The absence of significant mutational events of the p53 gene in the only two salivary gland tumors possessing radiation-related development risks, mucoepidermoid carcinoma and Warthin tumor

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Abstract: Since the risks of development of salivary mucoepidermoid carcinoma and Warthin tumor have been significantly greater among atomic bomb survivors in a dose-dependent manner, the p53 gene, an important proto-oncogene whose mutation has been related to radiation, was analyzed by DNA sequencing and immunohistochemistry in 37 cases of mucoepidermoid carcinoma collected from Niigata and Nagasaki and in 33 cases of Warthin tumor collected from Niigata. Immunohistochemically, p53 gene products were heavily demonstrated in most of the tumor cell nuclei of mucoepidermoid carcinomas but not in Warthin tumors. Mucoepidermoid carcinomas had some point mutations (codons 136-137, 144, 232, 234, and 241) but their incidences among the samples were not significantly high (2.7%-10.8%). In contrast, three point mutations (codons 143, 151, and 229) were commonly found in the Warthin tumor cases (80%-87%), but the former two mutations did not alter their amino acid composition. Thus, there were no p53 mutations which were shared by the two tumors. However, the mutations at exon 5 of mucoepidermoid carcinomas were significantly higher in the cases from Nagasaki than those from Niigata, although their highest frequencies at most were around 10%. The results suggest that point mutations of p53 gene, as far as exons 5-7 were concerned, do not play any obviously important roles in the radiation-based tumorigenic processes shared by mucoepidermoid carcinoma and Warthin tumor.


Key words: atomic bomb, mucoepidermoid carcinoma, p53, salivary gland, Warthin tumor

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Introduction

Little is known about the causes of salivary gland tumors. One of the most frequently investigated etiologic relationships concerns the association of Epstein-Barr viral (EBV) infections with lymphoepithelial carcinomas, although the pathogenic mechanism of EBV still remains poorly understood (1-3). Another line of evidence reported for salivary gland tumorigenesis is the later effect of exposure to ionizing radiation by atomic bombs (4-7) and therapeutic radiation (8-13). Based on the survey of atomic bomb survivors in Hiroshima and Nagasaki, Japan, we have already found a causal role for ionizing radiation in salivary gland tumorigenesis, particularly in mucoepidermoid carcinomas and Warthin tumors, incidences of which were shown to be dependent on the radiation doses (14-15).

It has generally been accepted that mutational events in proto-oncogenes play important roles in human tumorigenesis. Among the proto-oncogenes, p53 gene has been extensively investigated for its functional changes due to alteration in various kinds of human experimental animal tumors (16-17). However, there has been a significant amount of information about p53 mutations in salivary gland tumors, and their important role in their pathogeneses has been indicated (18-33). In addition, there have been a large number of reports on the relationship between frequent p53 mutations and ultraviolet radiation-induced tumorigenesis of human skin squamous cell carcinoma (34) or experimental
murine skin tumors (35-36).

However, it is unknown whether radiation-related salivary gland tumors have p53 mutations. The aim of this study was to investigate mutational conditions of the p53 gene in mucoepidermoid carcinomas and Warthin tumors, which have been closely related to radiation, in order to elucidate whether or not the developments of these two salivary tumors are dependent on the mutation of the p53 gene.

Materials and methods

Surgical materials

Thirty-seven surgical specimens of mucoepidermoid carcinoma were collected from the surgical pathology files of the Department of Pathology, Faculty of Dentistry, Niigata University (21 cases) and Nagasaki University Hospital (16 cases) during a 34-year period from 1965 to 1998 without any selection procedures. The specimens from Nagasaki included those of atomic bomb survivors, although individual information about the bombing victims was not available due to personal information protection regulations. Thirty-three specimens of Warthin tumor were collected from only the Niigata University files during the same period. Surgical materials were fixed in 10% formalin and routinely processed for embedding in paraffin. Serial sections cut at 4 μm were stained with hematoxylin-eosin (HE), and stained immunohistochemically with the antibody described below. The paraffin sections were also used for DNA extraction. For control studies, 5 surgical specimens of normal submandibular gland obtained in radical neck dissections were used in the same manner as described above. The experimental protocol for isolation and analyses of tumor cells was reviewed and approved by the Niigata University Graduate School of Medical and Dental Sciences Ethical Board.

Antibody

Bp53-11, a mouse monoclonal antibody (IgG2a) against human p53 gene product, which recognizes the NH2-terminal domain, was purchased from Progen Biotechnik GmbH (Heidelberg, Germany).

Immunohistochemistry

The avidin-biotin peroxidase complex (ABC) technique, using biotinylated rabbit anti-mouse IgG (1:500, Dako, Glostrup, Denmark) and peroxidase-conjugated streptavidin complex (1:500, DAKO), was employed for immunohistochemical staining for the p53 gene products (P53) (37). Briefly, sections were pretreated in 0.01M sodium citrate (pH 6.0) for 10 min at 120°C (wet autoclave) to disclose antigenic sites. The primary antibody was diluted to a concentration of 50 ng/ml. For visualization of reaction products, sections were treated with 3.3'-diaminobenzidine in the presence of 0.05% hydrogen peroxide, and the sections were counterstained with hematoxylin. For the control sections, the primary antibody was replaced with normal mouse IgG. Prior to the reaction with the primary antibody, sections were incubated in 0.002% hydrogen peroxide in methanol to block endogenous peroxidase activities. Then, the sections were further incubated with 5% skim milk/PBS for 1 hr at 37°C to block non-specific protein binding.

Polymerase Chain Reaction (PCR)

Total DNA was isolated from paraffin sections of mucoepidermoid carcinomas, Warthin tumors and normal submandibular gland tissues using the phenol-chloroform system. PCR was carried out in an Astec thermal cycler PC-800 (Astec Co., Ltd., Fukuoka, Japan) as follows: reaction products of the reverse transcription were diluted with 1x PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.5), 0.01% Triton X-100] to a final volume of 50 μl, which contained 100 ng each of forward oligonucleotide primers and reverse primers for exons 5-7 of p53, additional dNTPs (final concentration of 0.2 mM), and 2.5 units of Taq DNA polymerase (Takara Bio Inc., Otsu, Japan). The PCR primers for human p53 exon 5 were designed as follows: the sense primer was 5'-GGTGC TGCCG TGTTG-3' (12972-12991), and the antisense primer was 3'-ACGGG TCCCA GGCGG CCGGA-5' (13275-13294). Those for exon 6 were 5'-TGGTT GCCCA GGGTC CCCAG-3' (13271-13290, forward) and 3'-ACCAA CAGTCA CCGGG AGG-5' (13475-13493, reverse). Those for exon 7 were 5'-CTTCG CACAG GTCTC CCCCA-3' (13941-13960, forward) and 3'-CGGTG AACGG TGCGA CGTGT-5' (14117-14136, reverse). The thermocycling protocol during 35 amplification cycles after denaturation at 94°C for 4 min was as follows: denaturation at 94°C for 1 min; annealing at 60°C for 1 min; and extension at 72°C for 1 min. An exponential amplification for p53 was confirmed in the 35 cycles. The amplified DNA fragments were analyzed by electrophoresis on 3% agarose gels (26).

Direct Sequencing of PCR products for p53

All PCR products were subjected to cycle sequencing by using Thermo Sequenase Core Sequencing Kits with 7-deaza-dGTP (GE Healthcare Ltd./Amersham); 1 μl of template PCR products, which were purified with GFX PCR DNA and Gel Band Purification Kits (Amersham); and 2 μl (2 pm) of Texas red-labeled primers. After denaturation at 95°C for 5 min, the reaction mixture was placed on a thermal cycler for 25 cycles of denaturation at 95°C for 30 sec and annealing/extension at 60°C for 30 sec. The reaction products were dissolved in 3 μl loading dye by vortexing,
and then the mixture was concentrated with vacuum desiccators. Then, 3 μl of samples for each lane were loaded on a gel (7% Long Ranger (Nuseive FMC Bioproducts, Rocland, ME, USA)/6.1 M urea/1.2 × TBE buffer (10 mM Tris, 10 mM boric acid, and 2 mM EDTA)). The electrophoresis was performed in a fluorescent DNA sequencer (SQ-5500-S, Hitachi Ltd., Tokyo, Japan), and the sequencing data were analyzed by using the SQ-5500 analysis software, ver. 3.03 (Hitachi) (2, 38).

Statistical analysis

Statistical analysis was performed using Fisher’s exact test for the incidental difference of p53 mutational events in mucoepidermoid carcinomas between Nagasaki and Niigata patients. A P-value of less than 0.05 was considered statistically significant.

Results

Immunohistochemistry

In mucoepidermoid carcinomas, differentiation of carcinoma cells varied among cases, showing two major directions of differentiation to mucous cells and squamous epithelial cells. Their carcinoma cell nests also varied from solid to glandular or large-cystic with mucous contents. Bizarre and hyperchromatic nuclei tended to be more often observed among squamous epithelial cells or mucous cells around cystic structures but not so frequently in regularly aligned glandular cells and so-called intermediate cells with clear cytoplasm. The stroma was partially hyaline, while most of it was fibrous with a recognizable amount of fibroblastic stromal cells (Fig. 1a). Immunohistochemically, positive staining for P53 was obtained in most of the cases examined. The reaction products were restricted to tumor cell nuclei. In particular, atypical nuclei showed stronger staining intensities. However, extremely bizarre nuclei did not show positive reactions for P53 (Fig. 1b). All of the examined cases showed the same tendency.

The histology of Warthin tumors was characterized by glandular structures composed of a two-cell layer of eosinophilic ductal cells with oncocytic appearances and a dense lymphocytic stroma scattering lymphoid follicles. The glandular structures were often distended with serous contents with lines of tumor cells infolded in a complicated

![Fig. 1. Immunohistochemical localization of P53 in mucoepidermoid carcinoma and Warthin tumor. (a, b) Mucoepidermoid carcinoma, (c, d) Warthin tumor. (a, c) Hematoxylin and eosin (HE) stain, (b, d) immunoperoxidase stain for P53, hematoxylin counterstain, × 180. In mucoepidermoid carcinoma, P53 was localized within nuclei of carcinoma cells. The stainings were more intensive in bizarre nuclei, while some of them were not positive. In Warthin tumor, P53 was not demonstrated in tumor cells nor in stromal cells.](image-url)
manner, resulting in their papillary appearance (Fig. 1c). Immunohistochemically, tumor cells did not show any discernible staining for P53. Stromal lymphoid cells were not positive, either (Fig. 1d).

The results indicated that $p53$ gene products were over-expressed in mucoepidermoid carcinoma cells but not in Warthin tumor cells. This demonstrated that there seemed to be a significant difference in P53 turnover between benign and malignant tumors.

**PCR and sequencing**

Based on the histological evidence that over-expression of $p53$ gene products took place in mucoepidermoid carcinomas, we carried out PCR amplification of the $p53$ gene fragments from genomic DNA extracts of paraffin sections which were serial to those used for immunohistochemistry. Five to twenty sections, depending on the size, were dewaxed and extracted in a plastic tube. Total DNA yields averaged about 16 μg per tube. Under the PCR condition described in Materials and Methods, exons 5 to 7 were successfully amplified as shown in electrophoresis images of Fig. 2. Single clear bands with molecular masses of 323 bp, 223 bp, and 196 bp were obtained for exons 5, 6, and 7, respectively (Fig. 2).

For direct sequencing of the PCR products, they were further purified with GFX PCR DNA and Gel Band Purification Kits. The purified DNA samples of exons 5 to 7 were applied for sequenase reactions and sequencing procedures. The results from mucoepidermoid carcinoma samples are summarized in Table 1. In exon 5, two point mutations were found. They were CAA CTG $\rightarrow$ CAG GTG at codons 136-137 and CAG $\rightarrow$ TAG at codon 144. These mutations led to changes in the amino acid sequence from Gln-Leu to Gln-Val and Gln to a stop codon, respectively. The replacement of C with T at codon 144 was not always caused by complete deletion of C but by double small peaks of C and T, indicating a heterogeneous mutation at this point. The incidence of the former was 2.7% among 37 cases and that of the latter was 10.8%. There were no mutational events in exon 6. In exon 7, three point mutations were revealed from mucoepidermoid carcinoma samples. They were ATC $\rightarrow$ AGC at codon 232, TAC $\rightarrow$ TGC or CAC at codon 234, and TCC TGC $\rightarrow$ CC TGC AGT at codon 241-242. When translated into amino acids, they should have altered from Ile to Ser (3.2%), from Tyr to Cys or His (6.5%), and from Ser-Cys to nonsense sequences starting with Pro-Ala (3.2%). The replacement of A with G at codon 234 was not always caused by complete deletion of A, indicating a heterogeneous mutation at this point. All of these mutations were severe enough to affect their translation into amino acids. However, their incidence among mucoepidermoid carcinomas was not conspicuous. In addition, these mutations were not always C $\rightarrow$ T and CC $\rightarrow$ TT transitions, which were considered to be UV radiation specific (34, 36).

**Table 1. $p53$ mutations in 37 cases of mucoepidermoid carcinoma**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>Bases (Amino acid)</th>
<th>Mutation</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>136-137</td>
<td>CAA CTG (Gln Leu)</td>
<td>CAG GTG (Gln Val)</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>144</td>
<td>CAG (Gln)</td>
<td>TAG (stop)</td>
<td>10.8</td>
</tr>
<tr>
<td>7</td>
<td>232</td>
<td>ATC (Ile)</td>
<td>AGC (Ser)</td>
<td>3.2</td>
</tr>
<tr>
<td>7</td>
<td>234</td>
<td>TAC (Tyr)</td>
<td>TG C / CAC (Cys) / Cys (His)</td>
<td>6.5</td>
</tr>
<tr>
<td>7</td>
<td>241</td>
<td>TCC TGC (Ser Cys)</td>
<td>CC TGC AGT (Pro Ala .......)</td>
<td>3.2</td>
</tr>
</tbody>
</table>
In Warthin tumors, two point mutations were found from exon 5. They were located in codons 143 and 151. In the former, GTG was altered to GTC or GTA, and their incidences were equally 87% of 33 cases. The replacement of G with C at codon 143 was not always caused by complete deletion of G but by the presence of a small G peak, indicating that the mutation was heterogeneous. Similar to mucoepidermoid carcinomas, there was no exon 6 mutation in Warthin tumors. In exon 7, there was a highly coincidental (80%) mutation at codon 229, in which TGT (Cys) was replaced with AGT (Ser) or GGT (Gly). Since the replacement of T with G at codon 229 was associated with incomplete deletion of T, the mutations were regarded as heterogeneous. Although the coincidence of these point mutations was quite high, two of the three did not lead to any change in the amino acid sequence. The results from Warthin tumor samples are summarized in Table 2.

Discussion

In the present study, the over-expression of p53 gene products was only obvious in tumor cells of mucoepidermoid carcinoma but not in those of Warthin tumor. This result was not surprising, because the over-expression of p53 has been reported to be specific to lesions with cellular proliferation or malignancy (18-25). The over-expression of P53 in mucoepidermoid carcinomas has been well documented with its frequencies ranging from 53% to 67% (25, 28, 30-31), while the sequencing data of the p53 gene have been rather limited, showing some sporadic point mutations (27, 29, 32-33). In physiological conditions, p53 gene products are degraded soon after they are targeted to nuclei (39), hence, it is usually hard to detect P53 within a cell by conventional immunohistochemical methods. The intensive staining for P53 within nuclei of mucoepidermoid carcinoma cells thus indicates that the cells produced too much protein in comparison to those degraded by its lyases, which were within normal levels of expression. Another possible explanation is that the over-expressed P53 are mutant forms which are resistant to proteolytic cleavages. Thus, it was expected that we would analyze the p53 gene for mutations in mucoepidermoid carcinoma specimens with enhanced expression of its gene products, although it was uncertain whether the over-expressed p53 was functional in G1 arrest of the cell cycling as well as apoptotic pathways (40).

There have been no documents in the literature describing mutational events of the p53 gene in Warthin tumor. In the present study, however, we found p53 mutational points in Warthin tumor cases which were highly shared among the sample group, although those point mutations did not affect the amino acid translation so much, indicating that they were just genetic polymorphisms. As shown in the sequencing

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>Bases (Amino acid)</th>
<th>Mutation</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>143</td>
<td>GTG   (Val)</td>
<td>GTC / GTA (Val) / (Val)</td>
<td>87</td>
</tr>
<tr>
<td>5</td>
<td>151</td>
<td>CCC   (Pro)</td>
<td>CCT / CCA (Pro) / (Pro)</td>
<td>87</td>
</tr>
<tr>
<td>7</td>
<td>229</td>
<td>TGT   (Cys)</td>
<td>AAT / AGT (Ser) / (Gly)</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 2. p53 mutations in 33 cases of Warthin tumor

<table>
<thead>
<tr>
<th>Patients from exon 5</th>
<th>exon 6</th>
<th>exon 7</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagasaki (n=16)</td>
<td>25</td>
<td>0</td>
<td>15.4</td>
</tr>
<tr>
<td>Niigata (n=21)</td>
<td>0</td>
<td>5.6</td>
<td>4.8</td>
</tr>
<tr>
<td>Total (n=37)</td>
<td></td>
<td>23.1</td>
<td>16.2</td>
</tr>
</tbody>
</table>

Table 3. Comparison of incidence of p53 mutations in mucoepidermoid carcinomas between patients from Nagasaki and Niigata (%)
profiles, the mutations were not always homogenous but were heterogeneous in many of the instances. The clinical features of Warthin tumors are quite different from those of other types of salivary gland tumors. They often show bilaterally synchronous as well as metachronous development or unilateral multiple developments (41). These developmental characteristics indicate some background of reactive histogenesis, including delayed hypersensitivity or inflammation in Warthin tumors (14, 42). The present data indicate a possibility that immunological or inflammatory stimuli tend to be associated with some intrinsic background such as p53 gene polymorphisms in the pathogenesis of Warthin tumor.

Together with Warthin tumors, mucoepidermoid carcinomas have been shown to be related to radiation in their pathogenesis (14-15). Therefore, these two tumors were simply expected to share certain common genetic mutational backgrounds caused by radiation. However, in contrast to the Warthin tumor data of silent mutations, all of the point mutations in mucoepidermoid carcinoma cases were missense, leading to changes in the amino acid sequence of P53, but each of their frequencies was much lower than those found in Warthin tumors. The present results clearly showed that there was neither a common feature of genetic mutation between the two tumors nor UV-radiation specific mutations such as C → T and CC → TT transitions (34, 35), as far as exons 5-7 were concerned. In addition, the result that obviously atypical mucoepidermoid carcinoma cells were not always immunopositive for P53 was noted. This may have been due to the mutated amino acid sequences of the P53 NH2-terminal region, which could not be recognized by the antibody, Bp53-11 (43).

Different from the myelogenous or thyroid neoplasms that atomic bomb survivors suffered from soon after the Hiroshima-Nagasaki bombings (5) or the Chernobyl accident (44), direct and severe gene mutations after high dose radiation exposure have not been considered to be causative of salivary mucoepidermoid carcinomas and Warthin tumors. Our previous studies showed that these tumors developed after long intervals since irradiation by the atomic bombs, although their occurrences were highly dependent on the radiation doses of the patients (14-15). These two tumors are hence typical but rare examples of human diseases caused by delayed gene alterations due to radiation-induced genetic instability (45-46). It is unknown whether the molecular mechanisms for such gene alterations develop after a long interval from irradiation events. However, recent in-vitro studies have demonstrated several lines of evidence for the delayed gene mutation. Delayed lethal gene mutations were confirmed in Chinese hamster ovary cells or BALB/3T3 cells, which had undergone 30 mean population doublings after X-ray irradiation (47). Carls and Schiestl reported deletion of one specific DNA fragment in mice whose parents were irradiated (48), indicating that a high level of genetic instability was caused by a mutator phenotype transmitted through many cell divisions or over generations. More recently, chromosomal instability has also been shown in atomic bomb survivors with leukemia (49). We have also paid attention to the translocation t(11:19)(q21:p13), which had been found in mucoepidermoid carcinoma and rarely in Warthin tumor, as well (50). However, recently, Fehr et al have demonstrated that this translocation was not significant in the pathogenesis of Warthin tumors (51). Further investigations on the detailed molecular basis for genetic instability are necessary for a better understanding of the molecular basis of radiation-induced tumorigenesis shared by mucoepidermoid carcinoma and Warthin tumor.

The frequency and the severity of the p53 mutations in mucoepidermoid carcinomas were not so high, although they were greater than those in Warthin tumor. However, their incidence in mucoepidermoid carcinoma among the patients from Nagasaki, which contained atomic bomb survivors, was higher than that of the patients from Niigata. Unfortunately, it was not possible to collate those mutations with the radiation doses among the patients from Nagasaki in the present study. However, the present data suggest that a history of irradiation predisposes p53 gene mutations. A number of studies have dealt with the relationship between p53 mutations and ultraviolet-radiation-induced tumorigenesis of experimental murine skin tumors (35-36, 52) and human cancers (34, 53). Although the p53 gene mutations in exons 5-7 did not seem to be important in the pathogeneses of mucoepidermoid carcinoma and Warthin tumor in the present study, the definite enhancement of P53 expression in mucoepidermoid carcinoma indicates at least a metabolic disturbance of p53 gene products in mucoepidermoid carcinoma cells. It is therefore suggested that some delayed gene mutations caused by radiation took place somewhere in the upstream of p53 cascades, which stimulates over-expression of P53, although it still remains unknown whether the over-expressed P53 participate in the carcinogenesis of mucoepidermoid carcinomas.

Acknowledgments

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