

ACCELERATED PAPER

p53 mutations in lung cancers from Japanese mustard gas workers

Yukio Takeshima^{1,2,3}, Kouki Inai², William P. Bennett¹, Robert A. Metcalf⁴, Judith A. Welsh¹, Shuji Yonehara², Yuzo Hayashi⁴, Megumu Fujihara⁵, Michio Yamakido⁶, Mitoshi Akiyama⁷, Shoji Tokuoka³, Charles E. Lands⁸ and Curtis C. Harris^{1,9}

¹Laboratory of Human Carcinogenesis, ²Radiation Epidemiology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA, ³Department of Pathology, ⁴Department of Internal Medicine, Hiroshima University School of Medicine, Hiroshima 734, ⁵Department of Epidemiologic Pathology, ⁶Department of Radiobiology, Radiation Effects Research Foundation, Hiroshima 732, ⁷Department of Pathology, Hiroshima City Asa Hospital, Hiroshima 731-02 and ⁸Department of Pathology, Hiroshima Red Cross and Atomic Bomb Survivors Hospital, Hiroshima 730, Japan

⁹To whom requests for reprints should be sent

Mustard gas (MG) is a mutagenic and carcinogenic alkylating agent, and is a known risk factor for occupational lung cancer. Our hypothesis is that lung cancers from MG workers contain mutations (G:C to A:T transitions) as the result of MG-produced DNA promutagenic adducts in the p53 tumor suppressor gene. We analyzed 12 primary lung cancers from Japanese MG factory workers and 12 lung cancers from non-exposed individuals. Genomic DNA was isolated from archival paraffin-embedded tissues. Exons 5–8 were amplified by polymerase chain reaction using p53-specific primers, and sequenced by dideoxy termination methods. Six out of 12 lung cancers from MG workers contained a total of eight somatic point mutations: two cases had double G:C to A:T transitions; one had a G:C to T:A transversion; one case had an A:T to G:C transition; and two cases had single base deletions. Four of the six mutated purines occurred on the non-transcribed, DNA-coding strand. Out of 12 unexposed cases, there were six single base mutations in six cancers, and no double mutations. The p53 mutational frequency in the MG-exposed cases is similar to the non-exposed controls and the usual smoking-related lung cancers reported previously. However, the distinctive double mutations (G:C to A:T transition) observed in two cases are unusual and may be related to MG exposure.

Introduction

Between 1929 and 1945, poisonous gases including bis(β-chloroethyl)sulfide [i.e. sulphur mustard, mustard gas (MG*) or Yperite], chlorvinylarsine (i.e. Lewisite), diphenylcyanarsine, hydrocyanic acid, chloracetophene and phosgene were manufactured on Okuno-jima island, Japan (1). MG is a mutagenic and carcinogenic alkylating agent (2–7), and Japanese MG factory workers have an increased risk of lung cancer (8), as observed among German (9) and British MG workers (10). Central lung cancers are more commonly observed than peripheral lung cancers, and squamous cell carcinoma and

small cell carcinoma are the most common histologic types in Japanese MG cases (11). In US and British army veterans with battlefield exposure to MG in World War I, MG exposure also is correlated with an excess risk of lung cancer (12–14).

Mutations in the p53 tumor suppressor gene are common in human cancer (reviewed in 15–18). The presence of p53 mutations in approximately half of all human cancers allows comparison of the mutational spectra of various cancer types. The character and distribution of mutations constitute a mutational spectrum. *In vitro* studies show that chemicals can produce distinctive mutational spectra, suggesting ‘fingerprinting’ of genetic damage. Molecular epidemiological studies have generated hypotheses linking environmental carcinogen exposure to human cancer. For example, a mutational hotspot in the third base of codon 249 in human hepatocellular carcinoma is common in areas of high liver cancer incidence where people are infected with hepatitis viruses and are exposed to high levels of dietary aflatoxin B₁, but is uncommon in low-incidence areas where people are exposed to low levels of aflatoxin B₁ (17,19–22). A second example is the linkage between mutations at dipyrimidine sites and UV radiation. p53 mutations characteristic of this type of damage have been found in human skin cancers (23–25). These and other examples suggest that analysis of p53 mutations in human cancer may provide clues to cancer etiology.

There have been no reported analyses of oncogenes or tumor-suppressor genes in lung cancers from MG factory workers. Therefore, we investigated mutations in the p53 gene within archival lung tumor samples from Japanese MG factory workers to determine whether MG produces a distinctive mutational spectrum.

Materials and methods

Patient selection

Lung cancers from 25 male MG factory workers were collected during 1952–1990 at the Second Department of Pathology, Hiroshima University School of Medicine, Japan. After excluding poorly preserved tissue, 12 cases of lung cancer from MG workers were available for this study. Non-exposed controls among MG factory workers were not available, so primary lung tumor tissues from non-exposed males were obtained from the Radiation Effects Research Foundation, Japan, the Hiroshima University School of Medicine, the Hiroshima City Asa Hospital, the Hiroshima Red Cross Hospital, and the Atomic Bomb Survivors Hospital, all in Japan.

Non-MG-exposed controls were matched for type of specimen (autopsy or surgery) and histologic type (small cell carcinoma/non-small cell carcinoma). Clinical and occupational details are shown in Table 1. The occupational exposure categories were described previously (1).

Isolation of genomic DNA from paraffin-embedded tissues

Lung tissues were fixed with formalin and embedded in paraffin. Two paraffin blocks with lung tumor tissue (for somatic/mutational analysis) and normal peripheral lung tissues (for germline analysis) were collected for each patient.

Sections 50 μm thick were cut from each paraffin block; microtome blades were changed between blocks to prevent tissue carry-over. Tissue sections were dewaxed twice with xylene and once with 100% ethanol. Well-preserved tumor and non-tumor tissues were dissected from the 50 μm sections using a dissecting microscope and sterile needles. The average tissue sample was a 1 cm³ tumor dissected from one, two or three of the 50 μm sections. Genomic DNA was isolated by proteinase K digestion (0.5 mg/ml, final concentration).

* Abbreviations: MG, mustard gas; PCR, polymerase chain reaction.

Table I. *p53* mutations in lung cancers from a study of MG workers

Case no.	Age ^a	Histology ^b	Exposure history ^c	Smoking history ^d	Codon change ^e	DNA base change	Amino acid change
MG-exposed cases							
MG1	48	sm	3×35	20	252 254	CTC → CTI ATC → ATI	silent silent
MG2	62	sm	3×26	25	wild-type		
MG3	83	sm	3×72	37	244	GGC → TGC	Gly → Cys
MG4 ^f	65	sm	1×20	45	wild-type		
MG5	67	ad	2×11	41	wild-type		
MG6	74	ad	1×15	<1	wild-type		
MG7	59	adsq	1×36	80	263	AAT → AGT	Asn → Ser
MG8	83	adsq	1×84	13	wild-type		
MG9	80	sq	1×184	0	262 274	GGT → AGT GTT → ATT	Gly → Ser Val → Ile
MG10 ^g	57	sq	4×35	53	199–200 (1 base deletion)	GGAAAT → GGAAT	frameshift
MG11 ^h	59	sq	2×45	40	wild-type		
MG12 ^h	60	sq	1×24	29	274–275 (1 base deletion)	GTTTGT → GTTGT	frameshift
Non-exposed cases							
MN1	87	sm	–	36	wild-type		
MN2	72	sm	–	9	wild-type		
MN3	78	sm	–	28	173	GTG → ATG	Val → Met
MN4 ^f	63	sm	–	51	159	GCC → CCC	Ala → Pro
MN5	63	ad	–	?	wild-type		
MN6	58	sq	–	wild-type			
MN7	66	ad	–	?	wild-type		
MN8	88	ad	–	?	235	AAC → GAC	Asn → Asp
MN9	74	ad	–	?	249	AGG → AGI	Arg → Ser
MN10 ^f	58	sq	–	114	293–294 (1 base deletion)	GGGGAG → GGGAG	frameshift
MN11 ^f	71	sq	–	45	245	GGC → AGC	Gly → Ser
MN12 ^f	63	sq	–	56	wild-type		

^aAge at diagnosis.

^bHistology of lung cancer: sm, small cell carcinoma; sq, squamous cell carcinoma; ad, adenocarcinoma; adsq, adenosquamous carcinoma.

^cType of work × working duration (month). Type of work: 1. Yperite (MG) production; 2. Lewisite production; 3. laboratory, incineration, repair; 4. sneezing gas production.

^dPack-years of cigarette smoking.

^eMutations on the non-transcribed strand are shown.

^fSurgical cases.

followed by phenol/chloroform extraction, and ethanol precipitation as described previously (26). The precipitated DNA was resuspended in 100 µl of sterile water.

Polymerase chain reaction (PCR) and direct sequencing

Coding sequences and splice-site junctions were amplified by PCR (27–29) from genomic DNA (250–500 ng) using intron-specific primers as previously described (30). Protocol modifications included the use of nested primers in sequential rounds of amplification to produce sufficient template for sequencing (31,32).

Gel-purified DNA templates were sequenced directly using a modification of the dideoxy chain termination method of Sanger *et al.* (33). Template DNA was denatured at 98°C for 3 min, annealed with 3 pmol sequencing primer, and sequenced with Sequenase Version 2.0 Kit reagents (US Biochemical Corporation, Cleveland, OH), as previously described (34). On occasion, single-strand binding protein (US Biochemical) was used according to the manufacturer's protocols to produce cleaner sequencing ladders.

To eliminate the possibilities of *Taq* polymerase misincorporations, all mutations were confirmed by a second round of PCR, starting from genomic DNA and sequencing in both directions. When a point mutation was identified, the germline sequence of the mutated exon was analysed from genomic DNA isolated from non-neoplastic peripheral lung tissues.

Results

Six of 12 MG-exposed cases had *p53* point mutations; two cases had two mutations each, and four cases had single mutations. The eight mutations were distributed in exons 6, 7 and 8; there were two silent mutations, four missense mutations and two single-base deletions (Table I). The base substitutions

included five transitions and one transversion. Case MG1 had two G:C to A:T transitions at codons 252 and 254 (exon 7); neither transition caused an amino acid change (Figure 1). Case MG9 also had two G:C to A:T transitions in codons 262 and 274, causing glycine to serine and valine to isoleucine amino acid changes respectively (Figure 2). The remaining mutations included one G:C to T:A transversion in exon 7, codon 244; one A:T to G:C transition in exon 8, codon 263; and two single-base deletions in exon 6, codons 199 or 200 and in exon 8, codons 274 or 275. Of the six nucleotide substitutions, four of the altered purines occurred on the non-transcribed, DNA-coding strands. There were no transitions at CpG dinucleotide sites and no germline mutations.

Six of 12 non-MG-exposed control cases had *p53* mutations including five missense mutations and one single-base deletion (Table I). All six mutations occurred in exons 5, 7 and 8. The mutations included two G:C to A:T transitions: one in exon 5, codon 173; one in exon 7, codon 245. There was also one G:C to C:G transversion in exon 5, codon 159; one A:T to G:C transition in exon 7, codon 235; one G:C to T:A transversion in exon 7, codon 249; and a single-base deletion in exon 8, codon 293 or 294. There was one CpG site transition in case MN11, codon 245. Excluding CpG site mutations consistent with spontaneous deamination of 5-methylcytosine, there is evidence for DNA strand bias in that all of four mutated

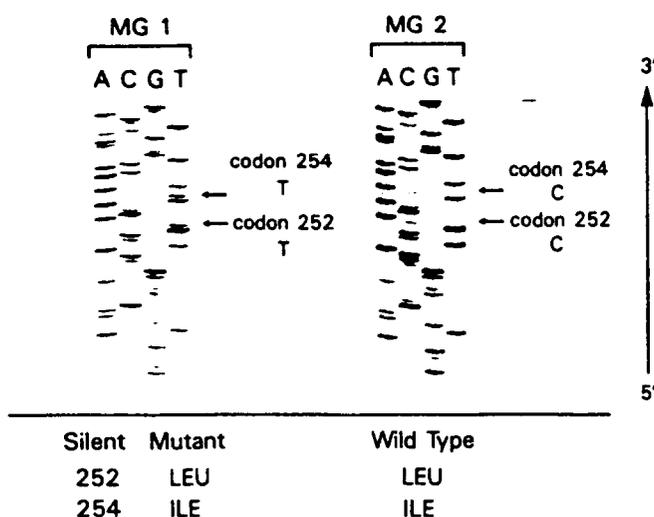


Fig. 1. Double G to A transitions in one lung tumor from a MG worker. Tumor *p53* sequences were amplified by PCR and sequenced directly. The nucleotide substitutions in the third bases of codons 252 and 254 (case MG1) are indicated by arrows. Neither caused an amino acid substitution (i.e. silent mutation). The wild-type sequence in case MG2 is shown for comparison.

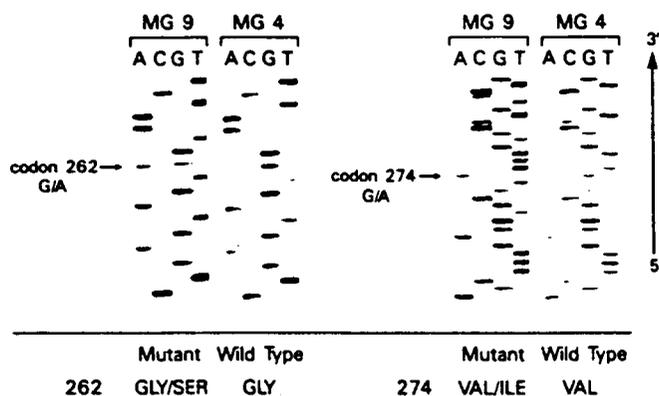


Fig. 2. Two *p53* missense mutations in one lung cancer from a MG worker. G:C to A:T nucleotide substitutions in the first positions of codons 262 and 274 from case MG9 are indicated by arrows. The mutation in codon 262 caused a glycine to serine amino acid change, and the mutation in codon 274 caused a valine to isoleucine amino acid change. The wild-type sequence in case MG4 is shown for comparison.

purines occurred on the non-coding, transcribed strand. In all cases, the germline sequences from DNA of paired non-tumor tissues were wild-type.

Discussion

MG is an alkylating agent, and its mutagenicity and carcinogenicity may be explained by this chemical property (reviewed in 35). MG reacts with water to produce sulfonium ions. This intermediate binds DNA and forms DNA-DNA interstrand crosslinks. Specific DNA adducts include *N*⁷-ethylthioethyl guanine, *N*⁷-ethylthioethyl adenine, *O*⁶-ethylthioethyl guanine and others (36). To our knowledge, there are no reported mutation analyses of MG-associated tumors or cell lines.

Alkylating nitroso agents induce predominantly G:C to A:T transitions. For example, G:C to A:T transition on the non-transcribed strand of the *p53* gene has recently been described

in human fibroblasts exposed to *N*-ethyl-*N*-nitrosourea (37). *O*⁶-Alkylguanine is a promutagenic alkylation adduct produced by these alkylating agents (reviewed in 38). *O*⁶-Alkylguanine is repaired by *O*⁶-alkylguanine DNA alkyltransferase (39,40). However, *O*⁶-ethylthioethyl guanine induced by MG is a poor substrate for this DNA repair system (41). Assuming that *O*⁶-ethylthioethyl guanine has the same mutational specificity as *O*⁶-alkylguanine, then this fact would further increase the possibility of a G:C to A:T transition.

Mutations suggesting alkylating damage occurred in both cases with double mutations. Case MG9 had two G:C to A:T transitions (both missense mutations) in codons 262 and 274 on the non-transcribed strand. Case MG1 had two G:C to A:T transitions in codons 252 and 254 (both silent mutations); in both codons the cytosine residue was on the non-transcribed strand. Because no polymorphisms have been reported at these two codons, and DNA from non-tumor tissues did not contain these base changes, both of these mutations are considered to be somatically acquired. Although the silent mutations observed in case MG1 may not have contributed to lung carcinogenesis, they may reflect the strong mutagenicity of MG, since double mutations are rare. About 350 *p53* mutations have been reported in lung cancer, and three double mutations have been documented thus far (42,43). Therefore, it is notable that among MG workers, two out of six cases with mutations had double mutations.

Among eight mutations observed in the MG exposure group, no transitions at CpG site dinucleotides were found. Such mutations can be attributed to spontaneous deamination of 5-methylcytosine (44). This observation is consistent with the hypothesis that the lung cancers reported here were induced by an exogenous carcinogen (i.e. MG and/or tobacco smoke).

G:C to T:A transversions are the most common missense mutations in lung cancers from tobacco smokers. The frequency of G:C to T:A transversions on the non-transcribed DNA strand in lung cancer has a positive correlation with the donor's history of tobacco smoking (45,46). Tobacco smoke contains a mixture of highly mutagenic chemicals, including tobacco-specific *N*-nitrosamines and polycyclic aromatic hydrocarbons (PAH) (47). Many electrophilic metabolic PAH derivatives, such as benzo[*a*]pyrene diol-epoxide, preferentially attack guanine regions and induce G:C to T:A substitutions. Strand bias may be explained by preferential repair of the transcribed strand of the *p53* gene by the transcription-repair complex (48,49). The predominant guanine to thymine substitutions are proposed to occur upon DNA replication through preferential insertion of adenine opposite the non-instructive promutagenic base (the A rule) (50). In the present study, all cases but one (case MG9) had a history of smoking, so we cannot exclude the effect of tobacco smoke on *p53* mutations. However, we found only one G:C to T:A transversion among the MG workers (Table I), instead of the two or three G:C to T:A transversions that would be expected, based on literature values (18). Although these numbers are small, the prominence of G:C to A:T transitions and the occurrence of double mutations in two cases suggest that MG exposure made some contribution to the development of these cancers.

Preneoplastic lesions, such as dysplasias and carcinomas *in situ*, are observed more frequently among the MG-exposed group than the non-MG-exposed group (51). This suggests that MG may cause widespread genetic changes in respiratory epithelial cells. Because *p53* gene alterations in lung cancer

are found in preinvasive lesions (32,52–54), additional analysis of dysplastic bronchial lesions may further reveal MG related genetic changes and may elucidate the timing of *p53* mutations in lung carcinogenesis.

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