

Proteomics-based Identification of Protein Gene Product 9.5 as a Tumor Antigen That Induces a Humoral Immune Response in Lung Cancer

Franck Brichory, David Beer, Francois Le Naour, Thomas Giordano, and Samir Hanash¹

Departments of Pediatrics [F. B., F. L. N., S. H.], Pathology [T. G.], and Surgery [D. B.], University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT

We used a proteomic approach to identify proteins that commonly induce an antibody response in lung cancer. Sera from 64 newly diagnosed patients with lung cancer, 99 patients with other types of cancer, and 71 noncancer controls were analyzed for antibody-based reactivity against lung adenocarcinoma proteins resolved by two-dimensional PAGE. Unlike controls, autoantibodies against a protein identified by mass spectrometry as protein gene product 9.5 (PGP 9.5) were detected in sera from 9 of 64 patients with lung cancer. Circulating PGP 9.5 antigen was detected in sera from two additional patients with lung cancer, without detectable PGP 9.5 autoantibodies. PGP 9.5 is a neurospecific polypeptide previously proposed as a marker for non-small cell lung cancer, based on its expression in tumor tissue. Using A549 lung adenocarcinoma cell line, we have demonstrated that PGP 9.5 was present at the cell surface, as well as secreted. Thus, the findings of PGP 9.5 antigen and/or antibodies in serum of patients with lung cancer suggest that PGP 9.5 may have utility in lung cancer screening and diagnosis.

INTRODUCTION

Autoantibodies to tumor antigens represent one type of markers that could be assayed in serum for the detection of cancer in individuals at risk. In lung cancer, as in other tumor types, the majority of tumor-derived antigens that have been identified that elicit a humoral response are not the products of mutated genes. These antigens include differentiation antigens and other proteins that are overexpressed in tumors, such as the oncogenic proteins L-Myc and C-Myc, which have been found to elicit autoantibodies in some patients (1–3). There is some evidence that the occurrence of autoantibodies to specific antigens in lung cancer may have prognostic relevance (4–8).

Autoantibodies against onconeural antigens have been reported in lung cancer (7, 9–12). The majority of patients with paraneoplastic syndromes, with anti-Hu autoantibodies, have SCLC² (13, 14). Anti-Hu autoantibodies have been detected in ~15% of patients with SCLC without neurological symptoms (15, 16). Other autoantibodies associated with neurological symptoms in lung cancer include anti-P/Q type voltage-gated calcium channel (17), anti-CV2 (18), and antisynaptotagmin antibodies (19) have been already described in patients with lung cancer.

It is not clear why only a subset of patients with a tumor type develop a humoral response to a particular antigen. Immunogenicity may depend on the level of expression, post-translational modification, or other types of processing of a protein, the extent of which may be variable among tumors of a similar type. Other factors that influence the immune response may include variability among individuals and tumors in MHC molecules. Cytokines, such as IL-1, IL-2, and

IL-6; tumor necrosis factor α , or IFN γ , are also known to affect the immune response and may vary in concentration between tumors or in circulation (20–22).

PGP 9.5 (ubiquitin COOH-terminal esterase L1, or UCHL1) is a ubiquitin COOH-terminal hydrolase that is widely expressed in neuronal tissues at all stages of neuronal differentiation and that has been suggested as a neuroendocrine marker (23, 24). PGP 9.5 has previously been associated with pulmonary neuroendocrine tumors and, less frequently, with NSCLC (25). More recently, using SAGE, Hibi *et al.* (26) demonstrated that the PGP 9.5 transcript was highly expressed in primary lung cancers and lung cancer cell lines but was not detectable in normal lung. These results suggested that increased expression of PGP 9.5 was specifically associated with lung cancer development and that PGP 9.5 may thus serve as a marker for lung cancer.

We have implemented a proteomic approach for the identification of tumor antigens that elicit a humoral response (27). We have used 2-D PAGE to simultaneously separate several thousand individual cellular proteins from tumor tissue or tumor cell lines. Separated proteins are transferred onto membranes, and sera from cancer patients are screened individually by Western blot analysis for antibodies that react against separated proteins. Proteins that specifically react with sera from cancer patients are identified by mass spectrometric analysis. In this study, we report the identification of autoantibodies to PGP 9.5 and the detection of PGP 9.5 antigen in serum of patients with lung cancer.

MATERIALS AND METHODS

Tissue and Serum Specimens. Tumor tissue and sera were obtained at the time of diagnosis, after informed consent was obtained. The experimental protocol was approved by the University of Michigan Institutional Review Board. Sera from 64 lung cancer patients were analyzed. This patient population consisted approximately equally of males and females with an age range of 46–82 years (median, 64.6 years). Of 64 cases, there were 40 with adenocarcinoma, 18 with squamous cell carcinoma, 4 with SCLC, and 2 with large cell carcinoma, all histologically confirmed. An additional group of 82 lung tumors (33 adenocarcinomas, 27 squamous cell carcinomas, 15 SCLC, and 7 neuroendocrine differentiated adenocarcinomas) and 16 samples of normal lung tissue adjacent to tumors were analyzed by 2-D electrophoresis. Sera from 99 patients with other types of cancer, including 44 with liver cancer, 11 with breast cancer, 14 with brain tumor, 23 with neuroblastoma, and 7 with melanoma, were also investigated. Noncancer controls included 61 healthy subjects without a prior history of cancer or autoimmune disease and 10 other subjects with chronic lung disease.

2-D PAGE and Western Blotting. Following excision, tumor tissue was immediately frozen at -80°C , after which an aliquot was lysed in solubilization buffer [8 M urea, 2% NP-40, 2% carrier ampholytes (pH 4–8), 2% β -mercaptoethanol, and 10 mM phenylmethylsulfonyl fluoride] and stored at -80°C until use. Cultured A549 lung adenocarcinoma cells were lysed by addition of 300 μl of solubilization buffer, harvested using a cell scraper, and stored at -80°C until use. One hundred seventy-five μg of the proteins derived from the extracts of either cultured cells or solid tumors were separated in two dimensions as described previously (28). The separated proteins were transferred onto a PVDF membrane. Protein patterns in some gels were visualized directly by silver staining and, for some membranes, by Coomassie blue staining. For hybridization with serum, membranes were incubated with a blocking buffer consisting of Tris-buffered saline, 1.8% nonfat dry milk, and

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¹ To whom requests for reprints should be addressed, at Department of Pediatrics, University of Michigan Medical Center, 1150 W. Medical Center Drive, A520 Medical Science Research Bldg I, Ann Arbor, MI 48109-0656. Phone: (734) 763-9311; Fax: (734) 647-8148; E-mail: shanash@umich.edu.

² The abbreviations used are: SCLC, small cell lung cancer; IL, interleukin; PGP 9.5, protein gene product 9.5; NSCLC, non-small cell lung cancer; SAGE, Serial Analysis of Gene Expression; 2-D, two-dimensional; PVDF, polyvinylidene fluoride; MALDI-TOF, matrix-assisted desorption ionization-time of flight.

0.01% Tween 20 for 2 h, and then were washed and incubated with serum at a 1:100 dilution for 1 h at room temp. After three washes with washing buffer (Tris-buffered saline containing 0.01% Tween 20), the membranes were incubated with a secondary antibody at a 1:1000 dilution for 30 min at room temperature, washed, and briefly incubated in ECL (Enhanced Chemiluminescence; Amersham Pharmacia Biotech, Piscataway, NJ).

Protein Identification. For protein identification by mass spectrometry, 2-D gels were stained using a modified silver staining method, and excised proteins were digested as described previously (29). A peptide mass profile was obtained using a Perseptive Biosystems MALDI-TOF Voyager-DE Mass Spectrometer (Framingham, MA). The peptide masses obtained were used for database searches for protein identification.³

PGP 9.5 Detection by Immunoblotting. An anti-PGP 9.5 rabbit polyclonal antibody (Biogenesis, Kingston, NH) was used at a 1:10,000 dilution in immunoblotting assays and was processed as for incubations with patient sera, with a horseradish peroxidase-conjugated donkey antirabbit IgG as secondary antibody (Amersham Pharmacia Biotech).

PGP 9.5 Cellular Localization. Eighty percent confluent A549 cells were cultured for 24 h in DMEM without FCS. The culture supernatant was subsequently recovered and concentrated using Centrprep 3 and Centricon 3 centrifugal filter units (Millipore Corp., Bedford, MA). Cultured cells were washed three times with PBS, and the proteins bound to the cell membrane were EDTA-extracted for 30 min at 4°C in PBS supplemented with 1 mM EDTA and a cocktail of protease inhibitors (Roche Molecular Biochemicals) and concentrated. Cultured cells were lysed by the addition of 300 µl of solubilization buffer and scraped. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA) prior to SDS electrophoresis and protein transfer to an Immobilon-P PVDF membrane for Western blotting analysis with anti-PGP 9.5 and anti- α -tubulin (Sigma, St. Louis, MO) antibodies.

RESULTS

Reactivity of Sera from Lung Cancer Patients with PGP 9.5. 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 autologous tumor tissue proteins. Serum from 9 of 64 patients with lung cancer (Table 1), including 6 sera from patients with adenocarcinoma, 2 with squamous cell carcinoma, and 1 with SCLC, exhibited IgG-based reactivity against a group of three proteins with an estimated molecular mass of 25 kDa and with a pI between 5.0 and 5.6 (Fig. 1). Positive sera were reactive against this group of proteins at the highest serum dilution tested, which was 1:1000. Tumor stage information was available for 30 patients with adenocarcinoma (22 with stage I, 4 with stage II, 3 with stage III, and 1 with stage IV). In this subset, two patients with stage I and one with stage II had autoantibodies to the group of three proteins, suggesting that the occurrence of antibodies was not a feature of advanced stage disease. Likewise, the two patients with squamous cell carcinoma and positive sera had stage I disease. Sera from lung cancer patients that exhibited IgG-based reactivity against this group of proteins exhibited reactivity that was specific to IgG1 among the IgG subtypes examined (IgG1–4; data not shown).

The identity of this set of proteins was determined by mass spectrometry after trypsin digestion and corresponded to PGP 9.5 (Fig. 2A). 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 (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 female nonsmoker) exhibited immunoreactivity against PGP 9.5 proteins.

Table 1 Anti-PGP 9.5 autoantibodies in subject sera

	Number of subjects	PGP 9.5 autoAb ^a 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

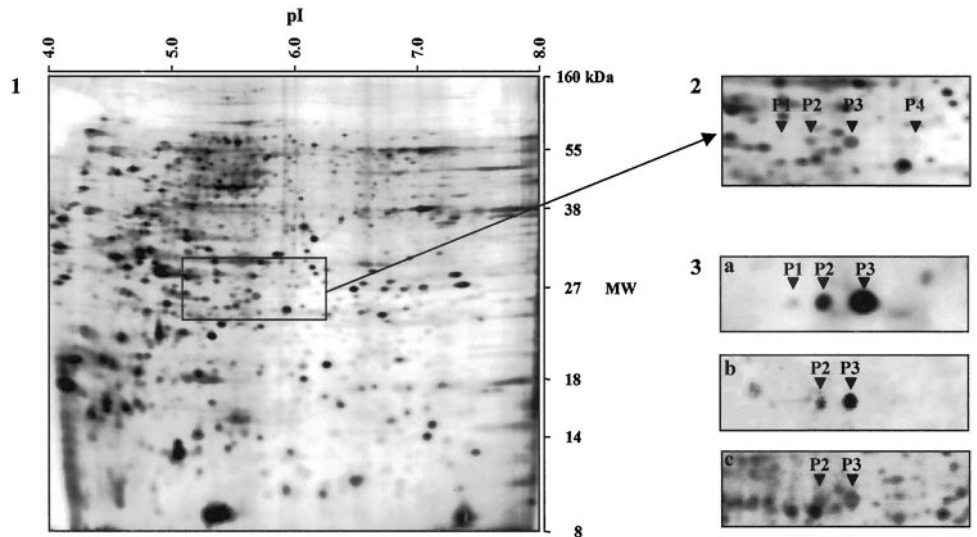
^a Ab, antibody.

To confirm the identity of the reactive proteins as PGP 9.5, A549 adenocarcinoma proteins were separated by 2-D PAGE, blotted onto PVDF membranes, and subsequently hybridized with an anti-PGP 9.5 polyclonal rabbit antiserum. Protein spots that reacted against patient sera and were identified as PGP 9.5 also reacted with the anti-PGP 9.5 polyclonal antibody (Fig. 2B). An additional protein spot, designated P4 (Fig. 2B), reacted with anti-PGP 9.5 polyclonal antibody. It was subsequently identified by mass spectrometry as a PGP 9.5 variant. Its lower abundance, compared with the other three variants, may account for its lack of reactivity with patient sera. Alternatively, this variant may lack the epitope that elicits reactivity with patient sera but still be reactive with the rabbit polyclonal antiserum.

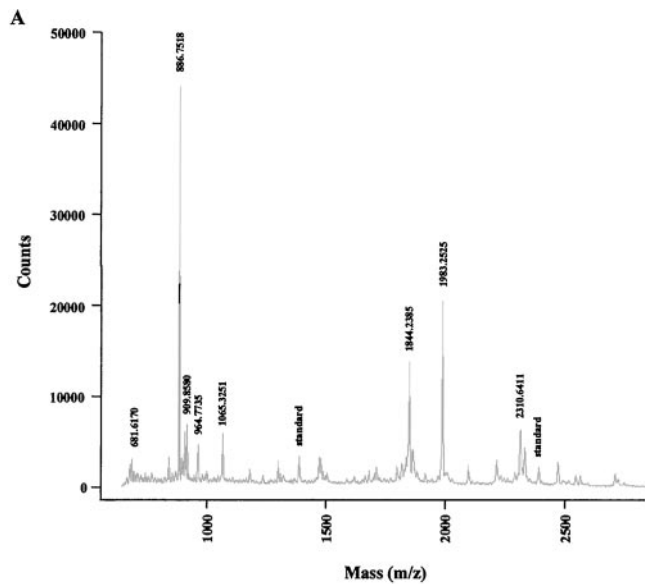
Expression of PGP 9.5 Protein in Lung Tissue. Increased levels of PGP 9.5 mRNA and protein have been reported previously in NSCLC tissue, based on SAGE and immunochemistry (26, 30). Given the occurrence of multiple variants of PGP 9.5 protein in A549 adenocarcinoma cell lysates, we sought to analyze PGP 9.5 expression in lung tumors and in normal lung, using 2-D PAGE to investigate differential expression of PGP 9.5 variants. We analyzed by 2-D PAGE and silver staining protein patterns corresponding to 82 lung tumors (33 adenocarcinomas, 27 squamous cell carcinomas, 15 SCLC, and 7 neuroendocrine differentiated adenocarcinomas) and adjacent normal lung tissue for 16 tumors. Tumors were scored as expressing PGP 9.5 based on visual detection of the constellation of spots characteristic of PGP 9.5 in the silver-stained 2-D patterns. PGP 9.5 protein was detected in 100% of small cell carcinomas, 63% (21 of 33) of adenocarcinomas, 85% (23 of 27) of squamous cell carcinomas, and 100% of neuroendocrine differentiated adenocarcinomas (Fig. 3). There was no significant correlation between the presence of PGP 9.5 and disease stage or histological subtype. There was a lack of PGP 9.5 protein in all 16 normal lung 2-D protein patterns analyzed (Fig. 3). The predominant variants of PGP 9.5 observed by silver staining were P3 and P2. There was a uniformly greater abundance of P3 relative to P2. The P1 and P4 variants were not detectable by silver staining. To increase the sensitivity of the analysis, blots prepared from lung tumor tissue and adjacent normal lung were hybridized with the anti-PGP 9.5 polyclonal rabbit serum. Eleven paired tumor and normal lung tissues were investigated in this series, consisting of 9 adenocarcinomas and 2 squamous cell carcinomas. Corresponding sera were screened for PGP 9.5 antigen and autoantibodies. In 8 of the 11 tumor samples (6 adenocarcinomas and 2 squamous cell carcinomas), PGP 9.5 was readily detected in tumor tissue and absent in normal lung tissue (Fig. 4). In most tumors, the predominant variants observed were P2 and P3, with a lower abundance of P1. Careful comparison of PGP 9.5 patterns in silver-stained 2-D gels and Western blots of the same tumors indicated preferential reactivity of the

³ <http://prospector.ucsf.edu>.

Fig. 1. 2-D PAGE and Western blot analysis of A549 lung adenocarcinoma cell proteins. *Panel 1* shows A549 2-D protein pattern after silver staining. The boxed area in *panel 1* is shown in *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, recognized by a polyclonal rabbit anti-PGP 9.5 antiserum, which also recognized P1–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, which showed reactivity against PGP 9.5 proteins. A close-up of the autologous tumor tissue 2-D protein pattern stained with silver nitrate (c) shows P2 and P3 PGP 9.5 variants.



anti-PGP 9.5 antibody with the P2 form of PGP 9.5. Unlike the A549 adenocarcinoma cell line, PGP 9.5 variant P4 was not detected in tumors. PGP 9.5 was faintly detected in the other three tumor samples. Furthermore, no correlation was found between PGP 9.5 protein level



m/z	Peptide Sequence	Start	End
681.6170	EFTER	188	192
886.7518	LGVAGQWR	5	12
909.8580	FSAVALCK	199	206
964.7735	QFLSETEK	101	108
1065.3251	LG FEDGAVLK	91	100
1844.2385	MPFPVNHGASSEDLLK	164	180
1983.2525	NEAIQA AHDAVAQEGQCR	121	138

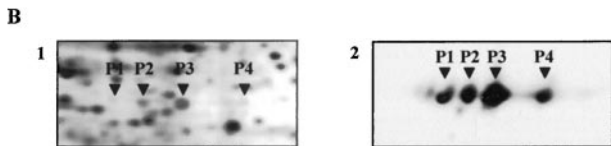


Fig. 2. Identification of reactive proteins as PGP 9.5 by MALDI-TOF mass spectrometry and by Western blot analysis. A, MALDI-MS spectrum obtained from spot P3 after trypsin digestion (top) and peptide sequences from PGP 9.5 matching with peaks obtained from MALDI-MS spectra (bottom). B, close-up of a silver-stained 2-D gel in the location of PGP 9.5 proteins (panel 1) and of a Western blot of the same area hybridized with an anti-PGP 9.5 polyclonal antibody (panel 2).

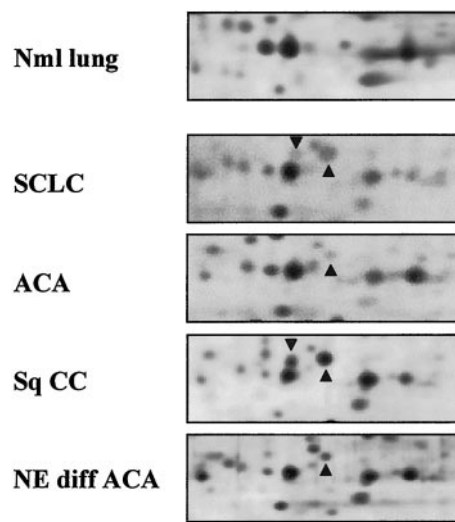


Fig. 3. Close up of representative 2-D PAGE protein patterns of lung tissues stained with silver nitrate. Protein extracts from normal lung (*Nml*), *SCLC*, lung adenocarcinoma (*ACA*), squamous cell lung carcinoma (*Sq CC*), and neuroendocrine differentiated adenocarcinomas (*NE diff ACA*) were separated by 2-D PAGE and stained with silver nitrate. No expression of PGP 9.5 variants could be observed in normal lung compared with the different lung cancer types. Arrowheads indicate the P2 (▼) and P3 (▲) PGP 9.5 variants.

in the tumor tissue and the occurrence of autoantibodies in the corresponding serum.

PGP 9.5 Cellular Localization. To determine the cellular distribution of PGP 9.5 and its occurrence as a secreted protein, we analyzed different protein compartments from the A549 adenocarcinoma cell line by Western blotting. Aliquots consisted of total cellular protein extracts, a membrane-associated protein fraction, and a secreted fraction. As a control for cell lysis that might result in release of PGP 9.5 into the culture medium, we monitored the presence of α -tubulin in the culture medium. PGP 9.5 was readily detected in all three fractions, whereas α -tubulin was absent in the culture medium (Fig. 5). Although all four PGP 9.5 variants were present in each fraction, a larger proportion of the P3 variant was found in the membrane fraction, relative to total cellular lysates and to the secreted protein fraction.

Occurrence of PGP 9.5 Protein in Patient Sera. Given the occurrence of PGP 9.5 in the secreted protein fraction of A549 lung adenocarcinoma cell line, we sought to determine whether PGP 9.5

could be detected in sera from lung cancer patients. The 64 sera from patients with lung cancer and 71 sera from noncancer controls, including 15 chronic smokers and 10 patients with chronic lung disease, were investigated for the presence of PGP 9.5 in serum. Ten- μ l aliquots of each serum were separated by SDS electrophoresis and analyzed by Western blotting using anti-PGP 9.5 polyclonal antibody. Two sera from lung cancer patients that were nonreactive against PGP 9.5 exhibited circulating PGP 9.5 (data not shown). PGP 9.5 was not detected in any of the control sera.

DISCUSSION

The proteomic approach for the identification of tumor antigens that elicit a humoral response, which we have applied to sera from newly diagnosed patients with lung cancer, uncovered antibodies against a group of four 25-kDa proteins identified as PGP 9.5 in 9 of 64 sera. The occurrence of multiple forms of PGP 9.5 is likely the result of post-translational modifications. Two additional sera from lung cancer patients, which were nonreactive against PGP 9.5, exhibited circulating PGP 9.5. Only one serum among the 71 noncancer controls exhibited IgG immunoreactivity to PGP 9.5, and PGP 9.5 antigen was not detected in any of the control sera investigated. PGP 9.5 was first identified as a specific marker for neurons and neuroendocrine cells (24). PGP 9.5 belongs to a family of ubiquitin COOH-terminal hydrolase isoenzymes that play a regulatory role in the ubiquitin system (31). PGP 9.5 has been implicated in the mechanism to remove ubiquitin from ubiquitinated proteins, thus preventing their degradation by proteasomes (32). Ubiquitination of cellular proteins and their targeting for subsequent degradation via ubiquitin-mediated proteolysis is an important mechanism that regulates the activity of a variety of genes, notably cell cycle genes (31, 33).

In our study we demonstrated by 2-D PAGE and Western blot analyses that 80% of the lung tumors we have studied contained detectable levels of PGP 9.5. In previous analyses by immunohistochemistry, PGP 9.5 was detected in 40–82.5% of NSCLCs and 50–90% of SCLCs (25, 30, 34, 35). Hibi *et al.* (26, 30) reported ectopic expression of PGP 9.5 in lung cancers by SAGE analysis and by immunochemistry. In primary NSCLCs, 54% of the cases had positive PGP 9.5 staining, and protein expression was associated with pathological stage (44% of stage I and 75% of stages II and IIIA).

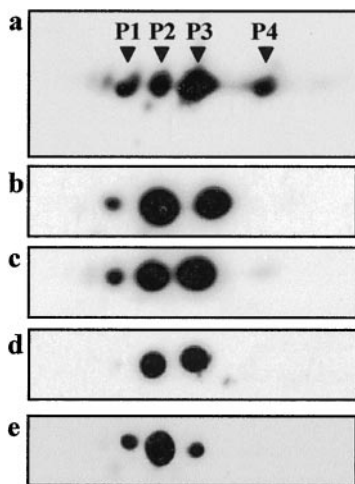


Fig. 4. Western blot analysis of PGP 9.5 protein in lysates of A549 (a), of three stage I adenocarcinomas (b–d), and of one stage III squamous cell carcinoma (e). Proteins extracts from lung tumors were separated by 2-D PAGE and hybridized with anti-PGP 9.5 antibody. In contrast to A549 lysates (a), in which variants P1–P4 are readily observed, there is a predominance of P2 and P3 in tumor tissue (b–e).

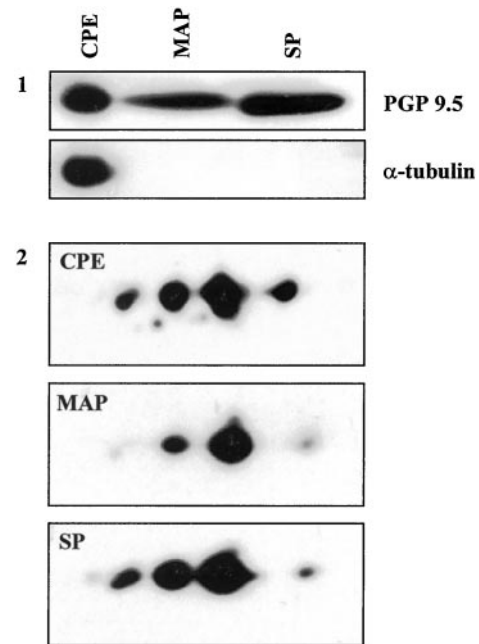


Fig. 5. Western blot analysis of proteins from A549 lysates, separated by 1-D (panel 1) and 2-D (panel 2) PAGE and hybridized with either anti-PGP 9.5 antibody or anti- α -tubulin (control) antibodies. PGP 9.5 is present in the secreted protein (SP) fraction, in the membrane-associated protein (MAP) fraction, and in cytosolic protein extract (CPE). Panel 2 shows that the four PGP 9.5 variants are present in each fraction, with P3 as the predominant variant in the membrane-associated protein fraction.

PGP 9.5 was observed in both SCLC and NSCLC cell lines, independent of neuronal differentiation. These results suggest that increased expression of PGP 9.5 may have an important role in oncogenic transformation of human lung epithelial cells. PGP 9.5 expression in tumor tissue is not limited to lung cancer. For example, PGP 9.5 was detected in pancreatic cancer, and it has been suggested that PGP 9.5 expression may serve as a marker for predicting outcome for patients with resected pancreatic tumors (36).

In contrast to prior findings pertaining to the occurrence of PGP 9.5 in tumor tissue, our study has demonstrated the occurrence of PGP 9.5 antibodies and, to a lesser extent, PGP 9.5 antigen in sera from patients with lung cancer. We have shown that PGP 9.5 was secreted in the lung cancer cell line A549 despite the lack of signal peptide. The presence of PGP 9.5 antigen in sera from patients with lung cancer could be the result of active secretion during lung tumor development and progression. Thus, our findings suggest that ectopic expression of PGP 9.5 and release into the serum are associated with a humoral response detectable in a subset of patients.

In addition to ectopic expression, other changes in a protein may induce a humoral response. p53 autoantibodies are associated with p53 gene missense mutations and p53 accumulation in tumors (37). In a recent study, we found that the occurrence of autoantibodies against annexins I and II in sera of patients with lung cancer was associated with an increase in the membrane-bound fraction of annexins and with increased circulating levels of IL-6, an important modulator of the immune response (38). We have investigated whether the presence of autoantibodies to PGP 9.5 was associated with high circulating levels of IL-6 and found no significant correlation between the two. Thus, the nature of factors, other than increased expression, that contribute to a humoral response against PGP 9.5 in some patients with lung cancer but not in others remains to be determined.

Given the common occurrence of autoantibodies to certain proteins in different cancers, as we have demonstrated with autoantibodies to annexins I and II and PGP 9.5 in lung cancer, assays for panels of such

circulating antibodies and/or their corresponding antigens may represent an important avenue for cancer diagnosis (4–8).

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