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 tumorigenetic processes shared by mucoepidermoid carcinoma and Warthin tumor.

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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 pathogenetic 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 pathogenesis 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 (IgG_{2a}) 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 1× 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'-GTTTC TTTGC TGCCG TGTTC-3' (12972-12991), and the antisense primer was 3'-ACGGG TCCCA GGGGT 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'-CTTGC CACAG GTCTC CCCAA-3' (13941-13960, forward) and 3'-CGGTG AACGG TGGGA 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 (62°C, exon 5) for 1 min; extension at 72°C for 1 min; and termination with a final cycle which involved annealing (60°C or 62°C, exon 5, for 1 min) and extension (72°C for 7 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, Buckinghamshire, UK). The sequence primers were synthesized based on the published data and labeled with Texas red at the 5'-end by using the 5' Oligonucleotide Texas Red Labeling Kit (Amersham). The labeled primers for *p53* exon 5 were 5'-TTCAA CTCTG TCTCC TTCCT-3' (forward); those for exon 6 were 5'-GCCTC TGATT CCTCA CTGAT-3' (forward); and those for exon 7 were 5'-CTTGC CACAG GTCTC CCCAA-3' (forward). One reaction mixture contained 3 µl of premixes (appropriate nucleotides/reaction buffer/Thermo Sequenase DNA polymerase); 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 μ l 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 $\times$ 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, $\times$ 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.

Fig. 2. PCR-products for *p53* gene, exons 5, 6 and 7 in mucoepidermoid carcinoma. (**left**) Exon 5, 323-bp, (**middle**) exon 6, 223-bp, (**right**) exon 7, 196-bp. Left lanes in each column, molecular weight standards. PCR products of three exons were successfully obtained from DNA samples extracted from paraffin sections of formalin-fixed surgical materials.

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 → CAG GTG at codons 136-137 and CAG → 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 → AGC at codon 232, TAC → TGC or CAC at codon 234, and TCC TGC → 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 → T and CC → TT transitions, which were considered to be UV radiation specific (34, 36).

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

Exon	Codon	Bases (Amino acid)	Mutation	Frequency (%)
5	136-137	CAA CTG (Gln Leu)	CAG GTG (Gln Val)	2.7
5	144	CAG (Gln)	TAG (stop)	10.8
7	232	ATC (Ile)	AGC (Ser)	3.2
7	234	TAC (Tyr)	TGC / CAC (Cys) / (His)	6.5
7	241	TCC TGC (Ser Cys)	_CC TGC AGT (Pro Ala)	3.2

Table 2. *p53* mutations in 33 cases of Warthin tumor

Exon	Codon	Bases (Amino acid)	Mutation	Frequency (%)
5	143	GTG (Val)	GTC / GTA (Val) / (Val)	87
5	151	CCC (Pro)	CCT / CCA (Pro) / (Pro)	87
7	229	TGT (Cys)	AGT / GGT (Ser) / (Gly)	80

In Warthin tumors, two point mutations were found from exon 5. They were located in codons 143 and 151. In the latter, CCC was changed to CCT or CCA. 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.

Mutational events of *p53* gene in mucoepidermoid carcinoma cases revealed as above were compared between patients from Niigata and Nagasaki, because the cases from Nagasaki should have included those from atomic bomb survivors. Since personal information about atomic bomb radiation doses was unfortunately not obtained, the comparison itself was not always worth performing. However, as shown in Table 3, all of the mutations in exon 5 were found in 4 patients from Nagasaki only. The mutations in exon 7 were found in 2 patients from Nagasaki and 1 from Niigata. The incidence of these point mutations was only 8-10% of the cases, so it was unlikely that *p53* mutations played key roles in the pathogenesis of mucoepidermoid carcinoma. However, when patients from Nagasaki and Niigata were compared, the incidence of mutational events in exon 5 of the *p53* gene was significantly higher ($P<0.05$) in patients from Nagasaki compared to those from Niigata. In regard to exon 7, no significant difference was demonstrated

between the two areas.

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 3. Comparison of incidence of *p53* mutations in mucoepidermoid carcinomas between patients from Nagasaki and Niigata (%)

Patients from	exon 5	exon 6	exon 7	Total
Nagasaki	25	0	15.4	31.3
(n=16)	(4)	(0)	(2)	(5)
Niigata	0	0	5.6	4.8
(n=21)	(0)	(0)	(1)	(1)
Total	10.8	0	23.1	16.2
(n=37)	(4)	(0)	(3)	(6)

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 tumor. 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 gene 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 pathogenesis 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.

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