membranes and extensively washed with 50 mmol/L of ammonium bicarbonate containing 50% acetonitrile, then vacuum-dried and incubated with 20 μL of 20 μg/mL trypsin (Promega) solution in 50 mmol/L of ammonium bicarbonate. Digestion was carried out overnight at 37°C and then interrupted by the addition of 5 μL of 10% formic acid solution. The resultant peptide mixtures were analyzed in an LTQ-FT mass spectrometer (Thermo-Finnigan) coupled to a nano Acquity nanoflow chromatography system (Waters). The liquid chromatography separation was done in a 25 cm column (Picofrit 75 μm inside diameter, New Objectives,

Table 2. Peptides identified for 14-3-3θ isoforms

Two-dimensional Spot	Unique peptides	Peptide probability	ΔMass	Sequence	Peptide position	Total MS events
Spot 1	9	0.3240	0.001	LAEQAER	12–18	**1**
		0.9994	0.000	AVTEQGAELSNEER	28–41	**3**
		0.9303	−0.001	NLLSVAYK	42–49	**1**
		0.9996	0.000	YLIANATNPESK	104-115	2
		0.5473	0.002	YLAEVACGDDR	128–138	1
		0.9997	0.003	QTIDNSQGAYQEAFDISK	140–157	10
		0.5695	0.001	EMQPTHPIR	159–167	2
		0.9999	0.002	TAFDEAIAELDTLNEDSYK	194–212	6
		0.9974	0.000	DSTLIMQLLR	213–222	2
Spot 4	6	0.9997	0.003	YLIANATNPESK	104–115	2
		0.5574	0.002	MKGDYFR	121–127	2
		0.9998	0.007	QTIDNSQGAYQEAFDISK	140–157	7
		0.5896	0.003	EMQPTHPIR	159–167	4
		0.9999	0.003	TAFDEAIAELDTLNEDSYK	194–212	4
		0.9930	0.003	DSTLIMQLLR	213–222	4

NOTE: Spot 1 and spot 4 were recognized by the 14-3-3 monoclonal antibody and lung cancer sera in A549 two-dimensional blots (Fig. 1D). Peptides at the NH$_2$-terminal portion identified only in spot 1 are in boldface. Three MS runs from three independent spot digestions were performed. ProteinProphet Score was 1.0 representing an error rate <1%.
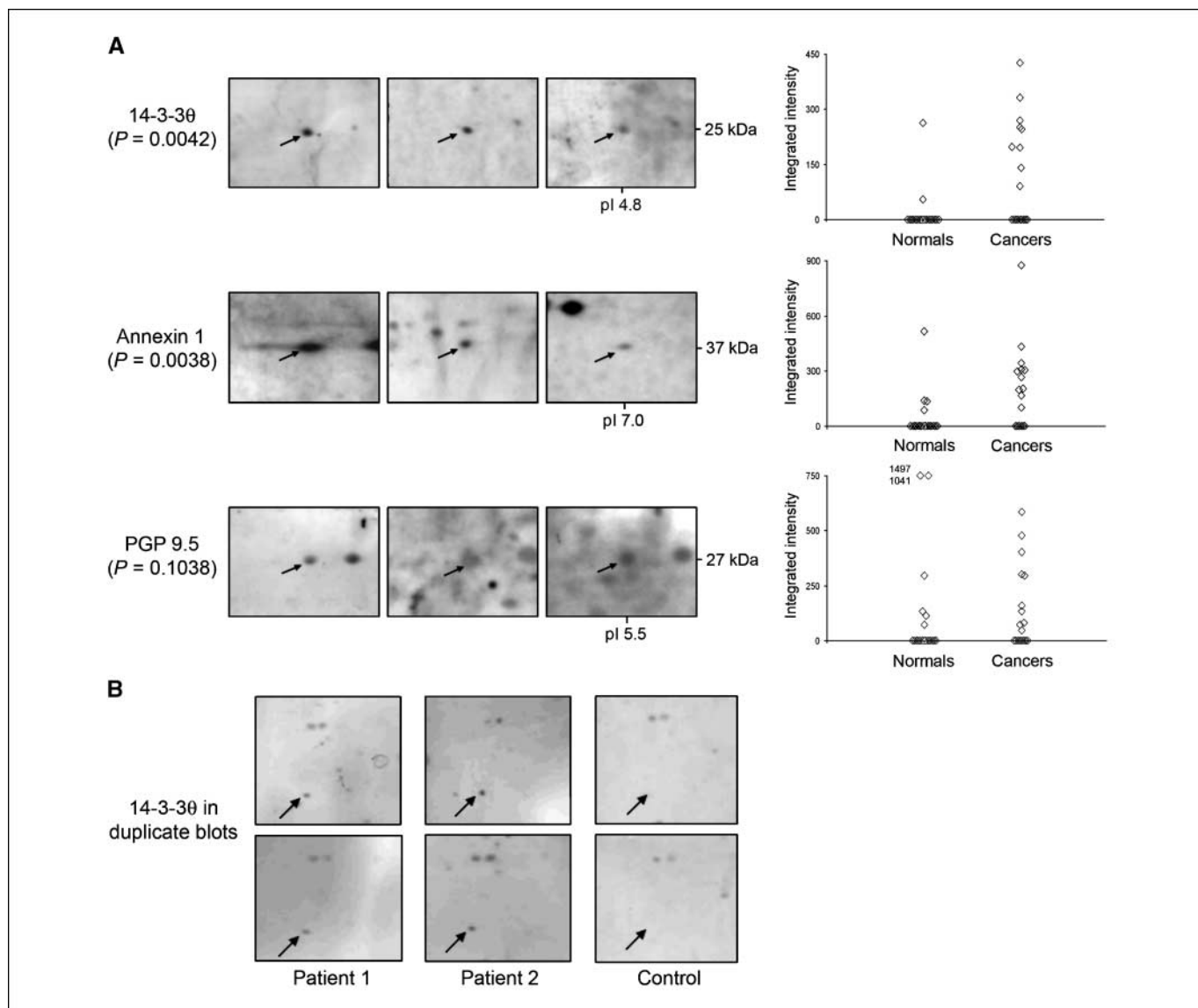
Figure 2. Panel of antigenic proteins recognized by lung cancer sera collected within a year prior to diagnosis. *A,* two-dimensional spots corresponding to the three antigenic proteins, 14-3-3θ, annexin 1, and PGP 9.5 (*arrows*) recognized by lung cancer sera. Significant *P* values were obtained for 14-3-3θ and annexin 1 in one-sided rank-sum tests. The integrated intensity for each protein is plotted for all 19 control and 18 cancer sera analyzed. Two controls exhibited high values for PGP 9.5 as indicated in the plot. *B,* 14-3-3θ spot reactivity in duplicate Western blot analysis of two lung cancer sera (patients 1 and 2) and one control.

packed in-house with MagicC18) using a 60 min linear gradient from 5% to 40% of acetonitrile in 0.1% formic acid at 250 nL/min. The spectra were acquired in a data-dependent mode in *m/z* range of 400 to 1,800, and the five most abundant +2 or +3 ions of each mass spectrometry (MS) spectrum were selected for tandem mass spectrometry analysis. Mass spectrometry variables were: capillary voltage of 2.2 kV, capillary temperature of 200°C, resolution of 100,000, and FT target value of 2,000,000. The acquired data was automatically processed by the Computational Proteomics Analysis System (13), using the X!Comet search algorithm. For databank searches, we only considered fully tryptic peptides with up to two miscleavages. Cysteine alkylation with iodoacetamide was considered as a fixed modification and oxidation of methionine as a variable modification. The minimum criteria for peptide matching was a PeptideProphet score > 0.2. Peptides that met these criteria were further grouped to protein sequences using the ProteinProphet algorithm at an error rate of 1% or less.

Serum specimens and subject characteristics. Three independent sets of sera were used in the present study. Informed consent was obtained for

all participants and the studies were all approved by appropriate institutional review boards. The first set consisted of sera from 19 newly diagnosed lung adenocarcinoma patients and 19 age and gender healthy nonsmoker subjects, collected through the Early Detection Research Network program at the University of Michigan. The second set consisted of sera from 26 lung cancer patients and 24 matched smoker subjects collected in a case-control study of lung cancer (the Environment and Genetics Lung Cancer Etiology study; ref. 14), from the Lombardy region of Italy. Controls were matched for smoking status (all smokers) and were similar by age and gender. The third set of sera were obtained from the Beta-Carotene and Retinol Efficacy Trial (CARET) cohort study (15), and included 18 prediagnostic sera and 19 heavy smokers matched controls. All cancer sera and all control sera in each cohort were individually tested. All lung cancer cases from whom blood was collected at the time of diagnosis had adenocarcinoma by histology. All analyses were conducted in batches with cases and controls equally included and vials were identified only with sample numbers.

Results

Autoantibody reactivity targeted to 14-3-3θ in sera from newly diagnosed subjects with lung cancer. Aliquots from 11 reversed-phase fractions of combined A549, H522, and H23 cell lysates were subjected to one-dimensional SDS electrophoresis and Western blotting to assess protein reactivity with lung cancer and control sera. Two independent sets of lung cancer sera, each with matched controls, were used. One set consisted of sera from 19 newly diagnosed subjects with lung adenocarcinoma and 19 healthy controls. A second set consisted of sera from 26 newly diagnosed subjects with lung adenocarcinoma and from 24 chronic smokers collected as part of a study of the genetic epidemiology of lung cancer and smoking (14).

The reactivity of specific bands was assessed using one-sided rank-sum tests. In the first set of sera from 19 lung adenocarcinoma subjects and 19 controls, a total of 103 reactive bands were observed. In general, more reactive bands were observed among cancer subjects than among controls. Nine bands were found to exhibit significantly greater reactivity among cancer sera relative to controls ($P < 0.05$). Two of these bands also exhibited greater reactivity among cancer subjects relative to chronic smokers in the second set ($P < 0.05$). One protein band which exhibited greater reactivity among cancer sera relative to controls from set 1 ($P = 0.023$) and from set 2 ($P = 0.015$; Fig. 1A and B), was identified with high confidence as 14-3-3θ by liquid chromatography tandem mass spectrometry analysis. Combining the data from both sets yielded highly significant differences between cancers and controls ($P = 0.0008$ one-sided rank-sum test). A receiver operating characteristic (ROC) curve was constructed using the single 14-3-3 band (Fig. 1C). A sensitivity of 27% and a specificity of 95% in discriminating newly diagnosed lung cancer sera from controls was obtained for this single band. Disease stage information was available for set 2 (Table 1). Seven of 10 cancer subjects presenting reactivity with 14-3-3 were at stage IB and IIB, indicating that reactivity was not limited to advanced stage lung cancer.

To obtain additional confirmation of the identity of the reactive band as 14-3-3θ, cell lysates were subjected to two-dimensional gel electrophoresis followed by Western blotting of individual membranes and incubation with 14-3-3 antibodies or with subject sera. A protein spot recognized by a monoclonal antibody to 14-3-3θ but not by monoclonal antibodies to 14-3-3γ, β, ε, and ζ, also showed reactivity with lung cancer sera (Fig. 1D). The anti–14-3-3θ
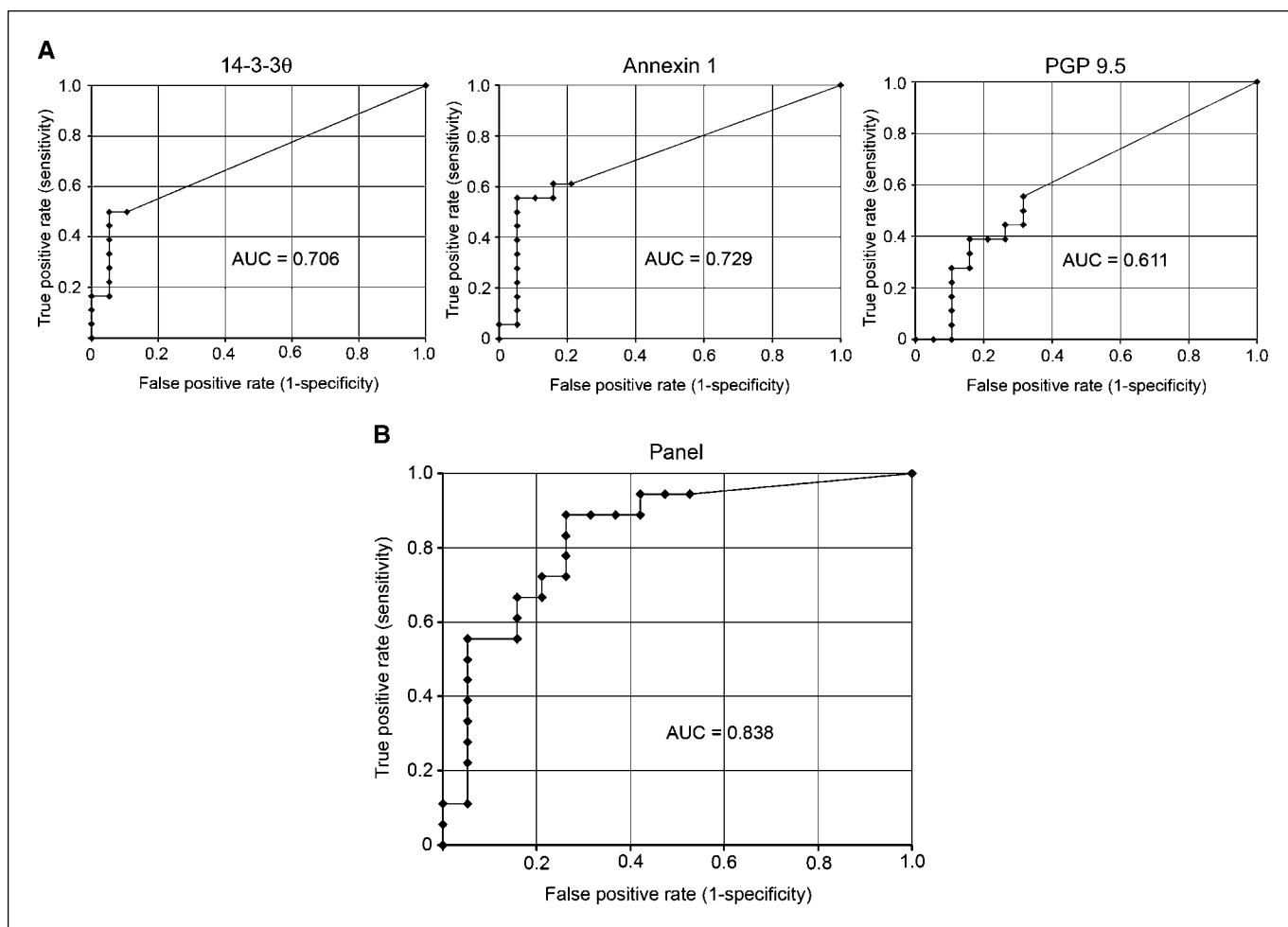
Figure 3. ROC curves for a panel of autoantibodies detected at a preclinical stage of lung cancer. Cell lysate two-dimensional Western blots were hybridized with sera from 18 lung cancer cases obtained within 1 year prior to the diagnosis of lung cancer and from 19 control subjects that did not develop lung cancer. *A,* ROC curves for individual antigenic proteins, 14-3-3θ, annexin 1, and PGP 9.5. The AUC is indicated. *B,* ROC curve for the panel of all three proteins combined by summing the ranks for each protein ($P = 0.00009$, one-sided rank-sum test).
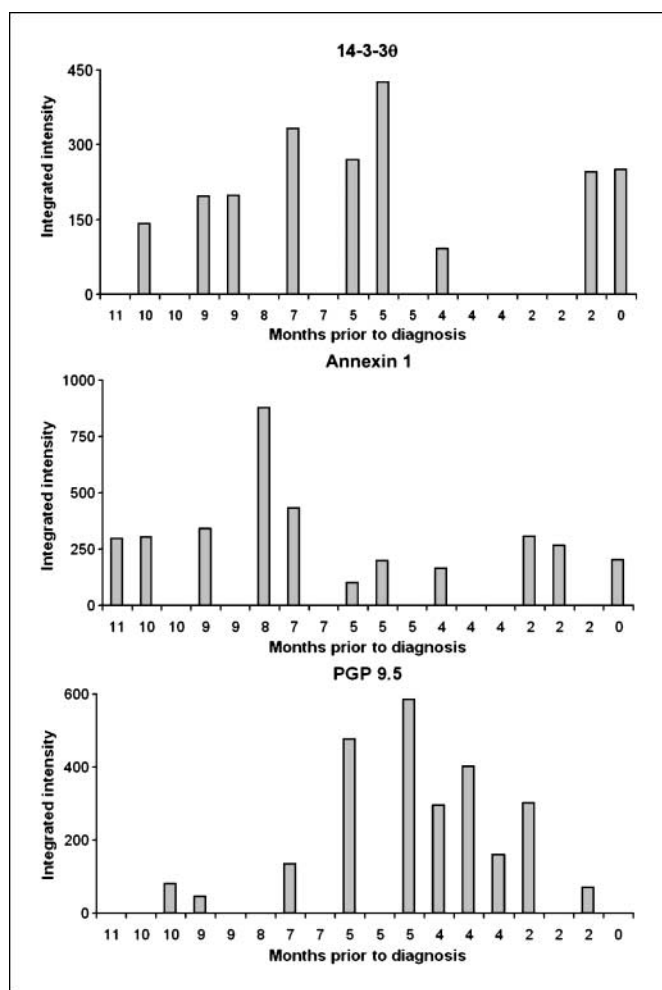
Figure 4. Autoantibody reactivity in relation to blood collection time prior to diagnosis. Integrated intensity for 14-3-3θ, annexin 1, and PGP 9.5 protein reactivity is plotted for individual cancer sera in relation to time prior to diagnosis when serum was collected.

monoclonal antibody also reacted with the one-dimensional band that was reactive with lung cancer patient sera. Interestingly, the anti–14-3-3θ monoclonal antibody reacted, in total, with four protein spots in two-dimensional blots but immunoreactivity with patient sera was largely limited to only one of the four which was the fastest migrating in the second dimension separation based on mass (Fig. 1D). We compared mass spectra between the fast-migrating form that was strongly reactive (spot 4) and a slower migrating form that was only weakly reactive with patient sera (spot 1). Comparison of the amino acid sequence coverage between the two forms showed that the weakly reactive form yielded spectra that matched the predicted NH_2-terminal sequence (Lys^{12}-Arg^{18}, Ala^{28}-Arg^{41}, and Asn^{42}-Lys^{49}), whereas the highly reactive form lacked spectra for the NH_2-terminal sequence of 14-3-3θ (Table 2). In contrast, comparison of five other peptides identified in both forms showed equivalent ion intensities indicating that lack of the NH_2-terminal peptide in the strongly reactive form was not due to limits of sensitivity of MS identification. These findings are consistent with the fast-migrating form with lower molecular weight being truncated, as we have previously observed for other tumor proteins that induce antibody reactivity (16).

Autoantibodies detected in subject sera collected prior to lung cancer diagnosis. To explore whether autoantibodies to 14-3-3θ are detectable at a preclinical stage, sera from participants in the CARET cohort study were analyzed (15). Specifically, sera were obtained from 18 subjects, collected within a year prior to a diagnosis of lung cancer, and from 19 subjects that did not develop lung cancer matched for age and sex, year of CARET enrollment, and the time blood was drawn in relation to enrollment. Sera were scored for reactivity against the fast-migrating 14-3-3θ isoform (spot 4) in two-dimensional Western blots of cell lysates blinded to case status. Significantly greater reactivity was observed among cancer sera relative to controls for 14-3-3θ (spot 4; $P = 0.0042$ one-sided rank-sum test).

In addition to 14-3-3θ, sera were scored for reactivity against annexin 1 and PGP 9.5, previously described by our group as inducing autoantibodies in lung cancer, based on analysis of sera from newly diagnosed lung cancer subjects (8, 9). Differential reactivity was observed for annexin 1 ($P = 0.0038$ one-sided rank-sum test), whereas PGP 9.5 protein reactivity did not achieve statistical significance ($P = 0.1038$; Fig. 2) in the analysis of prediagnostic sera.

Autoantibody reactivity against the three tumor antigens (14-3-3θ, annexin 1, and PGP 9.5) was also evaluated by ROC analysis. The areas under the curve (AUC) were 0.706, 0.729, and 0.611 for 14-3-3θ, annexin 1, and PGP 9.5, respectively (Fig. 3A). Four lung cancer sera, but none of the controls, exhibited reactivity against all three proteins. Only one of the lung cancer sera did not react against any of the proteins compared with eight controls. Combining data for 14-3-3θ, annexin 1, and PGP 9.5 proteins as a panel, by summing the ranks for each sample for each of the three proteins, yielded an AUC of 0.838 ($P = 0.00009$, one-sided rank-sum test; Fig. 3B). The relationship between reactivity and time prior to diagnosis for each protein is shown in Fig. 4. Reactivity against 14-3-3θ and annexin 1 was observed as early as 10 months prior to diagnosis, whereas reactivity against PGP 9.5 was observed more proximal to the time of diagnosis.

Discussion

We previously reported the occurrence of autoantibodies against annexin 1 and PGP 9.5 proteins among newly diagnosed subjects with lung cancer (8, 9). Here, we present evidence for the occurrence of autoantibodies to 14-3-3θ in sera from subjects with lung cancer. 14-3-3 proteins comprise a large family of highly conserved small, acidic polypeptides of 28 to 33 kDa that are found in all eukaryotic species. The human genome contains seven distinct 14-3-3 genes designated β, γ, ε, σ, ζ, η, and θ. The products of these genes exhibit distinct tissue localizations and functions (17). Expression of all seven 14-3-3 genes in lung cancer tissue has been examined by reverse transcription-PCR and Western blotting (18). In normal lung, two genes, ε and ζ, were found to be expressed. In biopsies of lung adenocarcinomas and squamous cell carcinomas, however, 14-3-3β, γ, σ, and θ were also found to be expressed.

14-3-3 proteins have been observed to be phosphorylated (19–21), cleaved (22), and acetylated (23). Here, we have identified a distinct 14-3-3θ isoform that was recognized by lung cancer sera, with evidence for truncation of the NH_2-terminal portion, based on MS analysis. 14-3-3 proteins were previously shown as phospho-serine/phosphothreonine-binding proteins, establishing their importance in cell signaling (24). Subsequently, 14-3-3 proteins have

been shown to contribute to the regulation of such crucial cellular processes as metabolism, signal transduction, cell cycle control, apoptosis, protein trafficking, transcription, stress responses, and malignant transformation (17, 25–27). Because targets of 14-3-3 proteins include components of both signal transduction and cell cycle regulatory pathways (such as Raf-1, protein kinase C, phosphatidylinositol 3-kinase, Bad, and Cdc25), it follows that 14-3-3 proteins regulate many cellular processes that are relevant to cancer development (28).

We sought evidence for the occurrence of autoantibodies to 14-3-3θ in prediagnostic sera, and for autoantibodies against annexin 1 and PGP 9.5, which we previously showed to be associated with autoantibodies at the time of lung cancer diagnosis. Annexin 1 is a 37-kDa glycoprotein that has been implicated in the control of cell growth (29, 30). It occurs both intracellularly and on the cell surface. PGP 9.5 belongs to a family of ubiquitin COOH terminal hydrolase isoenzymes that play a regulatory role in the ubiquitin system and is overexpressed in lung cancer (31, 32). Remarkably, a panel of these three markers exhibited 55% sensitivity at 95% specificity in comparisons of sera collected within a span of a year prior to the diagnosis of lung cancer and matched controls that were at high risk for lung cancer, predominantly due to smoking, but did not develop lung cancer. These findings provide supportive evidence for the occurrence of autoantibodies at the preclinical stage as previously shown for other antigens, notably p53 (33, 34), and point to the potential role of tumor antigen–directed autoantibodies in the early detection of lung cancer.

Acknowledgments

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Sandra R. Pereira-Faca, Rork Kuick, Eric Puravs, et al.

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