Expression of p53R2, newly p53 target in oral normal epithelium, epithelial dysplasia and squamous cell carcinoma

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Abstract

Recently, the p53R2 gene has been isolated and shown to play a crucial role in DNA repair after DNA damage. The p53R2 gene encodes the p53 inducible ribonucleotide reductase small subunit 2 homologue, which is part of the p53 pathway. However, the function of p53R2 in human cancer is still unclear. We investigated p53R2 mRNA expression in human oral normal epithelium, epithelial dysplasias and squamous cell carcinomas (SCCs). Surgical or biopsy-proven specimens of 10 normal epithelium, 48 epithelial dysplasias and 63 SCCs were collected in our department. Then, p53R2 was identified by in situ hybridization to visualize and localize the expression of specific mRNAs. The authors examined the p53 gene mutation by polymerase chain reaction-single strand conformation polymorphism analysis. p53, mdm2, p21WAF1/CIP1 and Ki-67 expression was detected by immunohistochemistry. p53R2 expression was detected in none of ten normal epithelium (0%), ten of 48 dysplasias (20.8%) and 33 of 63 SCCs (52.4%). In oral SCC, the expression of p53R2 was significantly associated with tumor size, lymph node metastasis and histological differentiation ($P = 0.014$, $0.046$ and $0.022$, respectively). p53R2 expression was significantly associated with p53 abnormality in epithelial dysplasia and SCC ($P = 0.034$ and $0.009$, respectively). Of 63 patients, 37 received preoperative radiochemotherapy. p53R2 mRNA expression was significantly associated with the pathologic response to radiochemotherapy ($P = 0.031$). This study suggested that p53R2 expression could be associated with oral carcinogenesis. The presence of p53R2 mRNA expression would be a predictive factor for tumor development, tumor cell differentiation and the sensitivity to radiochemotherapy in oral SCC.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for about 5% of all human cancers in developed countries, and one-half of all HNSCCs occur in the oral cavity [1]. Radiochemotherapy and surgery are the most important treatment modalities for the majority of these tumors [2]. Although recent advancements in surgical techniques and anticancer agents have improved the tumor regression and survival rate, the methods for determining the suitable treatment for individual patients have not been standardized [3–5]. However, some predictive biomarkers are likely to be useful in identifying patients who would benefit from more intensive treatment [6–9].
Several oncogenes and tumor suppressor genes previously have been implicated in the pathogenesis of various human cancers [10–13]. Cell cycle regulation is a complex process that involves these genes in a number of positive and negative feedback loops. Cell cycle-related oncogene activation and loss of tumor suppressor genes are therefore important during multistep carcinogenesis. Oral carcinogenesis is also achieved through a multistep process in which clinically recognizable precancerous stages occasionally occur [14,15]. Histologically, these precancerous lesions are characterized by variations in epithelial dysplasia from mild to severe.

Recently, the p53R2 gene has been isolated and shown to play a crucial role in DNA repair after DNA damage [16–18]. The p53R2 gene encodes the p53-inducible ribonucleotide reductase small subunit 2 homologue, which is part of the p53 pathway. Tanaka et al. [16] have shown that the p53R2 gene is induced by several stress signals activating p53, such as DNA-damaging agents and p14ARF, and that its product causes increased deoxyribonucleotide triphosphate (dNTP) production in the nucleus, which facilitates DNA repair. Those authors also reported that p53R2 could be localized on human chromosome 8q23.1 by fluorescence in situ hybridization, and suggested that the genomic instability often seen in tumors lacking wild-type p53 may reflect dysfunction of ribonucleotide reductase due to the failure of p53R2 induction [16]. Moreover, Yamaguchi et al. concluded that inactivation of either p53 or its transcriptional target, p53R2, should interfere with regulation of the p53-dependent DNA repair pathway and increase the sensitivity of cells to anticancer agents [18]. However, the function of p53R2 is still unclear, and there have been only one report about the clinical significance of p53R2 expression in human gastric cancer [19].

Therefore, we investigated p53R2 mRNA expression in human oral normal epithelium, epithelial dysplasias and squamous cell carcinomas (SCCs) by in situ hybridization (ISH). We also determined whether expression of p53R2 mRNA correlates with p53 abnormality, overexpression of other p53 target genes (mdm2 and p21WAF1/CIP1), or Ki-67 labeling index as a marker of cellular proliferation detected by immunohistochemistry (IHC). Moreover, in this study of patients receiving preoperative radiochemotherapy, the relationship between p53R2 mRNA expression and postoperative pathologic response was evaluated to determine the influence of p53R2 on the sensitivity to radiochemotherapy.

2. Materials and methods

2.1. Tissue preparation

Tissue samples of total 121 oral tissues, including 63 SCC, 48 epithelial dysplasias and ten normal epithelium were obtained from previously untreated patients in the First Department of Oral and Maxillofacial Surgery, Nagasaki University Hospital, attached to the School of Dentistry, between April 1999 and March 1999. All samples of oral SCC patients were biopsy specimens. Samples of normal oral epithelium were obtained after informed consent from ten patients undergoing routine surgical removal of their third molars. Tumor stage was classified according to the TNM classification of the International Union Against Cancer [20]. Tumor histologic differentiation and epithelial dysplasia were defined according to the classification of the World Health Organization [21]. The specimens were fixed in 10% buffered formalin for 24–48 h and embedded in paraffin wax.

2.2. In situ p53R2 mRNA hybridization

Serial sections 3 μm thick were prepared from the tissue blocks. Deparaffinized sections in xylene were rinsed in sterile H2O and pretreated with 30 μg/ml proteinase K for 15 min at 37 °C. The sections were prehybridized in ISH buffer for 3 h to block non-specific binding. The ISH buffer containing antisense double FITC-labeled p53R2 probe was added to each section, and the sections were covered with glass coverslips. The p53R2 mRNA antisense probe corresponding to bases 1059–1088 (GenBank accession no. XM042095: 5'-GTGTCTATTGCCGAAGTGGAAACCTACGTC-3') was manufactured by Biognostik, Germany. For hybridization, the slides were incubated in a humidified chamber at 30 °C overnight. Stringent washes were carried out twice for 15 min each at 39–41 °C in 0.1x saline–sodium citrate buffer. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide.
with 0.3% H₂O₂ in methanol for 20 min. ISH signal amplification was performed using an anti-FITC rabbit polyclonal antibody (FITC/HRP, Dako, Glostrup, Denmark) and catalyzed signal amplification (CSA) (GenePoint System, Dako, Carpinteria, CA). Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and counterstaining was performed with Mayer's hematoxylin. As positive controls, we used antisense RNA probe for poly d(T) and β-actin. Negative controls were performed by omission of the probe and hybridization of a sense control probe with comparable length and G/C content to the experiment probe. Only sections with increased expression of p53R2 mRNA compared to that of the sense control probe were considered positive. Specimens were recorded as positive or negative.

2.3. Detection of p53 mutation by polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis

DNA was extracted from paraffin-embedded samples. Depending on the size of the tumor, three 10-μm sections were cut from each sample. The sections were supplemented with 500 μl of extraction buffer (DEXPAT, Takara, Otsu, Japan), and the mixtures were boiled for 10 min and centrifuged, and DNA was found in the supernatant, not in the paraffin layer. PCR was performed for exons 5–9 of the p53 gene. The PCR reaction was carried out in a total volume of 50 μl, containing 5 μl of the extracted DNA solution, 20 pmol of each primer of primers, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.4 mM dNTP, and 1.25 units of Taq DNA polymerase (Premix Taq, Takara, Otsu, Japan). The reaction mixture was subjected to 40 cycles of denaturation at 94 °C for 1 min, annealing under conditions that varied as follows: exon 5, 53 °C for 1 min; exon 6, 57 °C for 1 min; exon 7, 66 °C for 2 min; exons 8 and 9, 55 °C for 2 min; and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The primer sequences were as follows. Exon 5: 5'-TACTCCCTCATGCTACACAA-3' and 5'-CAT CGCTATCTGAGCGCCG-3' (product size, 186 bp); exon 6: 5'-GTCTGGCCCTCCTCGACAT-3' and 5'-TCAGGCGGCTCATAGGGA-3' (product size, 111 bp); exon 7: 5'-TCTCTAGGTGGGTCCCT-GAC-3' and 5'-CAAGTTGCCTCCTGACCTGGA-3' (product size, 133 bp); exons 8–9: 5'-CTATCCT-GAGTAGTGGTATC-3' and 5'-CCAAAGACT-TAGTACCTGAG-3' (product size, 332 bp). The PCR products were analyzed by ethidium bromide agarose gel electrophoresis. The PCR products of the p53 gene were diluted 1:1 with 99% formamide containing 0.05% bromophenol blue and denatured at 95 °C for 5 min followed by rapid cooling on ice. Electrophoretic analysis was performed using a Multiphor Electrophoresis Unit at 600 V, 50 mA, 30 W, 4 °C for 80 min and an ExcelGel DNA Analysis Kit (Pharmacia Biotech, Uppsala, Sweden). After separation, the polyacrylamide gel was silver-stained in a Hoefer Automated Gel Stainer using a DNA Silver Staining Kit (Pharmacia Biotech).

2.4. IHC

Serial sections 3 μm thick were taken from the tissue blocks. Deparaffinized sections in xylene were soaked in 10 mM citrate buffer (pH 6.0) and placed in an autoclave at 121 °C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by the incubation with 0.3% H₂O₂ in methanol for 30 min. Immunohistochemical staining was performed using the Envision system (EnVision + , Dako, Carpinteria, CA). The primary antibodies used were directed against p53 (DO-7, Dako, Glostrup, Denmark; 1:100 dilution), mdm2 (SMP14, Dako, Glostrup, Denmark; 1:100 dilution), p21WAF1/CIP1 (SXI18, Dako, Glostrup, Denmark; 1:50 dilution) and Ki-67 (MIB1, Immunotech, Marseilles, France; 1:100 dilution). The sections were incubated with the monoclonal antibodies overnight at 4 °C. Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and the samples were counterstained with Mayer’s hematoxylin and mounted. Known positive-immunostaining slides were used as positive controls. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline. Results were evaluated according to the positive tumor or epithelial cell percentage. Specimens with more than 10% positive tumor or epithelial cells were considered positive for p53, mdm2 or p21WAF1/CIP1. The labeling index for immunostaining (LI) Ki-67 was calculated by count-
ing the positive cells among more than 1000 tumor or epithelial cells in randomly selected fields.

2.5. Assessment of tumor response to preoperative radiochemotherapy

Of the 63 oral SCC patients, 37 received preoperative radiochemotherapy to the primary tumor and lymphatics. Radiation was performed five times a week with 2 Gy at each session. The mean of total dose was 35.9 Gy (16–80 Gy). During the course of radiation therapy, the patients received cisplatin (80 mg/m² per body) or carboplatin (300–375 mg/body) and tegafur (300 mg/day) on days 21–28. Surgery was undertaken 3–4 weeks after the radiochemotherapy. Tumor response was evaluated by scoring the histopathologic response, based on the method described by Shintani et al. [22]. The histopathologic response was scored using semiserial sections of whole surgical specimens as follows: 0, no evidence of treatment effect; 1+, treatment effect involving up to one-third of the gross tumor mass; 2+, effect involving one third to two thirds of the gross tumor mass; 3+, treatment effect in more than two thirds of the gross tumor mass; 4+, treatment effect in the entire tumor with no viable carcinoma identified.

2.6. Statistical analysis

The categorical data were statistically analyzed by Fisher’s exact tests. The continuous data was summarized as mean ± SD. Significance was assessed by the Mann–Whitney U-test. A stepwise logistic regression model was used for the multivariate analysis of independent predictors of pathologic response to radiochemotherapy. Survival analysis was compared using the log-rank test. P values of less than 0.05 were considered to be significant.

3. Results

3.1. p53R2 expression

All samples were positive by ISH for both β-actin (Fig. 1A) and poly d(T) antisense probes. In normal oral epithelium, no p53R2 mRNA expression was detected (Fig. 1B). p53R2 mRNA was detected in ten of 48 epithelial dysplasias (20.8%) and 33 of 63 SCCs (52.4%) (Table 1). Hybridization with the p53R2 mRNA antisense probe was observed as any brown grains in the cytoplasm of epithelial cells or tumor cells. In oral epithelial dysplasia, the cytoplasmic positive epithelial cells were localized in suprabasal and upper layers (Fig. 1C). In oral SCC, the cytoplasmic positive tumor cells were localized in suprabasal and central region of tumor nest (Fig. 1D).

3.2. Relationship between p53R2 expression and p53 target gene status

Examination of 63 oral SCCs for mutations in p53 exons 5–9 by PCR–SSCP analysis demonstrated mobility shifts in 21 of 63 SCCs (33.3%). A representative PCR–SSCP polyacrylamide gel along with the gel picture of the amplimer of exon 5 is shown in Fig. 2A,B.

Immunohistochemically, in oral SCCs, p53 nuclear staining was most abundantly detected in the basal as well as the suprabasal regions (Fig. 3A). In oral epithelial dysplasia, p53 nuclear staining was most frequent in the epithelial basal region. Of the ten normal mucosa, none showed p53 expression. The data about the inactivation of the normal function of p53 by mutation and/or overexpression are summarized in Table 1. In oral SCC, the immunoreactivity of mdm2 was predominantly nuclear, with some degree of faint cytoplasmic staining (Fig. 3B). p21WAF1/CIP1 nuclear staining was most frequent in the suprabasal region, less frequent in the central region of tumor nests, and least frequent in the basal region (Fig. 3C). The proliferation antigen Ki-67 was detected in all of the 63 oral SCCs. Much higher levels of Ki-67 expression were seen in the basal and suprabasal regions than the central region of tumor nests.

The expression of p53R2 mRNA was significantly associated with p53 abnormality in oral epithelial dysplasia and SCC (P = 0.034 and 0.009, respectively; Table 2).

3.3. Relationship between p53R2 expression and clinicopathologic factors of oral SCC patients

As shown in Table 3, the expression of p53R2 mRNA in oral SCC was significantly associated with
Fig. 1. Expression of p53R2 mRNA in oral normal epithelium, epithelial dysplasia and SCC. In situ mRNA hybridization was performed on paraffin-embedded sections. (A) In situ hybridization for β-actin with antisense probe in normal mucosa (original magnification ×200). (B) In situ hybridization for p53R2 with antisense probe in normal mucosa (×200). (C) In situ hybridization for p53R2 with antisense probe in epithelial dysplasia (×200). (D) In situ hybridization for p53R2 with antisense probe in SCC (×200).

Table 1: p53R2 expression and p53 abnormalities in oral normal epithelium, epithelial dysplasia and SCC

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of cases</th>
<th>p53R2 mRNA expression (positive n (%))</th>
<th>p53 mutations by PCR–SSCP (positive n)</th>
<th>p53 IHC (positive n (%))</th>
<th>p53 abnormalities* (positive n (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exon 5</td>
<td>Exon 6</td>
<td>Exon 7</td>
<td>Exon 8–9</td>
</tr>
<tr>
<td>Normal epithelium</td>
<td>10</td>
<td>0 (0.0%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epithelial dysplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>40</td>
<td>8 (20.0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
<td>0 (0.0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Severe</td>
<td>4</td>
<td>2 (50.0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>10 (20.8%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SCC</td>
<td>63</td>
<td>33 (52.4%)</td>
<td>10</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

* p53 abnormalities include p53 mutations and/or immunohistochemical expression of p53 protein. NA, not analyzed due to insufficient DNA.
tumor size, lymph node metastasis and histological differentiation ($P = 0.014$, $P = 0.046$ and $P = 0.022$, respectively).

3.4. Relationship between pathologic response to radiochemotherapy and examined variables in oral SCC

Of 63 patients with oral SCC, 37 received preoperative radiochemotherapy. As shown in Table 4, the expression of p53R2 mRNA was the only one proven to be the independent predictor of the pathologic response to radiochemotherapy ($P = 0.031$), when pathologic response was grouped into ineffective (0 to 2 + of pathologic response) and effective (3 + to 4 + of pathologic response) categories.

3.5. Survival analysis

Lymph node metastasis was significantly associated with survival ($\chi^2 = 9.759$, $P = 0.002$). There was no significant relationship between the other examined variables and survival.

4. Discussion

We report here the first study of p53R2 mRNA expression in clinical samples of human oral tissue using ISH. Although a report from another group indicated that p53R2 was not a critical target of genetic alteration in gastric carcinogenesis [19], we observed p53R2 mRNA expression in 20.8% of epithelial dysplasias and 52.4% of SCCs, whereas none of normal epithelium was observed p53 mRNA expression. Therefore, p53R2 expression suggest a critical role in oral carcinogenesis. Some recent studies have shown that p53R2 may be a transcriptional target of the wild-type p53 protein in several human cancer cell lines [16,18]. Moreover, Byun et al. suggested that the expression of p53R2 might be regulated in a p53-independent manner in human gastric cancer [19]. On the other hand, Nakano et al. suggested that p53R2 expression is induced by mutated p53 protein that is defective for the activation of apoptosis but retains cell cycle arrest function [17]. Interestingly, our results showed a significant correlation between p53R2 mRNA expression and p53 abnormality. The analysis of p53 abnormality in this study demonstrated mutations by PCR–SSCP and

IHC analysis.