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Circulating anti-p53 antibodies in lung cancer and relationship to histology and smoking

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Anti-p53 antibodies were examined in the plasma of 112 lung cancer patients by ELISA in order to study the distributions in lung cancer patients and the determinants of these antibodies in relation to lung cancer. Twenty (17.9 %) lung cancer patients were found to have anti-p53 antibodies. The distribution of the antibodies by histological type was 7/48 (14.6 %) adenocarcinoma, 8/32 (25.0 %) squamous cell carcinoma, 3/7 (42.9 %) small cell lung cancer, 0/4 large cell carcinoma, 0/8 adenosquamous cell carcinoma and 2/13 (15.4 %) other types. By ethnicity, 8/44 (18.2 %) Caucasians, 4/20 (20.0 %) Hispanics and 8/48 (16.7 %) African-Americans were positive for anti-p53 antibodies, with no significant differences among the groups ($p = 0.5137$). The antibody positivity rates were higher in lung cancer patients 55 years or older (21.2 %) than in the patients under 55 years (7.4 %). The positive rates of the antibodies were 14.3 % in non-smokers, 16.7 % in ex-smokers and 19.1 % in current smokers, with heavy smokers (≥41 pack-years) having the highest positive rate (28.6 %), but none of these differences were statistically significant ($p > 0.05$). Seven controls who had anti-p53 antibodies were all ex-smokers or current smokers and some had occupational exposures. No anti-p53 antibodies were found in 41 non-smoking controls. These results suggest that the development of anti-p53 antibodies in pulmonary carcinogenesis and its association with smoking and other carcinogenic exposures deserve further study.

Keywords: lung cancer, anti-p53 antibody, histological type, ethnicity, smoking, pack-year.

Introduction

Lung cancer is currently the leading cause of cancer death and accounts for 30 % of all cancer death in the United States (Wingo *et al.* 1995). Cigarette smoking is the most important aetiological factor of lung cancer and accounts for more than 80 % of lung cancer cases. Cigarette smoke contains many known carcinogens, such as polycyclic aromatic hydrocarbons (PAHs). Recent study has revealed that benzo[*a*]pyrene diol epoxide (BPDE), one of PAHs in cigarette smoking, preferentially forms DNA-adducts along exons of the p53 gene in codons 157, 248 and 273, which are the major mutational hotspots in human lung cancer (Denissenko *et al.* 1996). Other environmental and occupational carcinogens, such as asbestos, have been associated with lung cancer. These carcinogens are capable of inducing mutations that can initiate pulmonary carcinogenesis.

p53 is a tumour suppressor gene, which encodes a 53 kDa protein that regulates

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cell proliferation and prevents the development of malignancies (Levine *et al.* 1991, Vogelstein *et al.* 1992). Mutation of the p53 gene has been the most common somatic genetic alteration found in a variety of cancers, including lung cancer. When the p53 gene is mutated by various carcinogens, such as those from cigarette smoking, it will express a mutant p53 protein which has lost its tumour suppressor function, resulting in uncontrolled cell growth (Levine *et al.* 1991, Vogelstein *et al.* 1992). The mutant p53 proteins have a much longer half-life than that of the wild-type protein and thus accumulate in tumour cells. The accumulated proteins can be released from the tumour cells, recognized by the immune system in humans as a foreign protein and induce a humoral response with the development of antibodies against the proteins. Thus, the detection of anti-p53 antibodies can be used as a possible biomarker for the occurrence of p53 gene mutation.

Anti-p53 antibodies in sera were first found in breast cancer patients in 1982 (Crawford *et al.* 1982). Since then, anti-p53 antibodies have been examined by several methods (Crawford *et al.* 1982, Davidoff *et al.* 1992, Winter *et al.* 1992, Angelopoulou *et al.* 1994, Wild *et al.* 1995) and detected in many kinds of cancers, including lung cancer (Crowford *et al.* 1982, Davidoff *et al.* 1992, Winter *et al.* 1992, Angelopoulou *et al.* 1994, Lubin *et al.* 1995a, Wild *et al.* 1995). Previous studies have revealed that the development of anti-p53 antibodies was dependent on the type and exons of the p53 gene mutation and the level of mutant p53 overexpression (Davidoff *et al.* 1992, Winter *et al.* 1992). The anti-p53 antibodies are induced by two immunodominant regions located at the carboxyl and amino termini of the p53 protein, outside the central mutational hot spot regions (Schlichtholz *et al.* 1992, 1994), and recognize the wild-type or mutant p53 proteins (Davidoff *et al.* 1992, Winter *et al.* 1992). The clinical significance of anti-p53 antibodies for various cancers has been extensively investigated. In lung cancer, some researchers have found that anti-p53 antibodies may even be used as an early biomarker of lung cancer because the antibodies can be detected a few months to years prior to the diagnosis of cancer (Lubin *et al.* 1995b, Trivers *et al.* 1996). The association between anti-p53 antibodies and the survival time of lung cancer patients remains somewhat controversial (Winter *et al.* 1993, Rosenfeld *et al.* 1997). Studies of anti-p53 antibody distribution in different populations and relationship to carcinogen exposures in lung cancer are limited.

In this study, we report the prevalence of anti-p53 antibodies in lung cancer patients, and the distribution of these antibodies in relation to demographic factors and carcinogen exposures.

Materials and methods

The subjects in this study were recruited from Harlem Hospital Center, Columbia-Presbyterian Medical Center and St Luke's and Roosevelt Hospital Center in the City of New York from 1992 to 1996. The primary lung cancer patients (112) were newly diagnosed and confirmed by pathology report and had not received any therapy, including radiotherapy, chemotherapy and surgical resection. The controls (172) used to establish ELISA cut-off value criteria were patients older than 20 years with a variety of medical conditions other than cancer, including pulmonary tuberculosis, asthma, pneumonia, hypertension and heart disease. After all subjects provided their informed consent, approximately 30 ml of blood was collected by routine venipuncture at the time of diagnosis and before treatment, and the subjects were interviewed in person by a trained interviewer. For subjects who only spoke Spanish, a trained bilingual interviewer conducted the interview and translated the answers into English.

A structured questionnaire was used to obtain information about demographic and residential characteristics, working experience, occupational exposure, smoking, alcohol consumption, vitamin supplements, diagnostic X-rays, medication and health status. Detailed information about smoking

Table 1. Characteristics of study population

	Cases	Controls	*p*
Total	112	172	
Age (mean ± SD)	62.09 ± 11.14	54.88 ± 14.86	0.0001
Gender			0.0055
Male	76 (67.9 %)	88 (51.2 %)	
Female	36 (32.1 %)	84 (48.8 %)	
Ethnicity			0.0001
Caucasian	44 (39.3 %)	33 (19.2 %)	
Hispanic	20 (17.9 %)	20 (11.6 %)	
Black	48 (42.9 %)	119 (69.2 %)	
Smoking[a]			0.0004
Non-smoker	7 (6.3 %)	41 (24.8 %)	
Ex-smoker	36 (32.4 %)	42 (25.5 %)	
Current smoker	68 (61.3 %)	82 (49.7 %)	
Occupational exposure[b]			0.0499
No	86 (86.9 %)	120 (76.9 %)	
Yes	13 (13.1 %)	36 (23.1 %)	
Histological type[c]			
Adenocarcinoma	48 (42.9 %)		
Squamous cell carcinoma	32 (28.6 %)		
Large cell carcinoma	4 (3.6 %)		
Small cell carcinoma	7 (6.3 %)		
Adenosquamous carcinoma	8 (7.1 %)		
Others	13 (11.6 %)		

[a] 1 case and seven controls lacked information on smoking.
[b] 13 cases and 16 controls lacked information on occupational exposure history.
[c] Others included one carcinoid, three mixed type, five non-small-cell carcinoma and four undifferentiated type.

status (smoking a cigarette or more per day for 6 months or more), smoking level (smoking packs per day), smoking years and pack-years smoked (packs per day × smoking years) was collected, and participants were categorized as current smokers (within 1 year), ex-smokers (stopped smoking 1 year ago) and non-smokers (never smoking). The subjects who were occupationally exposed to asbestos and its products, asphalt, coal tar, tar materials, and benzo[*a*]pyrene for 3 months or more in their lifetime were identified as occupational exposures. For ten lung cancer patients from Harlem Hospital who could not be interviewed, the information was collected from the Harlem Hospital Tumor Registry. For 20 randomly selected cases who had data available from both interview and the Tumor Registry, the information on demographic characteristics and smoking were essentially the same, suggesting that both sources were equally reliable. The study was approved by the Institutional Review Boards (IRBs) in the above three hospital or medical centres, respectively.

As noted, a 30 ml blood sample was collected from each participant into heparinized tubes, transported to the laboratory and coded. Plasma, buffy coat and red blood cells were separated by centrifugation, aliquoted and stored at –70 °C until assayed.

The anti-p53 antibodies were detected by a sandwich-type enzyme-linked immunosorbent assay (ELISA) based on matching microtitre plates coated either with glutathione-S-transferase (GST)-conjugated p53 protein (a fusion protein consisting of GST and p53 was made in an *E. coli* construction, extracted by French press rupture of the bacteria, followed by glutathione column purification using reduced glutathione as displacement) or GST protein alone (Oncogene Science Diagnostics, Cambridge, MA, USA). Briefly, 100 µl plasma samples (in duplicate) diluted at 1:50 were added to microtitre wells on separate plates that were precoated with GST-conjugated p53 protein or GST protein alone and incubated overnight at 4 °C. After washing, 100 µl of a conjugate solution of horseradish peroxidase-conjugated goat anti-human IgG was added to each well and incubated for 1 h at 37 °C. After washing again, 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate solution was added to each well and incubated for 5 min at room temperature followed by the addition of 100 µl sulphuric acid stop

Table 2. Anti-p53 antibodies in plasma by lung cancer patients after excluding ten cases whose information was collected from Tumor Registry.

	Lung cancer patients	Positive for p53 antibodies(%)	p^d
Total	112	20 (17.9)	
Histological type			0.7907
Adenocarcinoma	48	7 (14.6)	
Squamous cell carcinoma	32	8 (25.0)	
Large cell carcinoma	4	0 (0.0)	
Small cell carcinoma	7	3 (42.9)	
Adenosquamous carcinoma	8	0 (0.0)	
Others	13	2 (15.4)	
Age			0.1625
< 55	27	2 (7.4)	
≥55	85	18 (21.2)	
Gender			0.2403
Male	76	16 (21.1)	
Female	36	4 (11.1)	
Ethnicity			0.5137
Caucasian	44	8 (18.2)	
Hispanic	20	4 (20.0)	
African-American	48	8 (16.7)	
Smoking[a]			0.6158
Non-smoker	7	1 (14.3)	
Ex-smoker	36	6 (16.7)	
Current smoker	68	13 (19.1)	
Lifetime smoking history[b]			0.1486
0 pack-year	7	1 (14.3)	
1–40 pack-years	52	5 (9.6)	
41-pack-years	49	14 (28.6)	
Occupational exposure[c]			0.8038
No	86	16 (18.6)	
Yes	13	3 (23.1)	

[a] 1 lung cancer patient lacked smoking information

[b] 4 lung cancer patients lacked information on lifetime smoking history. Pack-year = smoking packs per day × smoking years.

[c] 13 lung cancer patients lacked occupational exposure information.

[d] Adjusted for age, gender, ethnicity, smoking or lifetime smoking history, occupational exposure and histological type.

solution. Then the absorbance of each well was read on a spectrophotometric plate reader at 450 nm. For each sample, the ratio of optical density on the mean of GST-p53 plate to the mean of GST alone plate was calculated. Known antibody-positive and antibody-negative controls (both were diluted human sera and confirmed by immunoblot and antigen competition against baculovirus-derived p53, provided by Oncogene Science Diagnostics, Cambridge, MA, USA) were included in each plate. The ELISA gave results that were in good agreement with those obtained by western blotting on 20 randomly selected subjects ($K = 0.7$, $p = 0.001$). In addition, repeat assays on 22 mid-range plasma samples showed only small ratio differences between the repeat results (mean = 0.32, SD = 0.34, range = 0.01–1.27) with no change in the positive-negative determination for any sample. Thus, this ELISA appears to be accurate and reproducible.

Data of the ratios of optical density on the GST-p53 plate to the GST alone plate were log transformed to normalize the distribution. Then the log mean and standard deviation were calculated and a cut-off value was chosen using log mean plus two standard deviations in the control group to

identify each sample as positive or negative. The differences of the ratio by ethnic groups in control group and the age between cases and controls were examined by Student's *t*-test. Chi-square and Fisher's exact test were used to analyse the differences of the population characteristics between the cases and controls. For multivariate analyses, a logistic regression model was adopted to evaluate the differences of anti-p53 antibody distributions between the cases and controls and among lung cancer patients. All *p* values were calculated for a two-sided test, and a value less than 0.05 was considered significant.

Results

Some characteristics of the study population are presented in table 1. Lung cancer patients were older than controls and had more males (67.9 %) in comparison with the control group where the proportion of males and females was nearly equal. In the lung cancer patients, Caucasians and African-Americans occupied similar proportions of about 40 %, however, African-Americans accounted for a larger proportion (69.2 %) of the control group. More lung cancer patients smoked than did controls, which was consistent with previous epidemiological studies.

There was no difference in the ratios on the GST-p53 plate to the GST alone plate among Caucasians (anti-log mean = 1.33), Hispanics (anti-log mean = 1.30) and African-Americans (anti-log mean = 1.35) in the control group ($p = 0.9415$). Thus, we merged the data of the three ethnic groups in the controls and calculated the mean and standard deviation of the ratios. The log mean of the ratios in the control group was 0.284 and log standard deviation 0.389. Thus, using the method of anti-log mean plus two standard deviations, we set up the cut-off value for positive p53 antibody ratio to be equal to or greater than 3.0.

Based on the ratio of 3.0 as a cut-off value, we found 20 of 112 lung cancer patients (17.9 %) were positive for anti-p53 antibodies and 7 of 172 controls (4.1 %) were positive. A significant difference between cases and controls was found ($p = 0.0329$), after adjusting for age, gender, ethnicity, smoking and occupational exposure.

Table 2 shows the distribution of p53 antibodies in lung cancer patients. By histological types, 25 % (8/32) of squamous cell carcinoma cases had anti-p53 antibodies in comparison with 14.6 % (7/48) of adenocarcinoma cases. Small cell lung cancer cases had the highest prevalence of anti-p53 antibodies at 42.9 % (3/7). We did not find any evidence of anti-p53 antibodies in large cell lung cancer cases (0/4) because of the small sample size. The p53 antibody positive rate was higher in male cases (21.1 %) than female cases (11.1 %) and in older cases (≥ 55 years old) (21.2 %) than in younger cases (< 55 years old) (7.4 %), but these differences did not reach statistical significance ($p > 0.05$). By ethnicity, the positive rates of anti-p53 antibodies among Caucasians (18.2 %), Hispanics (20.0 %) and African-Americans (16.7 %) were very close, and no significant difference was found.

There was a mild trend of increase of anti-p53 antibodies with smoking. One of 7 non-smokers (14.3 %), 6 of 36 ex-smokers (16.7 %) and 13 of 68 current smokers (19.1 %) among lung cancer patients had anti-p53 antibodies, though this was not statistically significant (table 2). Furthermore, we analysed the association between lifetime smoking history (pack-years) and anti-p53 antibodies and found heavy smokers (≥ 41 pack-years) had the highest positive rate of anti-p53 antibodies (28.6 %) in comparison with non-smokers (14.3 %) and light smokers with 1–40 pack-years (9.6 %).

Occupational exposure also affected the distribution in lung cancer patients. The patients who had an occupational exposure history (23.1 %) had a somewhat higher frequency of the antibodies than those who did not (18.6 %), but this difference did not have statistical significance ($p > 0.05$).

In the analysis combining smoking and occupational exposure, we found a joint effect between these two risk factors in lung cancer. Lung cancer patients with heavy smoking (≥41 pack-years) and occupational exposure had the highest positive rate (42.9 %) of anti-p53 antibodies as compared with the occupational unexposed lung cancer patients with never smoking (14.3 %), light smoking (10.5 %) and heavy smoking (27.5 %) history, although once again this was not statistically significant ($p > 0.05$).

Removing the ten cases whose information was obtained from the Tumor Registry alone had no effect on the analysis results.

All seven controls who had the antibodies against p53 were smokers. Among them, 4 of 42 (9.5 %) ex-smoker controls had low ratios (3.20–3.60) and 3 of 82 (3.7 %) current smoker controls had higher ratios (3.62, 8.48 and 10.55, respectively). We did not find any evidence of anti-p53 antibodies in 41 controls who never smoked.

Discussion

There are several studies that report the prevalences of anti-p53 antibodies from 8 % to 24 % in lung cancer patients (Winter *et al.* 1992, Angelopoulou *et al.* 1994, Schlichtholz *et al.* 1994, Wild *et al.* 1995, Rosenfeld *et al.* 1997). Our 18 % positive rate for anti-p53 antibodies in lung cancer patients is within this range. No statistical difference for anti-p53 antibodies among Caucasians, Hispanics and African-Americans indicates that different ethnic populations may have similar humoral responses following stimulation of their immune systems by mutant p53 proteins, although each population may be exposed to different quantities and qualities of lung carcinogens and consequently have different morbidity and mortality rates for lung cancer.

Different frequencies of p53 gene mutation and p53 protein overexpression among the histological types of lung cancer have been reported in many studies (D'Amico *et al.* 1992, McLaren *et al.* 1992, Brambilla *et al.* 1993, Top *et al.* 1995, Wild *et al.* 1995). A difference in prevalence of anti-p53 antibodies by histological types of lung cancer is also found in this study, which is consistent with the results of Wild *et al.* (1995). It is also of note that the distribution of anti-p53 antibodies among histological types of lung cancer in this study is similar to the frequency distributions of p53 mutation and p53 overexpression among the histological types of lung cancer in previous studies (figure 1). For example, small cell lung cancer with the highest frequency of anti-p53 antibodies (42.9 %) in this study is reported to have the highest frequency of p53 mutation (73.9 %) and p53 overexpression (66.0 %); adenocarcinoma of lung, showing a low frequency of the antibodies (14.6 %), is reported to have a low frequency of p53 mutation (34.4 %) and p53 overexpression (38.6 %). The similarity of these distributions among the histological types suggests that there is a correlation among p53 mutation, p53 overexpression and anti-p53 antibodies. Although the patterns of distribution of mutation, overexpression and antibody are similar by histological type, it should be noted that the absolute prevalence of the antibodies is generally lower than that of mutation or overexpression, regardless of type of cancer.

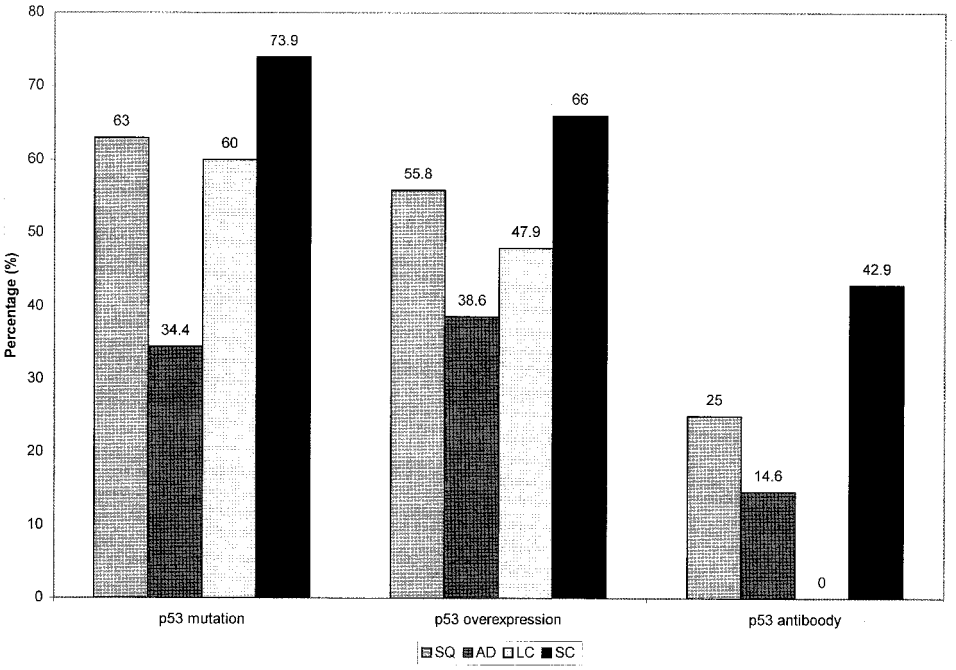

Figure 1. Distributions of p53 mutation, p53 overexpression and p53 antibodies by histological type of lung cancer. SQ: squamous cell carcinoma; AD: adenocarcinoma; LC: large cell carcinoma; SC: small cell carcinoma. Data on p53 mutation come from the references (Takahashi *et al.* 1991, D'Amico *et al.* 1992, Kishimoto *et al.* 1992, Winter *et al.* 1992, Horio *et al.* 1993, Mitsudomi *et al.* 1993, Top *et al.* 1995, Rosenfeld *et al.* 1997) and are the average from these references , SQ: 63.0 % (121/192); AD: 34.4 % (86/250); LC: 60.0 % (24/40); SC: 73.9 % (34/46); data on p53 overexpression come from the references (McLaren *et al.* 1992, Brambilla *et al.* 1993, Korkolopoulou *et al.* 1993, Dosaka-Akita *et al.* 1994, Top *et al.* 1995, Kondo *et al.* 1996) and are the average from these references, SQ: 55.8 % (163/292); AD: 38.6 % (83/215); LC: 47.9 % (23/48); SC: 66.0 % (31/47).

Cigarette smoke is closely associated with p53 mutation and p53 overexpression. Husgafvel-Pursiainen *et al.* (1996) reported that there were different frequencies of p53 mutation by smoking status with p53 mutations increasing from non-smokers (25 %) to ex-smokers (38 %) to current smokers (55 %). Kondo *et al.* (1996) found that p53 mutations increased with the lifetime smoking history. Dosaka-Akita *et al.* (1994) observed that smokers had a higher frequency of p53 overexpression than non-smokers, and Westra *et al.* (1993) demonstrated that the frequencies of p53 overexpression increased from non-smokers to ex-smokers to current smokers in adenocarcinoma of lung. In this study, we found a mild trend with the frequencies of anti-p53 antibodies increasing from non-smokers (14.3 %) to ex-smokers (16.7 %) to current smokers (19.1 %), and heavy smokers (41 pack-years and more) had the highest prevalence of the antibodies (28.6 %). Although our findings were not statistically significant, these results taken together with prior studies suggest that smoking may be associated with the development of anti-p53antibodies. More and larger studies will be needed to test this hypothesis.

Environmental and occupational exposures, such as asbestos, are the second leading cause of lung cancer. The association between p53 mutation and occupational exposures has been reported (Wang *et al.* 1995, Harty *et al.* 1996).

The association between occupational exposures and anti-p53 antibodies was also found in lung cancer patients in this study, although this was not a significant difference. In the analysis of the combined effects of lifetime smoking history and occupational exposure, the lung cancer patients who smoked for more than 40 pack-years and were occupationally exposed had the highest positive rate of the antibodies (42.9 %), suggesting that there is a biological interactive effect between occupational exposure and smoking. The failure to demonstrate the significance of this effect may be due to the small sample size in some subgroups. The association between occupational exposure and anti-p53 antibodies needs further study.

In this study, seven patients without cancers were found positive for anti-p53 antibodies. Several things could explain the positivity in these control patients. First, cigarette smoking may cause p53 mutation and lead to the development of anti-p53 antibodies prior to the development of cancer. Cigarette smoke contains many carcinogens and is associated with the p53 mutation and p53 overexpression (Westra *et al.* 1993, Dosaka-Akita *et al.* 1994, Husgafvel-Pursiainen *et al.* 1996, Kondo *et al.* 1996). The accumulated mutant p53 proteins could induce a humoral response to produce anti-p53 antibodies. Lubin *et al.* (1995b) and Trivers *et al.* (1996) found that the anti-p53 antibodies could be detected in ex-smokers or current smokers as early as 15 months prior to the diagnosis of cancers of the lung, breast and prostate. Wollenberg *et al.* (1997) even reported that the frequency of anti-p53 antibodies was higher in healthy smokers than in head and neck cancer patients. In the present study, the seven controls with anti-p53 antibodies were all ex-smokers (4/7) or current smokers (3/7). The presence of these anti-p53 antibodies may be associated with smoking alone or may indicate the future development of malignancy. Follow-up of these patients will be needed to see whether they develop cancer or not. On the other hand, no anti-p53 antibodies were detected in 41 non-smoker controls. Second, occupational exposures may elicit these antibodies. In one study (Trivers *et al.* 1995), 4 of 77 vinyl chloride-exposed workers without angiosarcoma of the liver (ASL) were positive for anti-p53 antibodies and two patients with ASL exposed to vinyl chloride had anti-p53 antibodies at 11.3 years and 4 months prior to the diagnosis, respectively. In our study, two of seven controls with the antibodies had an occupational exposure history. Third, there are several studies that report anti-p53 antibody detection in other diseases, such as benign pancreatic disease (Marxsen *et al.* 1994), systemic lupus erythematosus and other systemic rheumatic disease (Kovacs *et al.* 1997), and paraneoplastic disease of the nervous system (Gregson and Kaur 1997). Reviewing medical records for all controls in this study, we did not find evidence of these diseases in our controls, including those seven controls with anti-p53 antibodies. Fourth, some proteins may cross-react with p53 proteins. In the study of Schlichtholz *et al.* (1994), such a cross-reacting protein was suspected on the basis of protein mapping. Last, we cannot completely exclude the presence of false positives in this study because we used a statistical cut-off value, which allows for 2.5 % of false positives.

Another limitation of this study is the use of hospital patients without a cancer history as controls. The hospital patients may be different from healthy people in various ways. Some of the diseases that these patients have may be found to be associated with anti-p53 antibodies in the future. Further evaluation of the prevalence of anti-p53 antibodies in cancer patients should incorporate healthy, non-hospital controls. Nevertheless, in this study we have demonstrated a

significantly higher prevalence of anti-p53 antibodies in lung cancer patients in a pattern by histological types consistent with prior studies and the suggestion that this could be related to smoking or other carcinogen exposures. These results suggest that anti-p53 antibodies as a potential biomarker for the study of individuals with lung cancer or at-risk for the development of lung cancer deserve to be studied further.

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