**p53 Mutation, Murine Double Minute 2 Amplification, and Human Papillomavirus Infection Are Frequently Involved but not Associated with Each Other in Esophageal Squamous Cell Carcinoma**

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**ABSTRACT**

We examined the relationship between p53 mutation, murine double minute 2 (MDM2) gene amplification, and human papillomavirus (HPV) infection in 72 esophageal squamous cell carcinomas. We identified p53 mutations in 29 tumors (40.3%) by PCR-single-strand conformation polymorphism analysis and direct sequencing. Amplification of the MDM2 gene was detected by Southern blot hybridization in 13 (18.1%) of 72 tumor tissues and in 4 (33.3%) of 12 cultured esophageal squamous cell lines. All four cell lines with MDM2 amplifications showed overexpression of the MDM2 mRNA in Northern blotting. We observed HPV infection in 15 (20.8%) of 72 tumor tissues by specific PCR amplification and Southern blot hybridization. In most tumors, amplification of the MDM2 gene or infection of HPV was not associated with p53 mutations, except in four cases with p53 mutation and MDM2 amplification, and three cases with p53 mutation and HPV infection. Since p53 mutations, MDM2 overexpression, and HPV infection are all considered to abrogate the normal function of p53 protein, each of these genetic changes may be equally important in tumorigenesis. In addition, we found that patients with MDM2 amplification exhibited a significantly shorter survival period ($P = 0.0053$).

**INTRODUCTION**

Esophageal cancer is the sixth most common cancer in males and is one of the leading causes of cancer death in Japan (1). Recent molecular genetic studies revealed that accumulations of genetic alterations of both oncogenes and tumor suppressor genes play an important role in the tumorigenesis of ESC (2-4). Previously, we have shown loss of heterozygosity on several chromosomes such as 3p, 5q, 6p, 8p, 9p, 9q, 11p, 13q, 17p, 17q, 18q, and 19q, where tumor suppressor genes might be located (5). We also found mutations of the p53 gene in 47% of tumor tissues of Japanese ESC and a good correlation with the loss of 17p (4).

Among these genetic changes, mutation of the p53 gene was reported to be detected even at a very early stage of esophageal cancer (6-8), and loss of the normal function of the p53 gene is considered to be critically important in the development of ESC. However, in our previous study, only less than one half of the tumor tissues exhibited mutations of the p53 gene, and we speculated that another one half of the tumors should possess other genetic alterations which may be equivalent to mutational inactivation of the normal function of the p53 protein. For example, the product of the MDM2 gene is known to bind to p53 protein and inhibit its ability to activate transcription (9, 10). In some tumors such as sarcomas, amplification of the MDM2 gene plays an important role in tumorigenesis, causing loss of the normal function of the p53 gene (10, 11). Furthermore, the E6 proteins of HPV-types 16 and 18 are considered to have transforming ability by binding to p53 protein and degrading its function through a ubiquitin-dependent proteolysis system (12). Indeed, HPV infection is closely associated with the development of female genital epithelial cancers, and over 90% of cervical cancer biopsies contain high-risk HPV infection such as types 16 (HPV-16) and 18 (HPV-18) (13). A recent study showed that about 14% of resected surgical specimens of esophageal cancer were positive for HPV DNA by PCR analysis (14).

In this study, we have analyzed 72 tumor samples to determine the possible involvement of MDM2 amplification and HPV infection in addition to p53 mutation in tumorigenesis of ESC.

**PATIENTS AND METHODS**

**Tumor Samples and Cell Lines.** Tumor samples were obtained from 72 patients [including 32 patients previously described (4)] with ESC who underwent surgery at the Kyoto University Hospital. In each case, samples of the tumor and adjacent normal mucosal tissue were frozen immediately after surgical removal and stored at −80°C until isolation of DNA. Histological studies were also performed by the Clinicopatho-

Received 1/16/95; accepted 2/17/95.

1 This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture, Japan.

2 To whom requests for reprints should be addressed.

The abbreviations used are: ESC, esophageal squamous cell carcinoma; MDM2, murine double minute 2; HPV, human papillomavirus; SSCP, single-strand conformation polymorphism.
logical Department of the hospital, and all the tumors were confirmed to be ESC. Twelve ESC cell lines, which were established in our laboratory and designated as KYSE, were also used for analysis of amplification and expression of the MDM2 gene (15).

**DNA and RNA Preparation.** Normal tissues were removed from tumor samples by referring hematoxylin and eosin-stained frozen sections so that each tumor sample contained less than 20% normal tissue. High molecular weight DNA was extracted from tissues and ESC cell lines as described previously (16). Total RNA from cultured ESC lines was extracted by guanidine isothiocyanate solubilization of cells (16).

**PCR-SSCP and Direct Sequencing Analysis of the p53 Gene.** We previously screened p53 mutations in 32 tumors and detected 15 mutations (4). In the present study, we further screened 40 additional tumors from exons 2 to 11 (4, 17) using the same PCR-SSCP analysis. PCR was performed in 50 µl reaction mixture containing 100 ng genomic DNA, 20 pmol each primer, 200 µM each of dATP, dGTP, and dTTP, 20 µM dCTP, 0.1 µCi [³²P]dCTP (3000 Ci/mmol), and 0.5 unit Taq polymerase. We used 11 pairs of primers to amplify the entire coding region and the intron-exon junctions with 30 cycles of polymerase. We used 11 pairs of primers to amplify the entire sequence of the particular exons by direct sequencing of PCR products. The primer and template mixtures were heat denatured, and dideoxy chain termination sequencing reaction was performed using a BcaBEST sequencing kit (TAKARA).

**Analysis of the MDM2 Gene.** The Human MDM2 probe was kindly provided by Dr. B. Vogelstein. We subcloned a 969-bp Taq I fragment that spanned nucleotides 1–969 of the published cDNA sequence to remove the Alu repeated sequence and used it as a probe (10). Seventy-two tumor samples and normal mucosal tissues in addition to 12 ESC cell lines were analyzed for amplification of the MDM2 gene. Each DNA sample (5 µg) was completely digested with EcoRI and then fractionated by electrophoresis in an agarose gel and transferred to a nylon filter. Filters were hybridized with the MDM2 probe labeled with [³²P] by random priming and then rehybridized with a β-actin probe after stripping off the MDM2 probe.

**Analysis of HPV.** Seventy-two tumor samples were screened for integration of HPV DNA by PCR with consensus primers (sense: 5'-TGTCAAAAAAAAACCGTTGTGC-3'; antisense: 5'-GAGCTGTCGCTTAAATGGC-3') as described by Fujinaga et al. (19), using HeLa cells as a positive control. The amplified fragments were electrophoresed in a 4% agarose gel, and the gel was stained with ethidium bromide to take a photograph under UV light. We also analyzed the infection of HPV by Southern blot hybridization. The HPV-16 and -18 probes were obtained from the Japanese Resources for Cancer Study (Gene Bank). Each DNA sample (5 µg) was completely digested with BamHI and then fractionated by electrophoresis in an 8% agarose gel followed by transfer to a nylon membrane. The HPV-16 and -18 DNA probes were labeled by nick translation and hybridized to filters.

**Statistical Analysis.** The χ² test and Fisher’s exact test were performed for statistical analysis of the results. Survival curves were obtained using the Kaplan-Meier survival method, with statistical analysis performed using the generalized Wilcoxon test.

**RESULTS**

**p53 Mutations.** We identified p53 mutations in 14 (35%) of 40 tumors in the present study and in 29 (40.3%) of 72 tumors in total when combined with our previous results (4), which are summarized in Table 1. All normal tissue samples corresponding to each mutation showed normal mobility in PCR-SSCP, suggesting that these mutations were somatic mutations and not germ line mutations or genetic polymorphisms. We found 2, 8, 7, 3, 4, 3, and 2 mutations in and around exons 4, 5, 6, 7, 8, 9, and 10, respectively. Among these, 19 mutations (65.5%) were single-base substitutions causing amino acid substitution in p53 protein, and 6 (20.7%) were with premature termination codons created by a deletion, an insertion, and 4 single base substitutions. And 4 (12.5%) were single base substitution at splicing sites. The spectrum of the base substitutions is as follows: 12 (41.4%) were transitions and 15 (51.7%) were transversions. Among these, G:C to T:A transversion was predominant (11 cases, 34.4%). Although G:C to A:T transitions were observed in 9 cases (30.3%), only 3 cases were at CpG sites. These features of p53 mutations in ESC have confirmed the results of our previous study on a smaller number of patients (4).

**MDM2 Amplification and Expression.** Amplification of the MDM2 gene ranging from 3.7–9.4-fold was observed in 13 (18.1%) of 72 tumors and from 6.2–8.9-fold in 4 (33.3%) of 12 cell lines (Fig. 1). Amplification of the MDM2 gene in tumors was mainly observed in those without p53 mutations except in 4 cases (cases 12, 42, 47, and 57). Then, we examined the expression of the MDM2 gene in ESC cell lines because of the limited volume of fresh tumor specimens. All cell lines with...
amplifications (KYSE-30, KYSE-110, KYSE-170, and KYSE-450) showed the marked expression of the MDM2 mRNA. In contrast, in cell lines without the amplification, the amount of MDM2 mRNA was low or barely detectable under our conditions (Fig. 2).

Table 1  Somatic mutation of the p53 gene in ESC

<table>
<thead>
<tr>
<th>Case*</th>
<th>Exon (Codon)</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>MDM2 amplification*</th>
<th>HPV infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6 (220)</td>
<td>TAT \rightarrow TGT</td>
<td>Tyr \rightarrow Cys</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>6 (209)</td>
<td>AGA \rightarrow TGA</td>
<td>Arg \rightarrow stop</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>6 (224)</td>
<td>GAG \rightarrow TAG</td>
<td>Glu \rightarrow stop</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>5' end of intron 9</td>
<td>AGGT \rightarrow AGat</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>10 (342)</td>
<td>CGA \rightarrow TGA</td>
<td>Arg \rightarrow stop</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>5 (179)</td>
<td>CAT \rightarrow CGT</td>
<td>His \rightarrow Arg</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>5 (155)</td>
<td>ACC \rightarrow AAC</td>
<td>Thr \rightarrow Asn</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>5' end of intron 9</td>
<td>AGGT \rightarrow AGat</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>21</td>
<td>5 (157)</td>
<td>GTC \rightarrow TTC</td>
<td>Val \rightarrow Phe</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>23</td>
<td>5' end of intron 5</td>
<td>TGGT \rightarrow TGat</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>3' end of intron 5</td>
<td>agGT \rightarrow aaGT</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>25</td>
<td>7 (237)</td>
<td>ATG \rightarrow ATA</td>
<td>Met \rightarrow Ile</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>28</td>
<td>6 (209)</td>
<td>AGA \rightarrow AA</td>
<td>Stop at 220</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>6 (205)</td>
<td>TAT \rightarrow TGT</td>
<td>Tyr \rightarrow Cys</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>30</td>
<td>8 (266)</td>
<td>GGA \rightarrow GTA</td>
<td>Gly \rightarrow Val</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>42</td>
<td>8 (281)</td>
<td>GAC \rightarrow GAA</td>
<td>Asp \rightarrow Glu</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>43</td>
<td>7 (251)</td>
<td>ATC \rightarrow AGC</td>
<td>Ile \rightarrow Ser</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>45</td>
<td>4 (39)</td>
<td>GCA \rightarrow GTA</td>
<td>Ala \rightarrow Val</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>47</td>
<td>6 (201)</td>
<td>TTG \rightarrow TTT</td>
<td>Leu \rightarrow Phe</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>48</td>
<td>5 (146–7)</td>
<td>GGG \rightarrow GGAG</td>
<td>Stop at 148</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>50</td>
<td>9 (303)</td>
<td>AGC \rightarrow AAC</td>
<td>Ser \rightarrow Asn</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>56</td>
<td>5 (166)</td>
<td>TCA \rightarrow TAA</td>
<td>Ser \rightarrow stop</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>57</td>
<td>4 (34)</td>
<td>TCC \rightarrow ACC</td>
<td>Ser \rightarrow Thr</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>58</td>
<td>8 (282)</td>
<td>CGG \rightarrow TGG</td>
<td>Arg \rightarrow Trp</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>63</td>
<td>10 (352)</td>
<td>GAT \rightarrow CAT</td>
<td>Asp \rightarrow His</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>64</td>
<td>6 (203)</td>
<td>GTG \rightarrow TTG</td>
<td>Val \rightarrow Leu</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>66</td>
<td>7 (239)</td>
<td>AAC \rightarrow AAA</td>
<td>Asn \rightarrow Lys</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>70</td>
<td>8 (278)</td>
<td>CCT \rightarrow ACT</td>
<td>Pro \rightarrow Thr</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>71</td>
<td>5 (175)</td>
<td>CGC \rightarrow CAC</td>
<td>Arg \rightarrow His</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Mutations 2–30 were previously described (4).

+ , positive; –, negative.

**Infection of HPV.** We screened 72 tumors for infection of HPV DNA by PCR with consensus primers. To determine the type of HPV, we further examined all 72 tumors using Southern hybridization with HPV-16 and -18 DNA as probes. HPV DNA was detected in 15 tumors (20.8%) consistently by both methods, and all 15 cases were infected by HPV-16. These findings are consistent with those reported previously by Togawa et al. (14), who showed that the majority of HPV infection in ESC was HPV-16. Fig. 3 shows typical positive cases of infection of HPV. Among 15 positive cases, only 3 (cases 28, 43, and 63) were with p53 mutations and 2 were with MDM2 amplifications. There was no tumor exhibiting p53 mutation, MDM2 amplification, and HPV infection all together.

**Relationship of Clinicopathological Features.** The patients who underwent curative operations (63 patients) were analyzed for the relationship of clinicopathological features such as histological grade, lymph node involvement, and stage to genetic alterations which we studied here. Clinicopathological features of each tumor were not correlated with p53 mutation, MDM2 amplification, or HPV infection. However, when patients were divided into two groups (one negative for MDM2 amplification and another positive for it), the difference in survival period was significant (Fig. 4; mean survival period: 1249 ± 117 days and 425 ± 131 days for MDM2 amplification negative and positive, respectively, P = 0.0053). In contrast, patients with HPV infection did not show any difference in survival period from those without it. Furthermore, the patients...
p53 Mutation, MDM2 Amplification, and HPV Infection

Fig. 2 Expression of the MDM2 gene in ESC cell lines. a, Northern blotting with the MDM2 probe. b, Northern blotting with a β-actin probe for internal control. KYSE-30, -110, -170, and -450 showed MDM2 amplification of 6.2-, 8.5-, 8.9-, and 7.3-fold compared with a normal mucosal tissue, respectively, and also exhibited overexpression of the MDM2 mRNA. KYSE-590, -510, and -350 were without MDM2 amplification and exhibited very low expression.

Fig. 3 Detection of HPV in ESC by PCR using consensus primers (a) and Southern blot hybridization (b) with a HPV-16 probe. M, molecular weight marker; H, HeLa cell. Lane 1, case 10; Lane 2, case 25; Lane 3, case 38; and Lane 4, case 43. Cases 38 and 43 are positive for HPV infection. Arrows, bands specific to HPV.

with p53 mutation also did not show a significant difference from those without it.

**DISCUSSION**

We previously screened mutations of the p53 gene in Japanese ESC and found them in 15 (47%) of 32 tumor tissues (4). In this study, we further screened 40 additional ESC for the p53 mutations, and, in accordance with our previous study, we identified p53 mutations with a slightly lower frequency (14 of 40, 35%). In total, p53 mutations were detected in 40% (29/72) of the ESC tumors, which is consistent with results on non-Japanese patients (3).

In addition to p53 mutations, we also detected MDM2 amplification and HPV infection in a significant number of ESC tumors. It is reported that the MDM2 protein interacts with the p53 protein and inhibits p53-mediated transactivation, suggesting that some aspects of regulation of cellular proliferation by p53 can be abrogated by MDM2 (9). When the MDM2 gene is amplified and overexpressed, extended suppression of the function of the p53 protein may result in uncontrolled cell growth. So far, amplifications of the MDM2 gene have been reported in 17 (36.2%) of 47 sarcomas (10), and in 8% of glioblastomas and 11% of anaplastic astrocytomas in a series of 157 primary brain tumors (20). Although in a previous study, amplifications of the MDM2 gene in ESC tumors were not detected (21), the present study revealed amplifications and overexpressions of the MDM2 gene in 13 (18.1%) of 72 ESC and 4 (33.3%) of 12 ESC cell lines. The inconsistency between the findings reported here and those reported previously may be caused by a rather low frequency of MDM2 amplification in ESC and/or by the geographically and ethnically different populations of ESC that were studied. In 13 tumors with MDM2 amplification, only 4 were positive for p53 mutations, suggesting that in other 9 cases overexpression of the MDM2 protein may sequester the p53 protein and abrogate normal control of cell growth.

HPV infection in ESC was mainly studied by using in situ hybridization. Kulski et al. (22) detected HPV infection in 5 (50%) of 10 cases in Western Australia. Furihata et al. (23) demonstrated HPV infection in 24 (33.8%) of 71 cases in Japan, while Chang et al. (24) identified only 2 (3.9%) cases of HPV infection of 51 in the Linxian district of China. Togawa et al. (14) used a more sensitive PCR method and detected HPV infection in 14% of ESC gathered from various populations of the world. In this study, we identified infections in 15 (20.8%) of 72 cases using PCR and Southern blot hybridization. Among these 15 cases, only 3 had both p53 mutations and HPV infections, and a residual 12 were negative for p53 mutation. Lee et al. (25) also reported that there was an inverse relationship between HPV infection and p53 mutation in carcinoma of the vulva, where the E6 protein of HPV might inactivate the p53 function and play an important role in carcinogenesis, since the E6 protein of HPV-16 and -18 was demonstrated to stimulate the ubiquitin-dependent degradation of the p53 protein in vitro (12).

In total, 48 (66.7%) of 72 cases were positive for p53 mutation, MDM2 amplification, or HPV infection, all of which are supposed to abrogate the normal function of p53 protein. This suggests that loss of function of the p53 gene may be one of the most important genetic changes in tumorigenesis of ESC. Interestingly, in most cases, these genetic changes are observed...
p53 mutations did not. The overexpressed MDM2 protein may play a role in the progression of tumors by an unknown mechanism in addition to tumorigenesis by inactivation of the p53 protein.

ACKNOWLEDGMENTS

We thank Dr. B. Vogelstein for kindly providing the MDM2 probe and Dr. Y. Nakamura for the pYNH-15 probe. We also thank Dr. N. Nishida for valuable suggestions.

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p53 mutation, murine double minute 2 amplification, and human papillomavirus infection are frequently involved but not associated with each other in esophageal squamous cell carcinoma.

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