Assessment of the Mutations of p53 Suppressor Gene and Ha- and Ki-ras Oncogenes in Malignant Mesothelioma in Relation to Asbestos Exposure: A Study of 12 American Patients

Fumihiko KITAMURA1,4, Shunichi ARAKI1,4*, Yasunosuke SUZUKI2, Kazuhito YOKOYAMA1, Takeshi TANIGAWA1,5 and Ryu IWASAKI3

1 Department of Public Health and Occupational Medicine, Graduate School of Medicine, The University of Tokyo, 7–3–1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
2 Department of Community Medicine, Mount Sinai School of Medicine, 1 Gestave L. Levy Place, New York, NY 10029-6574, U.S.A.
3 Department of Gene Analysis, Mitsubishi Kagaku Bio-Clinical-Laboratories, Inc., 3–30–1, Shimura, Itabashiku, Tokyo 174-0056, Japan
Present:
4 National Institute of Industrial Health, 6–21–1, Nagao, Tama-ku, Kawasaki 214-8585, Japan
5 Institute of Community Medicine, University of Tsukuba, 1–1–1, Tennodai, Tsukuba-City, Ibaragi 305-8575, Japan

Received February 26, 2001 and accepted November 30, 2001

Abstract. In our previous study, we found no genetic alteration in exons 1 and 2 of Ha- and Ki-ras oncogenes nor in exons 5 to 9 of the p53 suppressor gene in seven Japanese malignant mesothelioma patients exposed to asbestos. To examine further whether malignant mesothelioma due to asbestos has genetic alterations in the p53 suppressor gene and in Ha- and Ki-ras oncogenes, we analyzed point mutations of these genes in paraffin embedded operative open biopsied samples of the primary tumor of malignant mesothelioma in twelve American patients. The genetic analysis was conducted by the PCR-SSCP (polymerase chain reaction single-strand conformation polymorphism) method in all patients and by sequencing analysis of DNA bases in the two patients with suspected gene mutation. The analysis of the p53 suppressor gene showed an amino acid converting mutation of exon 7 in one patient and a polymorphism of exon 6 in another patient; the former patient was a heavy smoker with a biphasic cell type. No genetic alteration was found in exons 1 and 2 of Ha- and Ki-ras oncogenes in any of the patients. The results suggest that the effects of asbestos on the p53 suppressor gene and Ha- and Ki-ras oncogenes in malignant mesothelioma are negligible. Further studies are needed to examine whether the observed mutation of the p53 suppressor gene is due to the combined effects of asbestos and smoking or to other unknown factors.

Key words: Malignant mesothelioma, Asbestos, p53 suppressor gene, ras oncogene, PCR-SSCP analysis, Sequencing analysis, Primary tumor

*To whom correspondence should be addressed.
**Introduction**

Malignant mesothelioma is caused by exposure to asbestos\(^1\), which can damage DNA through the production of reactive oxygen\(^2\). Single point mutation of the p53 suppressor gene has been reported in 2 of 4 malignant mesothelioma cell lines\(^3\) and in 2 of 20 cell lines\(^4\). In the former study, C\(\text{GC} \rightarrow \text{AC}\) transition in codon 175 (Exon 5) and G\(\text{GC} \rightarrow \text{GAC}\) transition in codon 245 (Exon 7) were demonstrated. In the latter, G\(\text{GC} \rightarrow \text{AGC}\) transition in codon 245 (Exon 7) and C\(\text{CT} \rightarrow \text{TCT}\) transition in codon 278 (Exon 8) were reported. No mutation of the Ki-ras oncogene has been observed in malignant mesothelioma cell lines\(^4\).

The mutation observed in the cell lines requires careful interpretation, as point mutation of genes can occur during serial passage *in vitro*\(^5\). Therefore, analysis of the genes of primary tumors is essential. Only one mutation in the p53 suppressor gene has been found in fifteen malignant mesothelioma patients, a G\(\text{GA} \rightarrow \text{TGA}\) transversion in codon 266 (Exon 8)\(^6\). Also, one silent mutation has been observed in the p53 suppressor gene of eleven primary malignant mesothelioma patients\(^7\). In other studies, no alteration in the Ha-ras oncogene was found in eleven primary tumors of malignant mesothelioma including those from cases with asbestos exposure\(^8\), and no alteration in the p53 suppressor gene was demonstrated in thirteen primary tumors of malignant mesothelioma, of which only two cases had a history of asbestos exposure\(^9\). We also failed to find mutations of the p53 suppressor gene and Ha- and Ki-ras oncogenes in seven primary tumors from Japanese malignant mesothelioma patients exposed to asbestos\(^10\).

In the present study, we analyzed the p53 suppressor gene and Ha- and Ki-ras oncogenes in the primary tumors of twelve American malignant mesothelioma patients ten of whom had a definite history of asbestos exposure. Genetic analysis was conducted using the PCR-SSCP (polymerase chain reaction single-strand conformation polymorphism) method in all patients. Sequencing of DNA was carried out in two patients with suspected gene mutation and the factors causing the mutations are discussed.

**Materials and Methods**

One of the present authors (Y.S.), a qualified pathologist, collected all open biopsied specimens from twelve patients with malignant mesothelioma for pathologic examination, together with data on the sex, age, asbestos exposure, cell types of malignant mesothelioma, and smoking history of the patients (Table 1). The period of tissue fixation using neutral formalin was less than two days.

DNA was extracted from the formalin-fixed and paraffin-embedded tissues of all the patients by the following standard method\(^11\): From a block of the tissue specimens, 5–10 \(\mu\)m sections were dissected. An area precisely corresponding to that of the tumor lesion was excised from the section, deparaffinized, and then digested with Proteinase K (Boehringer Mannheim GmbH, Mannheim). DNA was purified by means of phenol-chloroform extraction and ethanol precipitation, and was amplified by 30 to 40 cycles of polymerase chain reaction (PCR)\(^12\) through the use of a Gene Amplification PCR System 9600 (Perkin-Elmer Cetus, Norwalk, California). In the PCR, codons 1 to 31 (exon 1) and 34 to 93 (exon 2) of the Ha-ras oncogene were amplified, as mutation frequently occurs at codons 12, 13 and 61\(^13\).

---

**Table 1. Twelve American operative open biopsied patients with malignant mesothelioma**

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age at biopsy (yrs)</th>
<th>Asbestos exposure</th>
<th>Cell types of mesothelioma</th>
<th>Brinkman’s Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>56</td>
<td>+</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>56</td>
<td>+</td>
<td>Biphasic</td>
<td>1050</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>57</td>
<td>+</td>
<td>Epithelial</td>
<td>800</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>57</td>
<td>+</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>61</td>
<td>+</td>
<td>Biphasic</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>63</td>
<td>+</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>64</td>
<td>+</td>
<td>Fibrous</td>
<td>Unknown</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>67</td>
<td>+</td>
<td>Fibrous</td>
<td>320</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>68</td>
<td>+</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>45</td>
<td>Unknown</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
<tr>
<td>11</td>
<td>Female</td>
<td>66</td>
<td>+</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
<tr>
<td>12</td>
<td>Female</td>
<td>66</td>
<td>Unknown</td>
<td>Epithelial</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* Number of cigarettes per day multiplied by years of smoking.
Codons 1 to 37 (exon 1) and 48 to 80 (exon 2) of the Ki-ras oncogene were also amplified. Similarly, codons 126 to 307 (exons 5 to 9) of the p53 suppressor gene were amplified. The primers of the PCR used in this study are shown in the Appendix. The PCR products were further amplified with [α-32P]dCTP by 20 to 40 cycles in the PCR System 9600 for PCR-SSCP analysis, and were then subjected to electrophoresis at 35 watts using 5% polyacrylamide gels containing glycerol at 5% and 10%; usage at two different concentrations of glycerol in the gels was expected to yield higher sensitivity in the latter study, i.e. this could decrease the possibility of false negative results. The electrophoresis time was 2 to 3 hours, depending on the length of the amplified nucleotide. The gel was dried and exposed to X-ray film at room temperature for 3 to 24 hours with an intensification screen.

DNA was also extracted from the leucocytes of a healthy male volunteer, 29 years of age, and it was used as a normal control. Similarly, a DNA sample which had a GGC to GTC transversion at codon 12 (Exon 1) in Ha-ras oncogene and an AGA to ACA transversion at codon 280 (Exon 8) in the p53 suppressor gene and was obtained from a cell line of bladder cancer was used as a positive control. These DNA samples were analyzed by the same method as the samples taken from the mesothelioma patients.

Sequencing analyses of the Ha- and Ki-ras oncogenes and the p53 suppressor gene for the codons described above were conducted in two patients in whom mutations were suspected by the SSCP analysis (Patients 2 and 5, Table 1) on the DNA samples acquired from abnormal bands on gels. Sequencing reactions were carried out with Dye Terminator Cycle Sequencing kit and the products were analyzed using a 373A Sequencer (Applied Biosystems Inc., Foster City, California).

**Results**

**PCR-SSCP analysis**

Table 2 summarizes the results of PCR-SSCP and sequencing analysis of the 12 patients. Mobility shift from the normal band of the p53 suppressor gene was detected in exon 6 of Patient 5 and in exon 7 of Patient 2 (Fig. 1). Patient 2 was a heavy smoker (Table 1). No mobility shift of Ha- and Ki-ras oncogenes was found in any of the 12 patients by SSCP analysis.

**Sequencing analysis**

The results of the sequencing analysis of the p53 suppressor gene in Patients 2 and 5 are shown in Figure 2. Base changes in codon 213 in exon 6 (CGA → CGG, No amino acid converted) and codon 233 in exon 7 (CAC → CGC, Histidine → Arginine) were found in Patients 5 and 2, respectively.

**Discussion**

Following our previous study, we analyzed directly primary tumors of malignant mesothelioma to examine changes in the p53 suppressor gene and Ha- and Ki-ras oncogenes in ten American patients with a definite history of asbestos exposure and also in two American patients with an unknown history of asbestos exposure. The transition of CAC → CGC was found in codon 233 (Exon 7) of the p53 suppressor gene.
suppressor gene of a heavy smoker with a biphasic type of tumor. In another patient the change of CGA \( \rightarrow \) CGG in codon 213 of the p53 suppressor gene was also found.

Thus, we observed a mutation in the p53 suppressor gene without genetic alteration in the Ha- and Ki-ras oncogenes in one subject. The change in the other subject is considered to have resulted from polymorphism according to the IARC (International Agency for Research on Cancer) information on the polymorphism of p53\(^{16}\). These results, together with all other findings (Table 3)\(^{3, 4, 6-10}\), indicate that the effects of asbestos on the p53 suppressor gene and Ha- and Ki-ras oncogenes in malignant mesothelioma are negligible.

The mutation in the p53 suppressor gene was observed only in a heavy smoker in the present study. This suggests a combined effect of asbestos and smoking, although smoking has little or no effect upon the death rate in mesothelioma patients\(^{17}\). Alternatively, other unknown factors might have caused the mutation of the p53 suppressor gene in this patient. Further studies with a larger number of smokers are necessary to examine whether or not the mutation of the p53 suppressor gene is caused in malignant mesothelioma due to asbestos and what is the cause of the mutation, if any.

One of the p53 suppressor gene’s functions is the prevention of tumor onset, triggering G1 arrest (i.e. termination of cell cycles at the G1 stage before start of the DNA synthesis stage) and apoptosis of DNA-damaged cells. Thus, a mutation in the p53 suppressor gene is considered to lead to tumor onset\(^{18}\).

Analysis of the sequence of the p53 suppressor gene in humans has revealed five conserved regions, regions with a highly similar order of bases in common with various kinds of animals. Four of these regions fall within exons 5 through 8: region ii (Codons 117 to 142), region iii (Codons 171 to 181), region iv (Codons 234 to 258), and region v (Codons 270 to 286)\(^{19}\). It is commonly known that mutations of many kinds of natural onset tumors are clustered in these regions\(^{20}\).

Table 4 summarizes reports on p53 suppressor gene mutations in malignant mesothelioma\(^{3, 4, 6}\). Four of the six mutations reported were located in the conserved regions; the remaining two mutations, both of which were analyzed in primary tumors, were located outside the conserved regions. Thus, further studies are necessary to examine the effects of asbestos on the p53 suppressor gene both within and without the five conserved regions.

The CpG site of the p53 suppressor gene has a high mutability in natural onset tumors. This is because cytosine (C) in this pair is easily methylated and changed to thymine (T)\(^{21}\). On the other hand, four out of the six malignant mesothelioma cases reported had mutations at sites other than CpG in the p53 suppressor gene (Table 4). The results suggest that the mutation points are different between malignant mesothelioma and natural onset tumors.

The mutation found in the present study was in a biphasic type of tumor. Tumor types have not been reported in other cases except for one case with an epithelial type of tumor\(^{6}\), and the relationship between the mutation and the type of tumor cell remains to be studied further.

Manifestation of malignant mesothelioma is characterized by a long latency from the start of asbestos exposure. Clarification of oncogenesis at the gene level would provide a new tool for early detection and treatment of this malignancy.

Finally, it has been reported that polymorphism of the glutathione S transferase M1 gene and the N-acetyltransferase 2 gene, both of which code detoxic enzymes, plays an important role in the onset of malignant mesothelioma due to asbestos exposure\(^{22}\). Further studies into these findings should be also conducted.
Table 3. Seven Japanese autopsied patients examined in our previous study

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Death age (yrs)</th>
<th>Occupation</th>
<th>Asbestos exposure (yrs)</th>
<th>Type of Asbestos fiber</th>
<th>Cell type of mesothelioma</th>
<th>Brinkman’s Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>61</td>
<td>Plumber</td>
<td>20</td>
<td>Crocidolite</td>
<td>Biphasic</td>
<td>820</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>75</td>
<td>Carpenter</td>
<td>18</td>
<td>Crocidolite</td>
<td>Sarcomatous</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>90</td>
<td>Plumber</td>
<td>41</td>
<td>Crocidolite</td>
<td>Epithelial</td>
<td>550</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>67</td>
<td>Electrician</td>
<td>35</td>
<td>Crocidolite</td>
<td>Sarcomatous</td>
<td>533</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>54</td>
<td>Plumber</td>
<td>33</td>
<td>Chrysotile</td>
<td>Epithelial</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>52</td>
<td>Storekeeper</td>
<td>24</td>
<td>Chrysotile</td>
<td>Epithelial</td>
<td>384</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>64</td>
<td>Housewife**</td>
<td>10</td>
<td>Chrysotile</td>
<td>Biphasic</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of cigarettes per day multiplied by years of smoking. ** Washed clothes of an asbestos worker (her husband) for 10 years.

Table 4. Reports of p53 suppressor gene mutations in malignant mesothelioma

<table>
<thead>
<tr>
<th>Authors</th>
<th>Material</th>
<th>Codon</th>
<th>Mutation</th>
<th>Conserved region</th>
<th>CpG site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cote et al. (1991)**</td>
<td>Cell line</td>
<td>175</td>
<td>CGC → CAC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cote et al. (1991)**</td>
<td>Cell line</td>
<td>245</td>
<td>GGC → GAC</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Metcalf et al. (1992)**</td>
<td>Cell line</td>
<td>245</td>
<td>GGC → ΔGC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Metcalf et al. (1992)**</td>
<td>Cell line</td>
<td>278</td>
<td>CCT → TCT</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Segers et al. (1995)**</td>
<td>Primary tumor</td>
<td>266</td>
<td>GGA → TGA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Kitamura et al. (2001)**</td>
<td>Primary tumor</td>
<td>233</td>
<td>CAC → CGC</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

** References. ** Present study. +: Satisfied, –: Unsatisfied.

Fig. 2 Sequence of p53 suppressor gene.
Codon changes are underlined.
Acknowledgements

This paper is based in part on a dissertation submitted for the degree of Doctor of Medical Sciences (DrMSc) at the University of Tokyo. We thank Dr. Shunji Yamamori, Mr. Yoshiyasu Ogawa and other staff of Mitsubishi Kagaku Bio-Clinical-Laboratories, Inc., for their technical assistance. Thanks are also due to Dr. Hajime Sato, Department of Public Health and Occupational Medicine, Graduate School of Medicine, University of Tokyo, for his advice.

References

Appendix

Sequence of the primers[^10] of PCR used in this study (S=sense primer, A=antisense primer, respectively).

**p53**
- **exon 5**
  - S: 5'-TTCAACTCTGTCTCCTCTCCT-3'
  - A: 5'-CAGCCCTGTGCTCCTCCAG-3'
- **exon 6**
  - S: 5'-GCCTCTGTCTCCTCAGT-3'
  - A: 5'-TTAACCCCTCCTCCCAGAGA-3'
- **exon 7**
  - S: 5'-AGGGTGACTGGCGCTCTTCT-3'
  - A: 5'-AGGGGTCAGCGGCAAGCAGA-3'
- **exon 8**
  - S: 5'-AGCTTAGGCTCCAGAAAGGA-3'
  - A: 5'-TTTCTTCTTTGCTGGGGAG-3'
- **exon 9**
  - S: 5'-CACTAAGCGAGGTAAAGCAAG-3'
  - A: 5'-CGGCATTTTGTGTTAGAC-3'

**Ha-ras**
- **codon 12,13**
  - S: 5'-TGAGGAGCGATGACGAATA-3'
  - A: 5'-TTCGTCCACAAATGGTTCT-3'
- **codon 61**
  - S: 5'-AGGTGGTCTTATGAGGGAG-3'
  - A: 5'-TGGAAGTCTCCTCAAGAAGACTT-3'

**Ki-ras**
- **codon 12,13**
  - S: 5'-GGCCTGCTGAAAATGACTGA-3'
  - A: 5'-GTCTGACCAGTAATAGGC-3'
- **codon 61**
  - S: 5'-GGAGAAACCTGCTCTTGAGG-3'
  - A: 5'-CACAAAGAAGCCCTCCCA-3'