p53 Mutations in non-small-cell lung cancers occurring in individuals without a past history of active smoking

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Summary Accumulating evidence suggests that the p53 gene is a good target for molecular epidemiological studies. We previously reported an association between the presence of p53 mutations and lifetime cigarette consumption. Although over 675 p53 mutations have been reported in lung cancers in the literature thus far, very little is known about the nature of such changes in lung cancers in the absence of a smoking background. In the present study, we therefore analysed 69 non-small-cell lung cancer specimens from individuals without any history of active smoking and identified p53 mutations in 26% of the cases. Statistical analysis of the present cohort of non-smokers also showed absence of significant relationship between p53 mutations and age, sex, histological type or disease stage. Comparison of mutational spectra between the present results in non-smokers and previously reported mutations in smokers clearly demonstrated G:C to T:A transversions to be significantly less frequent in non-smokers than in smokers (OR 5.35, 95% CI 1.77–16.12). Interestingly, G:C to C:G and G:C to A:T mutations were also observed in tumours of non-smokers at similar frequencies to G:C to T:A mutations, suggesting that these mutations can occur relatively frequently in the absence of active smoking. This study is, to our knowledge, the largest so far analysing a well-defined cohort of non-smokers in a single laboratory.

Keywords: p53; mutation; smoking; lung cancer

The p53 gene is mutated in a large proportion of most human cancers, including those developing in the lung, with over 6000 examples of p53 mutations reported so far (Hainaut et al, 1997). Accumulating evidence suggests that the frequency and spectrum of p53 mutations may represent fingerprints left by specific carcinogens and that the p53 gene is a good target for molecular epidemiological studies aimed at determining risk factors for neoplasia (Hollstein et al, 1991; Greenblatt et al, 1994).

Mutations in the p53 gene appear to be the most frequent molecular change thus far identified in lung cancer (Naylor et al, 1987; Rodenhuis et al, 1987; Harbour et al, 1988; Takahashi et al, 1989, 1991; Chiba et al, 1990; D'Amico et al, 1992; Hibi et al, 1992; Miller et al, 1992; Suzuki et al, 1992, 1994; Washimi et al, 1995; Kondo et al, 1996; Nagatake et al, 1996a and b). We previously reported that p53 mutations occur in all histological subtypes of human lung cancer, with frequencies of ~75% in small-cell lung cancer (SCLC) and ~50% in non-small-cell lung cancer (NSCLC), and that wild-type p53 can function as a potent in vitro and in vivo growth suppressor in such lesions (Takahashi et al, 1989, 1991, 1992; Suzuki et al, 1992). Previous mutational analyses of the p53 gene have shown that the most prevalent p53 mutation in lung

cancers is the G:C to T:A transversion, which is uncommon in other cancers, such as colon carcinomas (Chiba et al, 1990; Takahashi et al, 1991; D'Amico et al, 1992; Miller et al, 1992; Mitsudomi et al, 1992; Suzuki et al, 1992; Takeshima et al, 1993). Numerous conventional epidemiological studies have provided evidence for a strong association between lung cancer and cigarette smoking (Muler, 1939; Doll and Hill, 1950; Levin et al, 1950; Wynder et al, 1950; US Public Health Service, 1964; US Department of Health and Human Services, 1989; Shopland et al, 1991), and we have previously shown that the presence of p53 mutations is also closely linked with lifetime cigarette consumption (Suzuki et al, 1992).

To date, over 675 mutations in lung cancers have been deposited in the database of p53 somatic mutations in human tumours and cell lines (Hainaut et al, 1997), but only eight instances could be retrieved as those identified in individuals without a smoking history (released January 1997). As smoking is presumably a major determinant regarding the nature of genetic changes occurring in lung neoplasia, we conducted the present study to identify characteristics of p53 mutations in lung cancers without this strong influence by examining 69 NSCLC specimens from individuals without any history of smoking. A comparison with the mutational spectrum in smokers was included.

MATERIALS AND METHODS

Tumours

Sixty-nine NSCLC samples from patients without a past history of active smoking were obtained at the time of surgery at either Aichi

Table 1 *p53* mutations in lung cancers occurring in non-smoking individuals

Case	Age (years)	Sex	Histology[a]	Stage	Codon	Base change
L389	71	F	AD	IV	135	TG<u>C</u> to TA<u>C</u>
L122[b]	56	F	AD	I	136	<u>C</u>AA to <u>G</u>AA
L265[b]	67	F	ADSQ	I	136	<u>C</u>AA to <u>G</u>AA
L335	55	M	AD	IV	158	C<u>G</u>C to C<u>T</u>C
L292[b]	63	F	AD	III	166	T<u>C</u>A to T<u>G</u>A
L227[b]	71	F	AD	III	179	<u>C</u>AT to <u>G</u>AT
L125[b]	43	F	AD	III	179	<u>C</u>AT to <u>G</u>AT
L230	39	M	AD	III	194	C<u>T</u>T to C<u>G</u>T
L489	72	M	SQ	III	211	<u>T</u>TT to <u>C</u>TT
L10[b]	42	F	ADSQ	I	238	T<u>G</u>T to <u>GG</u>T
L416	42	F	AD	II	242	TG<u>C</u> to TA<u>C</u>
L504	61	F	AD	II	249	AGG to AG<u>T</u>
L428	68	M	AD	I	249	AG<u>G</u> to AG<u>T</u>
L480	70	M	AD	I	249	AG<u>G</u> to AG<u>T</u>
L215[c]	61	F	AD	I	250	<u>CC</u>C to <u>TT</u>C
L244	74	F	SQ	I	272	GT<u>G</u> to GT<u>A</u>
L424	58	F	AD	II	282	<u>C</u>GG to <u>T</u>GG
L184[b]	74	F	AD	I	int 4	a<u>g</u> to a<u>a</u>

[a]Histological subtype: AD, adenocarcinoma; SQ, squamous cell carcinoma; ADSQ, adenosquamous carcinoma. [b]Previously reported by Takagi et al (1995). [c]Previously reported by Suzuki et al (1992).

Table 2 Relationships between clinical characteristics and *p53* mutations in lung cancers occurring in non-smoking individuals

Clinical feature	No. of cases	No. positive (%)	Odds ratio (95% CI)	
			Univariate	Multivariate
Age (years)				
< 62	35	9 (25.7)		
≥ 62	34	9 (26.5)	1.30 (0.44–3.83)	1.13 (0.35–3.64)
Sex				
Male	11	5 (45.4)		
Female	58	13 (22.4)	2.42 (0.65–8.90)	2.26 (0.57–9.03)
Histology				
Adenocarcinoma	61	14 (22.9)		
Squamous cell carcinoma	6	2 (33.3)		
Adenosquamous carcinoma	2	2 (100)	1.47 (0.25–8.79)[a]	1.00 (0.14–7.25)[a]
Tumour size				
pT1	22	8 (36.3)		
pT2	40	9 (22.5)		
pT3	4	1 (25.0)		
pT4	3	0 (0)	1.90 (0.79–4.57)	NI[b]
Nodal involvement				
pN0	40	9 (22.5)		
pN1	13	5 (38.5)		
pN2	16	4 (25.0)	0.87 (0.46–1.65)	NI[b]
Disease stage				
I	35	8 (22.9)		
II	9	3 (33.3)		
IIIA/IIIB	21	5 (23.8)		
IV	4	2 (50.0)	1.16 (0.69–1.95)[c]	1.13 (0.66–1.92)[c]

[a]Histology was dischotomized for analysis (squamous cell carcinoma vs other histological types). [b]NI, not included. [c]For an increase of one disease stage.

Cancer Center Hospital or National Chubu Hospital in Aichi prefecture, Japan, and stored at –80°C until analysis at Aichi Cancer Center Research Institute. The lung tumours were histologically typed according to the World Health Organization's histological classification. Mutations in some of the cases have been reported in our previous studies (Suzuki et al, 1992; Takagi et al, 1995).

Analysis of *p53* mutations

Polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP) analysis was performed to detect *p53* mutations in the region between exons 5 and 8 using genomic DNA. The primer pairs used in this study were as follows: e5s, 5'-AGCAAGCTTGACTTTCAACTCTGTCTCCTT and e5as,

5′-AGCGGATCCACCAGCCCTGTCGTCTCTCCA for exon 5; e6s, 5′-AGCAAGCTTAGGCCTCTGATTCCTCACTGA and e6as, 5′-AGCGGATCCCCAGAGACCCCAGTTGCAAAC for exon 6; e7s, 5′-AGCAAGCTTAAGGCGCACTGGCTTCATCTT and e7as, 5′-AGCGGATCCGCACAGCAGGCCAGTGTGCAG for exon 7; and e8s, 5′-AGCAAGCTTAGGACCTGATTTCC-TTACTGC and e8as, 5′-AGCGGATCCTGCACCCTTGGTCTC-CTCCAC for exon 8. For exons 5, 7 and 8, the PCR products labelled with [^{32}P]dCTP were electrophoretically separated by 6% non-denaturing polyacrylamide gel at 40 W for 3.5 h at 5°C with no cooling fan, while electrophoresis of the PCR products of exon 6 was performed at 30 W for 4.5 h at 5°C with a cooling fan. The PCR conditions used to amplify genomic DNA were identical to those detailed in our previous report (Horio et al, 1993). Genomic DNAs demonstrating an altered mobility shift in this PCR-SSCP analysis were further analysed by sequencing, and the identified mutations confirmed by separate PCR and subsequent sequence analysis as described previously (Horio et al, 1994).

Statistical analysis

Univariate and multivariate regression analyses were performed using SAS/Windows Ver. 6.11 statistical software (SAS Institute, Cary, NC, USA) to examine the possible relation of the presence of *p53* mutations to various clinical characteristics, including age, sex, histology and disease stage. Relative risks and 95% confidence intervals for the dependent parameter were estimated conditionally on the determinants (age, sex, histology and disease stage). In addition, logistic regression analysis was also conducted to examine the possible relation of the *p53* mutational spectra to lifetime cigarette consumption.

RESULTS

Detection of *p53* mutations in lifetime never-smokers

PCR-SSCP and sequencing analyses identified mutations in exons 5–8 of the *p53* gene to be present in 18 (26%) of 69 non-small-cell lung cancer specimens from patients without any past history of active smoking (Tables 1 and 2). Among the 18 *p53* mutations identified here, 17 were single nucleotide substitutions and the remaining one was a CC:GG to TT:AA tandem double mutation

Table 3 Spectrum of *p53* mutations in lung cancers occurring in non-smoking individuals

Mutation	Non-smokers (%) (*n* = 18)	Smokers^a (%) (*n* = 50)	Odds ratio (95% CI)
G:C to A:T	27.7	22.0	1.41 (0.46–4.31)
G:C to T:A	22.2	48.0	5.35 (1.77–16.12)
G:C to C:G	27.7	10.0	0.67 (0.19–2.39)
A:T to G:C	5.5	2.0	0.68 (0.04–11.11)
A:T to C:G	11.1	4.0	0.68 (0.09–4.93)
A:T to T:A	0	2.0	NA^b
Deletion	0	12.0	NA
CC:GG to TT:AA	5.5	0	NA

^{a}Compilation of *p53* mutations in smokers previously identified at the Aichi Cancer Center (Suzuki et al, 1992; Takagi et al, 1995; YT, HO, TT and TT, unpublished observation) and Hammon Center (Chiba et al, 1990; Mitsudomi et al, 1992). ^{b}NA, not available because of inability to attain convergence within 25 interations.

(Table 3). G:C to A:T transitions were observed in five (28%) cases, four being found at non-CpG sites. G:C to C:G and G:C to T:A transversions were observed in 28% and 22%, respectively, of the cohort.

Relationship between *p53* mutations and clinical characteristics in lifetime never-smokers

Adenocarcinomas, known to be the least related to smoking among the histological subtypes of lung cancers (Hanai et al, 1988), constituted the majority (61 of 69) of the studied cohort, and 14 (23%) of these had *p53* mutations (Table 2). Although *p53* mutations were found more frequently in squamous cell carcinomas (33.3% vs 23%), the difference was not statistically significant.

Statistically significant differences were not apparent with stratification according to age, gender, tumour size, nodal involvement, distant metastasis or disease stage. Multivariate analysis using a logistic regression model also showed absence of correlation between the presence of *p53* mutations and clinical characteristics including age, gender, histology and disease stage.

Comparison of *p53* mutational spectrum between lifetime never-smokers and individuals with smoking histories

We next investigated whether there are any distinctions between *p53* mutations in lung cancers occurring in individuals with and without smoking histories (Table 3). Although the database of *p53* somatic mutations in human tumours and cell lines available for conducting molecular epidemiological analyses is exceedingly large (Hollstein et al, 1991; Greenblatt et al, 1994; Hainaut et al, 1997), data on smoking histories are only limited. We therefore compiled data for *p53* mutations occurring in NSCLCs of smokers, which were previously identified in the laboratories of Aichi Cancer Center and Hammon Center, yielding 68 *p53* mutations within the region corresponding to that investigated here (exons 5–8) (Chiba et al, 1990; Mitsudomi et al, 1992; Suzuki et al, 1992; Takagi et al, 1995; YT, HO, TT and TT, unpublished observation). Comparison of the mutation spectra revealed that G:C to T:A transversions, the most prevalent type of *p53* mutations in lung cancers of smokers, to be significantly less frequent in their counterparts in non-smokers (OR 5.35, 95% CI 1.77–16.12) (Table 3). We noted that G:C to C:G transversions occurred three times as frequently in non-smokers (28%) as in smokers (10%); the difference was not statistically significant. No other statistically significant differences were observed between never-smokers and smokers, including G:C to A:T transitions.

DISCUSSION

The present study, conducted to assess the mutational characteristics of *p53* in lung cancers in the absence of the major influence of smoking, provides to our knowledge the only available data for a reasonably large cohort of lung cancers occurring in individuals without a past history of active smoking. *p53* mutations were detected in 26% of the NSCLC specimens examined, this relatively low frequency for non-smokers being consistent with our previous observation of a positive association between the presence of *p53* mutations and lifetime cigarette consumption (Suzuki et al, 1992). Statistical analysis of the present cohort of non-smokers also showed absence of significant relationship between

p53 mutations and age, sex, histological type or disease stage. Comparison of the mutation spectra between non-smokers and smokers revealed a significant difference regarding the frequencies of G:C to T:A transversions (OR 5.35, 95% CI 1.77–16.12). While a number of previous studies, in which most of the specimens were presumably from smokers, demonstrated G:C to T:A transversions as the most prevalent and characteristic type of mutations in lung cancers (Chiba et al, 1990; Takahashi et al, 1991; D'Amico et al, 1992; Miller et al, 1992; Mitsudomi et al, 1992; Suzuki et al, 1992; Takeshima et al, 1993), the present study clearly indicates that this type of mutation occurs significantly less frequently without the influence of smoking. In fact, G:C to A:T transitions and G:C to C:G transversions were found at similar frequencies.

In contrast to our results, Takeshima et al (1993) previously reported a predominance of G:C to A:T transitions in lung cancers of non-smoking atomic bomb survivors, identifying four mutations each in the analysis of nine non-smoking atomic bomb survivors and eight non-smoking control cases. It is notable that the mutation frequency (47%) reported by Takeshima et al (1993) is almost twice as high as that (26%) observed in the present study. The discrepancy could be due to an unintended bias in their analysis because of the inclusion of *p53* mutations in atomic bomb survivors and the compilation of the data from other laboratories.

In addition to the significantly lower frequency of G:C to T:A transversions, the CC:GG to TT:AA tandem double mutation identified may also provide an insight into one possible genesis of lung cancers in non-smoking individuals. Loeb and co-workers have clearly shown that while this tandem double mutation, a hallmark of damage to DNA by UV irradiation, is not produced by DNA polymerases or viral reverse transcriptases or by exposure to chemical carcinogens, it can be caused by oxidative damage to DNA (Reid and Loeb, 1993; Tkeshelashvili et al, 1993). G:C to C:G and G:C to T:A transversions, which were also found to be present at relatively high frequencies in the lung cancers of our non-smoker cohort, are also known to be induced by reactive oxygen species in certain circumstances (Moriya et al, 1991; McBride, 1992). The present findings suggest that oxygen radical species, which can be generated by various mechanisms, including normal cellular processes (Fridovich, 1983), might have a role in the development of lung cancers. Further studies are obviously required to elucidate any relationship between DNA damage due to reactive oxygen species and the pathogenesis of lung cancers.

It should be noted, however, that a number of other possibilities must be considered, such as passive smoking and exposure to environmental pollutants. In this regard, it is interesting that G:C to T:A transversions were present in three (75%) of the four male patients carrying *p53* mutations in contrast to only one (7%) in 14 female patients, the difference being significant (*P* = 0.019 by Fisher's exact probability test). The present finding is of interest when we consider the fact that smoking at the work place is still common in Japan, while until recently most women had usually spent most of their lives at home as housewives. Accordingly, one could speculate that these male patients may have been high-exposure passive smokers for many years (Matsukura et al, 1984).

A number of conventional epidemiological studies have shown that among the four major histological subtypes of lung cancers, the adenocarcinoma is the least related to smoking, yet its incidence is steadily increasing in various countries, including Japan and USA (Watanabe et al, 1987; Travis et al, 1994). Future carefully designed molecular epidemiological studies, with larger cohorts,

are required to identify risk factors and provide a basis for better strategies of lung cancer prevention in non-smoking individuals.

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