[CANCER RESEARCH 52, 734–736, February 1, 1992]

Advances in Brief

p53 Mutations in Non-Small Cell Lung Cancer in Japan: Association between Mutations and Smoking[1]

Hiroko Suzuki, Takashi Takahashi,[2] Tetsuo Kuroishi, Motokazu Suyama, Yutaka Ariyoshi, Toshitada Takahashi, and Ryuzo Ueda

Laboratories of Chemotherapy [H. S., Ta. T., R. U.], Epidemiology [T. K.], and Immunology [To. T.], Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan; Department of Internal Medicine [Y. A.] and Department of Surgery [M. S.], Aichi Cancer Center Hospital, Chikusa-ku, Nagoya 464, Japan

Abstract

The *p53* gene has been implicated as a tumor suppressor gene involved in the pathogenesis of lung cancer. Our previous study revealed that the *p53* gene is frequently mutated with a distinct nucleotide substitution pattern in small cell lung cancer specimens in Japanese patients. In this study, we examined 30 primary, resected non-small cell lung cancer samples in Japanese patients using complementary DNA-polymerase chain reaction and sequencing. Mutations changing the *p53* coding sequence were found in 14 of 30 tumor samples (47%), while G:C to T:A transversions which are uncommon in other cancers such as colon cancer were the most frequently observed mutations, in agreement with an earlier report on non-small cell lung cancer in American patients. Furthermore, the present study shows for the first time that in univariate and multivariate analyses, the presence of *p53* mutations is closely associated with lifetime cigarette consumption.

Introduction

The incidence of lung cancer is dramatically increasing in Japan and is expected to become the leading cause of cancer deaths in the near future (1). Unfortunately, as many as 90% of patients diagnosed with lung cancer still die of their disease, but recent progress in the molecular biology of lung cancer has reached a point where it can provide a basis for novel strategies in the prevention and treatment of this disease (2, 3). Accumulating evidence indicates that changes both in dominant oncogenes and in tumor suppressor genes are probably necessary for malignant transformation of normal bronchial epithelial cells. Among such genetic abnormalities, the *p53* gene appears to be the most frequent target (4–8). Our previous study revealed that a different nucleotide substitution pattern exists in small cell lung cancer in Japan when compared to that in the United States, suggesting the involvement of a distinct mutagenic process in each of these two populations (7).

Thus, the current study was initiated (*a*) to determine the frequency, location, and nature of *p53* mutations in NSCLC[3] in Japan and to compare them with those of NSCLC in the United States and (*b*) to correlate *p53* mutations with clinical data. Our findings showed that *p53* mutations in NSCLC tumors in Japanese patients are similar to those in American patients, although *p53* mutations at a prominent hot spot (codon 273) known in the United States were not observed in cases in Japan. We also found for the first time that the presence of *p53* mutations is closely associated with lifetime cigarette consumption.

Materials and Methods

Tumor Samples, Clinical Data, and Statistical Analyses. Thirty tumor specimens from 30 patients with NSCLC were obtained during surgery at the Aichi Cancer Center in Japan. Tumors were quick frozen and stored at −135°C until use. DNA and RNA were prepared from tumor samples as described previously (9). For clinical and postsurgical pathological stages, the new international staging system for lung cancer was used (10). Univariate and multivariate analyses of factors potentially related to *p53* mutations were performed by means of the logistic regression model to investigate the single and joint effects of these factors. All molecular genetic studies were done without knowledge of the clinical data.

cDNA/PCR Amplification and Sequencing. First-strand cDNA was synthesized using 25 μg of total cellular RNA with random hexanucleotide primers, and subsequent PCR was performed with one-fiftieth of the synthesized cDNA, as described previously (11). The primers used to amplify the entire open reading frame of *p53* cDNA were 5′-AGTCAAGCTTGACGGTGACACGCTTCCCTGGATT and antisense, 5′-AGTCGAATTCTCAGTGGGGAACAAGAAGTGGAGA. cDNA/PCR amplification was performed for 30 cycles at 94°C (1 min), 58°C (2 min), and 72°C (6 min) followed by 10 min of extention at 72°C. After digestion with *Hin*dIII and *Eco*RI, the PCR products were cloned into the *Hin*dIII-*Eco*RI site of pGEM7Zf(+) (Promega, Madison, WI). The plasmid DNA prepared from pooled clones was sequenced using *p53* specific primers and a DSP-240 DNA processor (Seiko, Tokyo, Japan) by the dideoxy method, as described previously (7). Identified mutations were confirmed by separate cDNA/PCR amplification and subsequent sequencing.

Genomic DNA/PCR Amplification and Sequencing. For tumor samples which exhibited large deletions at the exon-exon boundaries in *p53* cDNAs, we analyzed the putative abnormal splicing junctions. One μg of genomic DNA was amplified with appropriate primers (each set specific for the mutation involved). The genomic DNA/PCR amplification consisted of 30 cycles (94°C for 1 min, 58°C for 2 min, 72°C for 6 min) after an initial denaturation step (94°C for 5 min). Direct sequencing for genomic DNA/PCR products was performed using nested primers as described previously (11).

Results and Discussion

Identification of *p53* Mutations in NSCLC Tumor Specimens. We examined exons 4 through 9 of *p53* in cDNA prepared from 30 NSCLC tumor specimens which were surgically removed as part of potentially curative resections from 30 patients at the Aichi Cancer Center. *p53* mutations were found in 14 of these 30 NSCLC tumors (47%) (Table 1). Eleven of the 14 samples contained missense mutations, while 1 tumor sample

Received 10/2/91; accepted 12/4/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a Grant-in-Aid for the Comprehensive Ten-Year Strategy for Cancer Research from the Ministry of Health and Welfare, Japan; Grant-in-Aids for Cancer Research from the Ministry of Education, Science, and Culture and the Ministry of Health and Welfare, Japan; a grant from the Cancer Research Institute, Inc., New York; and a grant from the Imanaga Memorial Foundation, Japan.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; cDNA, complementary DNA; PCR, polymerase chain reaction.

Table 1 *p53 gene mutations in NSCLC*

Tumor sample	Codon	Nucleotide substitution	Amino acid substitution
L111	154	GGC to GTC	Gly to Val
L124	157	GTC to TTC	Val to Phe
L110	175	CGC to CTC	Arg to Leu
L116	179	CAT to CGT	His to Arg
L112	194	CTT to CGT	Leu to Arg
L213	245	GGC to TGC	Gly to Cys
L107	248	CGG to CAG	Arg to Gln
L206	249	AGG to ATG	Arg to Met
L215	250	CCC to TTC	Pro to Phe
L203	275	TGT to TTT	Cys to Phe
L168	282	CGG to TGG	Arg to Trp
L114[a]	216–218[b]	3-base pair deletion	Val to deletion
L126[a]	Intron 3	AG to TG	Inframe deletion of codons 33–125
L108[a]	Intron 7	5-base pair deletion	Inframe deletion of codons 262–269

Tumor samples with no mutations:
L7, L12, L109, L119, L122, L125, L127, L167, L169, L183, L199, L207, L210, L212, L214, L223.

[a] Both mutant and wild-type *p53* mRNA were expressed in these tumor samples.
[b] A three-base pair inframe deletion (GTG) was identified in the GTG triplet at codons 216 to 218.

(L114) had a 3-base pair inframe deletion in the coding sequence. In 2 tumors (L108, L126), large inframe deletions at the exon-exon boundaries in the cDNAs suggested the involvement of abnormal splicings (11). Therefore, we also performed genomic DNA/PCR amplification and subsequent direct sequencing. L108 contained a 5-base pair deletion in the splice acceptor consensus of intron 7 (GAGTAG), which resulted in the use of a cryptic acceptor site at codon 269. L126 had an intronic point mutation in the splice acceptor site of intron 3 (AG to TG) which caused exclusion of the entire exon 4 sequence from the resulting cDNA.

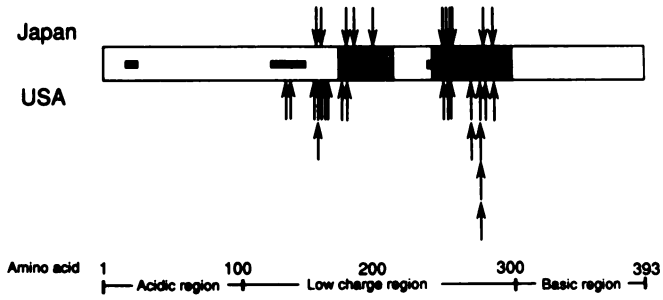

Fig. 1. *p53* missense mutations in NSCLC tumors of the Japanese patients in this study are shown above the schematic diagram for p53 protein compared with findings in NSCLC tumors of American patients (6). The five conserved regions (■) and the two predicted large T-antigen binding regions (□) are indicated (14). *Arrows*, points of mutation.

In general, the type of mutations reflects the mutagens involved. For example, G:C to T:A transversions can be caused by benzo[*a*]pyrene which is contained in cigarette smoke (12 G:C to T:A transversions, which are uncommon in *p53* mutations in other types of human cancers (13), were observed in NSCLC tumors at a frequency of 50%. Our results are in agreement with those previously reported for lung cancer, suggesting a common mutagen involved in lung cancer (5–7).

The mutations found in NSCLC were scattered throughout the low charge region in the central part of the *p53* protein (Fig. 1) (14). We note that together with the data of our previous analysis of small cell lung cancer (7), no *p53* mutations at codon 273 which is a known hot spot in American patients (6) have been found in Japan thus far (0 of 26). These observations may suggest a possible involvement of a distinct mutagenic process between these two populations as discussed below, although the number of cases studied is still too small to draw this conclusion definitely.

Table 2 *Correlation between clinical features and p53 mutations in NSCLC*

Clinical feature[a]	*p53* status		Univariate		Multivariate	
	Mutant	Normal	Odds ratio	*P*	Odds ratio	*P*
Age	62 ± 11.7[b]	62 ± 11.5	0.999[c]	0.986	0.971[c]	0.502
Sex						
Male	12	9				
Female	2	7	0.214	0.092	0.798	0.860
Clinicopathological status						
T_1	5	3				
T_2	6	12				
T_3	3	1	0.911	0.937	NI[d]	NI
N_0	7	8				
N_1	3	4				
N_2	4	4	1.105	0.908	NI	NI
Stage						
I	7	7				
II	2	4				
IIIA	4	5	0.955	0.956	0.954	0.970
Histology						
Squamous cell	8	4				
Adenocarcinoma	6	11				
Large cell	0	1	0.247[e]	0.064	0.642[e]	0.678
Smoking (cigarettes/day years)	1026 ± 504[b]	424 ± 425	1.003[f]	0.009	1.003[f]	0.038

[a] Data on 30 patients tabulated.
[b] Mean ± SD.
[c] For an increase of 1 year in age.
[d] NI, not included.
[e] Histology was dichotomized for analysis (squamous cell carcinoma *versus* other histological types).
[f] For an increase of 1 unit in lifetime cigarette consumption (1 cigarette/day for 1 year).

Correlation of *p53* Mutations with Clinical Data. This study was unblinded after completing analysis of *p53* mutations. Statistical analysis for the *p53* mutations was then performed to correlate with the clinical data (Table 2). Mutations in the *p53* gene were found even in small tumors (T_1) without any nodal metastases (N_0). There were no significant differences in the frequency of mutations with respect to tumor size (T), nodal involvement (N), or clinical stage. The frequency varied among the different histological subtypes of NSCLC (67% in squamous cell carcinoma and 35% in adenocarcinoma), a finding which generally agrees with a previous report in which American patients with *p53* abnormalities were shown at 65% in squamous cell carcinoma and 33% in adenocarcinoma (6).

We could not find any statistically significant association between the presence of *p53* mutations and age, sex, clinical stage, or histology. In univariate analyses, however, the amount of lifetime cigarette consumption (cigarettes/day × years) was closely associated with *p53* status ($P < 0.01$; Table 2). Using the logistic regression model, multivariate analysis was performed to investigate the independence of "lifetime cigarette consumption" as a predictive factor for mutation. This "lifetime cigarette consumption" was the only factor shown to be closely related to *p53* mutations, after adjusting for other covariates ($P < 0.05$; Table 2). For instance, the odds ratio for *p53* mutations to develop in smokers who consumed 20 cigarettes/day for 30 years can be estimated at a 5.3-fold increase over nonsmokers based on the computation (15)

$$\text{Odds ratio} = \exp(\ln(1.003) \times 20 \text{ cigarettes/day} \times 30 \text{ yr})$$

It is now clear that the *p53* gene is the most frequent target among the known genetic alterations in lung cancer (Refs. 4–8; this study). As various epidemiological studies have also shown that major fractions of lung cancer can be attributed to smoking (16, 17), identifying the association between smoking and *p53* mutations in this study is of particular interest and further supports the current public health efforts against smoking.

Our findings contradict the report by the NCI-Navy group which stated that there was no significant association between smoking and the *p53* mutations in NSCLC tumor samples of American patients (6). We do not know the exact reasons for such a discrepancy, but one possible explanation may be differences in the genetic features between the two populations as related to carcinogen metabolism or DNA repair activity. In this regard, it should be noted that the incidence of lung cancer among Japanese Americans is 2 to 3 times lower than that of white Americans, a figure comparable to that among Japanese (18).

The results of this study, together with previous reports by us and by other researchers, indicate that the *p53* gene is a good target for molecular epidemiological studies of various human cancers (7, 8, 13, 19, 20). Future studies carefully designed to search for the risk factors such as carcinogen exposure or higher sensitivity to certain carcinogens should provide a basis for the ultimate strategies in cancer prevention.

Acknowledgements

We are grateful to J. Minna for helpful discussions and for exchanging information prior to publication, to H. Ogawa for helpful discussions, and to P. Chumakov for providing the human *p53* genomic sequence.

References

1. Statistics and Information Department. Vital Statistics 1988 Japan, Vol. 1, pp. 252–267. Tokyo: Ministry of Health and Welfare, 1990.
2. Minna, J. D., Battey, J. F., Brooks, B. J., Cuttitta, F., Gazdar, A. F., Johnson, B. E., Ihde, D. C., Lebacq-Verheyden, A-M., Mulshine, J., Nau, M. M., Oie, H. K., Sausville, E. A., Seifter, E., and Vinocour, M. Molecular genetic analysis reveals chromosomal deletion, gene amplification, and autocrine growth factor production in the pathogenesis of human lung cancer. Cold Spring Harbor Symp. Quant. Biol., *51:* 843–853, 1986.
3. Minna, J., Nau, M., Takahashi, T., Shutte, J., Chiba, I., Viallet, J., Kaye, F., Whang-Peng, J., Oie, H., Russel, E. and Gazdar, A. Molecular pathogenesis of lung cancer. *In:* D. E. Bergsagel and T. W. Mak (eds.), Molecular Mechanisms and Their Clinical Applications in Malignancies, pp. 63–83. Orlando, FL: Academic Press, 1990.
4. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. *p53*: a frequent target for genetic abnormalities in lung cancer. Science (Washington DC) *246:* 491–494, 1989.
5. Iggo, R., Gatter, K., Bartek, J., Lane, D., and Harris, A. Increased expression of mutant forms of *p53* oncogene in primary lung cancer. Lancet, *335:* 675–678, 1990.
6. Chiba, I., Takahashi, T., Nau, M., D'Amico, D., Curiel, D., Mitsudomi, T., DL, B., Carbone, D., Piantadosi, S., Koga, H., Reissmann, P., Slamon, D., Holmes, E., and Minna, J. Mutations in the *p53* gene are frequent in primary, resected non-small cell lung cancer. Oncogene, *5:* 1603–1610, 1990.
7. Takahashi, T., Takahashi, T., Suzuki, H., Hida, T., Sekido, Y., Ariyoshi, Y., and Ueda, R. The *p53* gene is very frequently mutated in small-cell lung cancer with distinct nucleotide substitution pattern. Oncogene, *6:* 1775–1778, 1991.
8. D'Amico, D., Carbone, D., Mitsudomi, T., Nau, M., Fedorko, J., Russell, E., Johnson, B., Buchhagen, D., Bodner, S., Phelps, R., Gazdar, A., and Minna, J. High frequency of somatically acquired *p53* mutations in small cell lung cancer cell lines and tumors. Oncogene, in press, 1991.
9. Takahashi, T., Obata, Y., Sekido, Y., Hida, T., Ueda, R., Watanabe, H., Ariyoshi, Y., Sugiura, T., and Takahashi, T., Expression and amplification of *myc* gene family in small cell lung cancer and its relation to biological characteristics. Cancer Res., *49:* 2683–2688, 1989.
10. Mountain, C. F. A new international staging system for lung cancer. Chest, *89:* 225s–233s, 1986.
11. Takahashi, T., D'Amico, D., Chiba, I., Buchhagen, D. L., and Minna, J. D. Identification of intronic point mutations as an alternative mechanism for *p53* inactivation in lung cancer. J. Clin. Invest., *86:* 363–369, 1990.
12. Mazur, M., and Glickman, B. Somat. Cell. Mol. Genet., *14:* 393–400, 1988.
13. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. *p53* mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
14. Soussi, T., Caron de Fromentel, C., and May, P. Structural aspects of the p53 protein in relation to gene evolution. Oncogene, *5:* 945–952, 1990.
15. Breslow, N. E., and Day, N. E. Statistical Methods in Cancer Research, Vol. 1. Lyon, France: International Agency for Research on Cancer, 1980.
16. Royal College of Physicians. Smoking and Health. Summary and Report of the Royal College of Physicians of London on Smoking in Relation to Cancer of the Lung and Other Diseases. London: Pitman Publ. Corp., 1962.
17. Shopland, D. R., Eyre, H. J., and Pechacek, T. F. Smoking-attributable cancer motality in 1991: is lung cancer now the leading cause of death among smokers in the United States? J. Natl. Cancer Inst., *83:* 1142–1148, 1991.
18. International Agency for Research on Cancer and International Association of Cancer Registries. Cancer Incidence in Five Continents. Vol. 5, p. 868. Lyon, France; International Agency for Research on Cancer, 1987.
19. Bressac, B., Kew, M., Wands, J., and Osturk, M. Selective G to T mutations of *p53* gene in hepatocellular carcinoma from southern Africa. Nature (Lond.), *350:* 429–431, 1991.
20. Hsu, I., Metcalf, R., Sun, T., Welsh, J., Wang, N., and Harris, C. Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. Nature (Lond.), *350:* 427–428, 1991.

p53 Mutations in Non-Small Cell Lung Cancer in Japan: Association between Mutations and Smoking

Hiroko Suzuki, Takashi Takahashi, Tetsuo Kuroishi, et al.

Cancer Res 1992;52:734-736.