BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 95–100 (1997)
ARTICLE NO. RC977425

Mutations of the p53 Gene in Human Lung Cancer from Chromate-Exposed Workers

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Received September 4, 1997

We examined p53 mutations in 20 cancer samples from 19 chromate workers with lung cancer by Polymerase chain reaction-Single strand conformation polymorphism analysis and direct sequencing. Six missense mutations were identified in 4 (20%) of the 20 chromate lung cancer samples. Fewer mutations were found in the patients with lung cancers who had been exposed to chromate than in those who had not. However, the pattern of p53 mutations in lung cancer patients exposed to chromate differed from that of common lung cancers in 3 respects. There were no apparent G to T transversions, which are common base changes in lung cancers. Half of the mutational sites (3/6) had changes of AT base-pairs, and 2 of 4 mutational tumor samples had double missense mutations. Our results suggested that chromate exposure may induce point mutation of the p53 gene. © 1997 Academic Press

Key Words: **p53 gene, Mutation, Chromate exposure, Lung cancer.**

Carcinogens such as tobacco smoke, asbestos, radon, chromium, nickel, and bis(chloromethyl)ether have been demonstrated to be important risk factors in the development of lung cancer (1, 2). Recently, molecular biology has elucidated relationships between carcinogen exposure and changes in cancer-related genes in humans. In human lung cancer, tobacco smoke is associated with p53 and Ki-*ras* mutations (3, 4, 5), and radon and atomic bomb exposures are related to p53 mutations (6, 7).

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Chromium, primarily used in alloys, refractories, and the production of pigmentations, is derived primarily from chromate ore. Lehman first reported lung cancer in German chromate workers (8). According to the IARC Working Groups, the evidence for carcinogenicity of chromium(VI) compounds is sufficient only heavy exposure conditions as encountered in the chromate production, chromate pigment production and chromate plating industries. (9, 10). They are known to cause chromosomal aberrations, mutations, and transformations in cultured mammalian cells (9, 11). However, their genetic effects in humans are only partially understood.

We encountered 19 chromate workers who developed lung cancer. In this study, we examined p53 mutations in these 19 lung cancer patients, and found that chromate did not selectively attack the p53 gene in human lung cancers, but the possibility existed that chromate exposure induced point mutation of the p53 gene.

MATERIAL AND METHODS

Patients. Twenty tumor and normal lung tissue samples were obtained from 19 chromate workers with lung cancer during surgery or at autopsy at The Second Department of Surgery, School of Medicine, the University of Tokushima, Tokushima Municipal Hospital, Iwamizawa Rousai Hospital, and The Second Department of Surgery, School of Medicine, Hokkaido University. The patients' clinical information was as follows. Their mean age was 54.5±11.2 (40-75) years old. Five were non-smokers and 14 were smokers with a mean Brinkman index of 622±320 (360-1440). They were exposed to chromate for an average of 21.7±9.1 years (8-38). All 19 patients were male. The types of cancer included 17 squamous cell carcinomas, 1 adenocarcinoma, and 2 small cell carcinomas. We used the Union International Contre le Cancer (UICC) TNM staging system to the stage disease (12) as follows: Stage I, 8; Stage II, 4; Stage IIIA, 5; Stage IV, 3. Tumor histology was determined according to the World Health Organization (WHO) classification of lung tumors (13).

Polymerase chain reaction(PCR)-Single strand conformation polymorphism(SSCP) analysis. DNA extraction from formalin-fixed,

0006-291X/97 $25.00

paraffin-embedded specimens: Sections (5 μm) were cut from formalin-fixed paraffin-embedded tissue blocks containing chromate lung cancer samples, then they were stained with hematoxylin and eosin. The regions containing tumors were marked. From these regions, 5 sections of 10-μm were cut and placed in an Eppendorf reaction tube (1.5 ml). These sections were deparaffinized in graded xylene and alcohol series. To each of the samples, 400 μl of lysis buffer containing 150 mM NaCl, 15 mM sodium citrate, 1% SDS, and 0.1 mg/ml of proteinase K was added. The samples were vigorously shaken for 24 to 36 h at 48 °C. After phenol-chloroform extraction, DNAs were precipitated with cold ethanol overnight at −20 °C. After centrifugation, the pellets were dried and resuspended in 25 to 50 μl of distilled water.

PCR-SSCP: The DNA concentrations of the samples were adjusted to within a range of 0.01 to 0.1 mg/ml. The oligonucleotide primers used for the amplification of exons 5-8 were designed on the basis of the published sequences, as follows:

Exon 5: 5′-primer; AACTCTGTCTCCTTCCTC
3′-primer; GCCCCAGCTGCTCACCATCG
Exon 6: 5′-primer; CCTCACTGATTGCTCTTAGG
3′-primer; ACCCCAGTTGCAAACCAGAC
Exon 7: 5′-primer; CTCCTAGGTTGGCTCTGACT
3′-primer; CAAGTGGCTCCTGACCTGGA
Exon 8: 5′-primer; CCTATCCTGAGTAGTGGTAA
3′-primer; GTCCTGCTTGCTTACCTCGC

The primer pairs were labeled with [γ-^{32}P] dATP as described previously (14). The DNA was amplified by 35 cycles of PCR (94 °C, 55 °C, 72 °C, for 0.5, 0.5, 1.0 min.) in a total volume of 5 μl containing 0.25 μM of each of the 5′-end-labeled primers, 62.5 μM dNTP, 0.25 unit of *Taq*-polymerase, 0.01-0.1 μg of DNA and PCR buffer. The PCR products were diluted 10-fold in 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. The samples were heated at 80 °C for 5 min, to denature the DNA, then 1 μl of a heated sample was loaded onto a 6% nondenaturing polyacrylamide gel containing 90 mM Tris-borate and 2 mM EDTA with or without 5% glycerol. Electrophoresis was performed at room temperature at 40W. After electrophoresis, the gel was transferred to Whatman 3MM paper, dried, and autoradiographed with Kodak X-Omat AR film at room temperature for 1 hour (15).

Direct sequencing. The extra bands revealed by SSCP analysis were excised. The DNAs were extracted from the extra bands, and amplified by PCR. The PCR products were purified using a MERMAID Kit (United States, BIO 101 Corporation), and directly sequenced using the TAQuence Cycle-Sequencing Kit (United States, Biochemical Corporation). Separately amplified PCR products from the same sample were directly sequenced at least twice.

Statistical analysis was performed by means of the chi-square test and the t-test (unpaired).

RESULTS

PCR-SSCP Analysis of the p53 Gene in Lung Cancer Patients with Chromate Exposure

The DNAs of 4 (20%) of the 20 tumor samples from 19 lung cancer patients with chromate exposure (sample number 9, 13, 14 and 21) had different mobilities from those of normal lungs. Representative cases of the PCR-SSCP analysis of exon 7 are shown in Fig. 1. Bands of 2 lung cancer patients (13, 21) had slightly different mobilities from those of normal lungs (N).

Direct Sequencing of the Fragments with Mobility Shifts by PCR-SSCP Analysis

To identify nucleotide sequence changes in DNA samples with mobility shifts by PCR-SSCP, a single-strand DNA fragment was eluted from a small piece of dried polyacrylamide gel in the region corresponding to the band with abnormal mobility. This fragment was directly sequenced. The sequencing ladders of 2 DNA fragments (sample 13 and 21), which gave mobility shifts in exon 7 by SSCP analysis, are shown in Fig. 2. Sample 13 had an A→T transversion in the first position of codon 249. Sample 21 had a T→A transversion in the second position of codon 243. Two other DNA fragments (sample 9 and 14) were sequenced. Sample 9 had both a C→T transition in the second position of codon 278 and an A→C transversion in the second position of codon 286. Sample 14 had a C→T transition in the first position of both codon 194 and 222.

Mutations of the p53 Gene in Lung Cancer Patients with Chromate Exposure

Point mutations were identified in a region spanning exons 5 to 8 of the p53 gene in 4 (20%) of 20 tumor samples from 19 patients. Two of 4 tumor samples with point mutations had double missense mutations. All 6 point mutations produced an amino acid change. There were 3 transitions of GC base-pairs and 3 transversions of AT base-pairs, but G to T transversions, which are common in lung cancer in tobacco smokers, were not found (Table 1). Normal tissue around the tumors with point mutations had wild-type p53 sequences.

p53 Gene Mutations and Clinical Features of Lung Cancer Patients with Chromate Exposure

There was no significant difference in the period spent working in chromate factories among workers with (21.9 ± 9.0 years, n=4) and without (22.0±11.8 years, n=15) p53 gene mutations, although 2 workers with double mutations of the p53 gene had worked for relatively long periods in chromate factories (29 and 35 years). We did not find any significant association between the presence of p53 mutations and age (58.3±10.1 vs. 53.3±11.7), Brinkman index (190±256 vs. 489±395), histology or disease stage. Though 5 of 19 patients had double or triple cancers, only one of these patients had p53 mutations (Table 1).

DISCUSSION

Several epidemiologic surveys performed in the U.S.A., Great Britain and other countries have con-

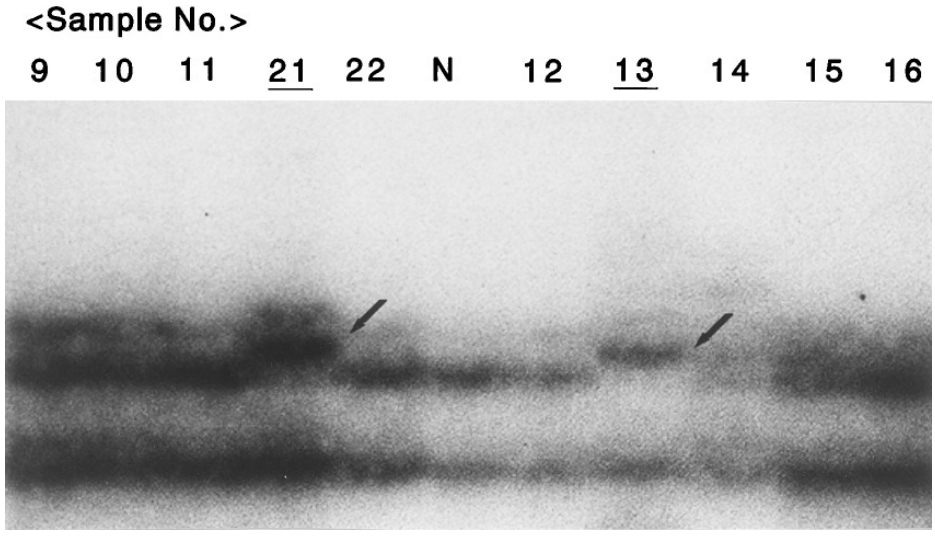

FIG. 1. SSCP analysis of DNA fragments, carrying exon 7 of the p53 gene. Electrophoresis was performed in a 6% polyacrylamide gel with 5% glycerol, at 40 W, at room temperature. The bands (arrows) of the underlined samples (13 and 21) showed different mobilities from those of normal lung samples (N: normal lung tissue).

firmed that the risk of lung cancer in chromate-production workers is higher than that in the general population. The risk has been reported to range from 2.0 to 18.3 times that of general population, and the mean latency periods reported in 8 studies ranged from 15 to 32 years, with a mean ± SD of 20 ± 6 years (9, 16). In Japan, a significantly elevated risk of lung cancer was found among workers in chromate-producing industries (relative risk, 9.2 and 18.3) (9). Further, Satoh et al. demonstrated that the longer the period spent working in chromate factories, the higher the relative risk for respiratory cancer (17).

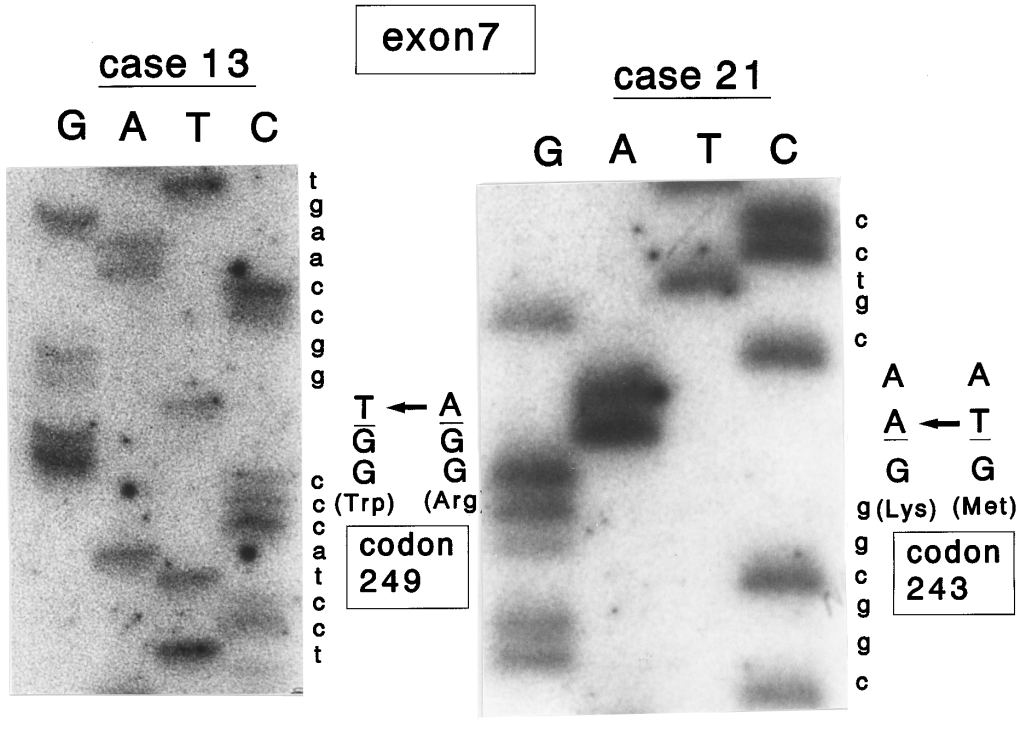

FIG. 2. Nucleotide sequence analysis of the DNA samples (13 and 21) which showed a mobility shift by SSCP. Sample 13 had an A → T transversion in the first position of codon 249. Sample 21 had a T → A transversion in the second position of codon 243.

TABLE 1

Mutation of the p53 Gene in Chromate Lung Cancer Patients

Sample number	Age (years)	B.I.[a]	Chromate exposure (years)	Histology[b]	pTNM, stage	Duplicated cancer	p53 mutation		
							Codon	Base change	Amino acid change
S-1	47	520	13	Sq	T2N2M0, IIIA	—			
S-2	48	0	28	Ad	T1N0M0, I	—			
S-3	46	810	26	Sq	T1N0M0, I	Duplicated			
S-4	61	0	38	Sq	T2N1M0, II	—			
S-5-sm	44	360	15	SCLC	T1N3M1, IV	Triplicated			
S-5-sq	44	360	15	Sq	T1N0M0, I	Triplicated			
S-6	67	0	33	Sq	T3N2M0, IIIA	—			
S-9	**67**	**0**	**35**	**Sq**	**T1N0M0, I**	—	**278**	**CCT → CTT**	**Pro → Leu**
							286	**GAA → GCA**	**Glu → Ala**
S-10	75	Smoking[c]	18	Sq	T3N2M0, IIIA	—			
S-11	45	750	21	Sq	T1N1M0, II	—			
S-12	59	572	32	Sq	T1N0M0, I	—			
S-13	**67**	**0**	**19**	**Sq**	**T1N0M0, I**	**Duplicated**	**249**	**AGG → TGG**	**Arg → Trp**
S-14	**50**	**540**	**29**	**Sq**	**T2N2M0, IIIA**	—	**222**	**CCG → TCG**	**Pro → Ser**
							194	**CTT → TTT**	**Leu → Phe**
S-15	74	980	23	Sq	T2N2M1, IV	—			
S-16	40	390	13	Sq	T2N2M1, IV	—			
S-17	56	450	14	Sq	T1N0M0, I	—			
S-18	55	1440	31	Sq	T1N2M0, IIIA	—			
S-19	45	370	8	Sq	T1N0M0, I	Triplicated			
S-21	**49**	**240**	**12**	**SCLC**	**T1N1M0, II**	—	**243**	**ATG → AAG**	**Met → Lys**
S-22	40	660	22	Sq	T2N1M0, II	Duplicated			

[a] B.I.: Brinkman index.
[b] Sq: squamous cell carcinoma; Ad: adenocarcinoma; SCLC: small cell carcinoma.
[c] He smoked, but the amount of smoking was undetermined.

Intoxication by chromium is mainly due to hexavalent chromium. This is probably because only the hexavalent form can cross biological membranes (11). Cr(VI) compounds produce a variety of genetic effects, such as the inhibition of DNA repair synthesis, DNA damage, mutations, sister-chromatid exchanges, micronuclei, and chromosomal aberrations (11). Several studies have revealed that chromium(VI) is directly mutagenic in bacteria, as it induces frameshift errors and, to a greater extent, base-pair substitutions both at G-C and A-T base-pairs (11). In eukaryotes, K_2CrO_4 concentration-dependently increases mutation induction at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells (18, 19, 20, 21). Morphological transformation of mammalian cells in vitro is also obtained by treatment with chromium(VI) (22). These data support the hypothesis that chromate(VI) mutagenicity induces transformation via the somatic mutation of proto-oncogenes or tumor suppressor genes. We focused on the frequency and the patterns of mutations in the p53 gene observed in lung cancer patients who had been exposed to chromate.

In this study, mutations of the p53 gene were identified in 4 (20%) of the 20 chromate lung cancer samples. Although we expected to find more p53 mutations in chromate lung cancer patients because of chromate, tobacco smoke, or the synergistic effects of the 2, mutations were less common in patients with lung cancers who had been exposed to chromate than those who had not (20% vs about 50%) (4, 5, 23, 24, 25). De Flora et al. demonstrated that the ability of human pulmonary alveolar macrophages homogenates to reduce chromium(VI) in smokers was significantly higher than in nonsmokers, and that chromium(VI) and tobacco smoke may act antagonistically (26). Suzuki et al. have reported that the odds ratio for p53 mutations to develop in heavy smokers (those with a Brinkman index over 600) can be estimated to increase 5.3-fold over that in nonsmokers (4). Although there were 14 smokers in this study (74%), there were only 5 heavy smokers (26%), Infrequency of p53 mutations in this study may be due to the antagonistic effect of tobacco smoke and low proportion of heavy smokers. As 4 chromate lung cancer patients with p53 mutations were not heavy smoker (B.I.=0, 0, 540, 240, Table 1), the pattern of p53 mutations may be due to chromate exposure. The possibility exists that the sensitivity of the PCR-SSCP method may have been responsible for the low frequency of p53 mutations detected in this study. However, Hayashi et al. reported that the sensitivity of PCR-SSCP for detecting point mutations was more

TABLE 2

Nature of p53 Gene Base Substitution Mutations of Chromate and Common Lung Cancers

Geographic area	Number of mutations	Mutations at G:C			Mutations at A:T			References
		→A:T	→T:A	→C:G	→T:A	→G:C	→C:G	
USA and Europe	43 (13 vs. 30)[a]	12 (28%)	19 (44%)	6 (14%)	3 (7%)	1 (2%)	2 (5%)	(27)
Japan	69 (12 vs. 57)	22 (32%)	25 (36%)	7 (10%)	1 (1%)	11 (16%)	3 (5%)	(4, 24, 25)
Chromate (our study)	6 (1 vs. 5)	3	0	0	2	0	1	

[a] (number of Small cell carcinoma vs. number of non-Small cell carcinoma)

than 89% for 300- to 400-bp fragments, and the specificity was 100% (15). The frequency of p53 mutations detected by PCR-SSCP in our previous studies (lung cancer and breast cancer samples) was in accordance with those in other studies (5, 14). We believe therefore that the PCR-SSCP method used in our study is a well-established procedure.

The pattern of p53 mutations in lung cancer patients who have been exposed to chromate was different from that of patients with common lung cancers in 3 respects (Table 2). G:C to T:A transversions in the coding strand of the p53 gene in chromate lung cancer patients were not found, although these are the predominant nucleotide changes (36-44%) in lung cancers (4, 24, 25, 27). Among p53 mutations in common lung cancers, GC base-pairs are predominantly attacked (78-86%) and AT base-pairs changes are infrequently altered (14-22%) (4, 24, 25, 27). In chromate lung cancer patients, AT transversions accounted for 3 of 6 mutations (50%), although these alterations are very rare (4/69 case, 6%) in common lung cancers in Japan (4, 24, 25). Double mutation of the p53 gene in the same patient have rarely been found in common lung cancers (4, 23, 24, 25, 28). In contrast, double mutations were found in 2 of 4 alterations in chromate lung cancers. Two lung cancer patients with double mutations had been exposed to chromate for a relatively long period (29 and 35 years). Hayes et al. reported that an excess risk of lung cancer is associated with exposure to chromate for more than 30 years (29). It is possible that excess chromate exposure induces mutation of the p53 gene.

This study revealed that there was no association between p53 mutations and the period spent working in chromate factories. Exposure to chromate varies according to the stage of the process involved in producing chromium. As chromate workers are switched to several processes throughout their career, it is difficult to ascertain the extent to which chromate exposure is a direct result of working in the chromium industry. Studies have demonstrated that biological reductants including riboflavin (vitamin B2), ascorbic acid, and glutathione play an important role in the induction of chromate-induced DNA damage (30, 31, 32), and that antioxidants such as vitamin E protect cells from chromate-induced DNA damage (33). These reports suggest that variations in the manner by which chromium is activated or inactivated may be associated with the risk of developing chromate lung cancers.

This study is the first molecular analysis of chromium-associated human lung cancer. Although chromate lung cancer patients harbored less p53 mutations than patients with common lung cancer, the patterns of p53 mutations differed between these two groups of patients. As chromate lung cancer patients with p53 mutation were not heavy smokers, these patterns of p53 mutations were suggested to have been due to chromate exposure. This suggests that chromate exposure may induce point mutations in the p53 gene. As p53 mutations induced by chromate exposure were infrequent, it is possible that lung carcinogenesis induced by chromate exposure may be associated with alterations of other cancer-associated genes. As chromate induces a variety of genetic effects, such as the inhibition of DNA repair synthesis, DNA damage, mutations, sister-chromatid exchanges, micronuclei and chromosomal aberrations, it is necessary to test samples from chromate lung cancer patients for mutations in other cancer-associated genes, gene instability, LOH, and chromosomal aberrations in an effort to elucidate the mechanisms involved in carcinogenesis in chromate workers.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (044-54346).

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