Expression of p21<sup>WAF1/CIP1</sup>, p53, and Ki-67 Proteins in Malignant Ameloblastomas and Ameloblastomas

Souichi Yanamoto, Goro Kawasaki, Akio Mizuno
Division of Oral and Maxillofacial Surgery, Department of Developmental and Reconstructive Medicine, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Abstract
Objectives: The cyclin-dependent kinase inhibitor p21 has been identified as a downstream effector or target of the p53 tumour suppressor gene. The aims of this study were to immunohistochemically assess the proliferative activity and to clarify the possible roles of p21 and p53 genes in oncogenesis of malignant ameloblastoma and ameloblastoma.

Patients and Methods: Expression of p21, p53, and cellular proliferation was investigated using the Ki-67 immunohistochemical labelling index in specimens from 2 patients with malignant ameloblastomas and 10 patients with ameloblastomas.

Results: Expression of p21 was immunohistochemically detected in 50% of malignant ameloblastomas, 0% of ameloblastomas, and 100% of healthy oral mucosa. Expression of p53 was immunohistochemically detected in 100% of the malignant ameloblastomas, 40% of ameloblastomas, and 0% of healthy oral mucosa. The p53-labelling index of malignant ameloblastomas was significantly higher than that of other ameloblastomas. There was no significant difference between Ki-67 antigen reactivity in p53 protein-positive tumours and negative tumours.

Conclusion: These findings suggest that expression of p21 and p53 proteins could be associated with oncogenesis in ameloblastoma. The expression of p53 could be associated with malignant transformation rather than cellular proliferative activity. However, the correlation between p21 and p53 has not yet been sufficiently clarified in malignant ameloblastoma and ameloblastoma.

Key Words: Ameloblastoma, Oncogene protein p21 (ras), protein p53, Ki-67 antigen

Introduction
Although ameloblastoma is classified as a benign odontogenic tumour of the central jaw bones, it shows higher proliferation and local invasion potential compared with other odontogenic neoplasms. Ameloblastomas are often recurrent, but are rarely malignant. Ameloblastoma consists of proliferating odontogenic epithelium, which has 2 main histological patterns, follicular and plexiform.

Malignant ameloblastoma, which is a rare tumour, is defined as a neoplasm showing cytological features of malignancy within the primary growth in the jaws and/or metastasis. It has been suggested that these ameloblastomatous tumours resemble the epithelial odontogenic apparatus such as the enamel organ or dental lamina — however, the mechanisms of their oncogenesis and malignant transformation remain unknown.

Recently, it was reported that tumour suppressor genes are associated with the proliferation of tumour cells as cell cycle regulators in a variety of human neoplasms, including oral neoplasms. The p21 gene was identified as an important target for the
p53 suppressor gene and wild-type p53 activated fragment-1 (WAF1) from studies looking for downstream effectors of p53.\textsuperscript{10,11} Although loss of p53 functions can result from mutations in the p53 tumour suppressor gene, which have been identified in oral cancers, studies of p21 and its relation with p53 are few.\textsuperscript{12,13} In addition, there have been no studies of the expression of p21 in ameloblastomas.

In this study, expression of p21 and p53 proteins in malignant ameloblastomas and ameloblastomas were examined by immunohistochemistry. The Ki-67 immunohistochemical labelling index (LI) was used as a marker of cellular proliferation. The aim of the study was to immunohistochemically assess the proliferative activity and to clarify the possible roles of p21 and p53 genes in oncogenesis of malignant ameloblastoma and ameloblastoma.

**Patients and Methods**

**Tissue Preparation**

Specimens were surgically removed from 2 patients with malignant ameloblastoma and 10 patients with ameloblastoma attending the Division of Oral and Maxillofacial Surgery, Nagasaki University Graduate School of Biomedical Sciences, Japan, between April 1990 and March 1998. Diagnosis of the tumours was made according to the World Health Organization (WHO) histological typing of odontogenic tumours.\textsuperscript{1}

The histological patterns of ameloblastoma observed in this study were follicular and plexiform. The follicular tumours showed typical islands of epithelia with central stellate reticulum-like cells. The plexiform tumours showed typical anastomosing strands of epithelium-forming cords with sparse stellate reticulum-like cells. Malignant ameloblastomas had been identified where cytological features of malignancy were shown by the primary growth in the jaw.

The specimens were fixed in 10% buffered formalin for 24 to 48 hours and embedded in paraffin wax. Serial sections 3 µm thick were taken from the tissue blocks and examined by immunohistochemistry. Samples of healthy oral mucosa, obtained after informed consent from 10 patients undergoing routine surgical removal of their third molars, served as controls.

**Immunohistochemical Staining**

Deparaffinised sections in xylene were soaked in 10 mM citrate buffer (pH 6) and processed in an autoclave at 121°C for 5 minutes for antigen retrieval. Endogenous peroxidase was blocked using 0.3% H₂O₂ in methanol for 30 minutes. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex method.

The primary antibodies used were p21\textsuperscript{WAF1/CIP1} Mo Ab SX118 (DAKO, Glostrup, Denmark) at a 1:50 dilution, p53 Mo Ab DO-7 (DAKO) at a 1:100 dilution, and Ki-67 Mo Ab MIB1 (Immunotech, Marseille, France) at a 1:100 dilution. The sections were incubated with the monoclonal antibodies overnight at 4°C. Reaction products were visualised by immersing the sections in diaminobenzidine (DAB) solution, and counterstaining with hematoxylin.

**Evaluation of Staining**

The sections were assessed independently by 2 investigators. Nuclear staining of neoplastic cells at greater than 5% was considered positive evidence of p21, p53, and Ki-67. The LI for immunostainings was calculated by counting the positive cells among more than 1,000 neoplastic cells in randomly selected fields.

**Statistical Analysis**

The percentage of positive cases was statistically analysed by chi-square test. The LI was summarised as the mean ± SD. Statistical significance was assessed by Student’s t test. P values of <0.05 were considered to be significant.

**Results**

The clinical and histological findings of the 12 patients are shown in Table 1. In all normal oral mucosa, p21 nuclear staining occurred most frequently in the suprabasal region, less in the upper region, and least in the basal region. Expression of the p21 protein was not observed in any of the ameloblastomas, but was seen in 1 of the 2 malignant ameloblastomas. Nuclei positive for the p21 protein were observed mainly in the suprabasal tumour cells of malignant ameloblastomas (Figure 1). The p21-LI of normal oral mucosa was significantly higher than that of ameloblastoma and malignant ameloblastoma.
Table 1. Clinical and histological findings of 12 patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Location</th>
<th>Histological pattern</th>
<th>p21 staining</th>
<th>p53 staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>M</td>
<td>Mandibular ramus</td>
<td>Follicular</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>F</td>
<td>Maxillary molar</td>
<td>Plexiform</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>M</td>
<td>Mandibular molar</td>
<td>Plexiform</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>M</td>
<td>Mandibular premolar</td>
<td>Follicular</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>F</td>
<td>Maxillary premolar</td>
<td>Follicular</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>M</td>
<td>Mandibular incisor</td>
<td>Follicular</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>54</td>
<td>M</td>
<td>Mandibular premolar</td>
<td>Follicular</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>69</td>
<td>M</td>
<td>Mandibular incisor</td>
<td>Follicular</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>41</td>
<td>M</td>
<td>Mandibular molar</td>
<td>Plexiform</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>M</td>
<td>Mandibular molar</td>
<td>Follicular</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>52</td>
<td>F</td>
<td>Mandibular molar</td>
<td>Malignant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>71</td>
<td>M</td>
<td>Mandibular incisor</td>
<td>Malignant</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Immunoreactivity of p53 was not detected in normal oral mucosa. Expression of the p53 protein was scattered among the nuclei of the tumour cells in 4 of 10 ameloblastomas (40.0%), including 3 of a follicular pattern and 1 of a plexiform pattern, and 2 malignant ameloblastomas. Nuclei positive for the p53 protein were observed mainly in the basal tumour cells of the follicular pattern, in the tumour cells neighbouring the basement membranes of the plexiform pattern (Figure 2), and in the tumour cells neighbouring the basement membranes of malignant ameloblastoma (Figure 3). The mean of p53-LI was 12.1% in ameloblastoma. The difference in the means of p53-LI between the follicular and plexiform patterns was not significant. The p53-LI of malignant

Figure 1. Malignant ameloblastoma. (a) Hematoxylin and eosin staining (original magnification x 40); (b) crowded tumour cells and high mitotic activity (hematoxylin and eosin; original magnification x 200); (c) nuclei positive for p21WAF1/CIP1 protein were observed mainly in the suprabasal region of ameloblastic tumour cells (avidin-biotin-peroxidase complex method; original magnification x 200).
Figure 2. Ameloblastoma of plexiform pattern. Nuclei positive for p53 protein were observed mainly in the tumour cells neighbouring the basement membranes of the plexiform pattern (original magnification x 100).

Figure 3. Malignant ameloblastoma. Nuclei positive for p53 protein were observed mainly in the basal region of ameloblastic tumour cells (original magnification x 200).

Ameloblastoma was significantly higher than that of other ameloblastomas (Table 2).

Ki-67 reacted with the nuclei in normal oral mucosa and neoplastic odontogenic epithelium, and was especially evident in basal tumour cells with the follicular pattern, in the cells neighbouring the basement membranes with the plexiform pattern, and malignant ameloblastoma.

The mean Ki-67-L1 was 2.7% in normal oral mucosa, 4.2% in ameloblastoma, and 12.2% in malignant ameloblastoma; the mean in neoplastic odontogenic epithelium was significantly higher than that in normal oral mucosa. In ameloblastomas, the mean Ki-67-L1 for the follicular pattern was significantly higher than that of the plexiform pattern. Although the mean Ki-67-L1 of malignant ameloblastoma tended to be higher than that of ameloblastoma, there was no significant difference (Table 2). Although the mean Ki-67-L1 of the p53 protein-positive ameloblastomas tended to be higher than that of negative cases, this was not significantly different (Table 3).

Discussion

Ameloblastoma is a rare benign neoplasm of the jaw. In particular, malignant ameloblastoma is a very rare tumour in which the pattern of ameloblastoma and cytological features of malignancy are shown in the primary tumour, regardless of whether it has metastasised. Several investigators have published data on the proliferation indices of ameloblastoma, by applying proliferating cell nuclear antigen (PCNA) and/or Ki-67, but there appears to be little data on p21 and p53 expression. Although the sample size of this study is small, it is useful to assess and speculate on the roles of p21 and p53 in malignant ameloblastoma and ameloblastoma.

Mutation of the p53 gene and/or accumulation of the p53 protein have been reported to be associated with increased cellular proliferation and malignant transformation in a variety of human tumours. Although the present study was performed using a monoclonal antibody reactive with wild-type and mutant-type p53 proteins, the level of wild-type p53 protein was extremely low and wild-type p53 protein has a short

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Number of positive cases</th>
<th>Labelling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p21</td>
<td>p53</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>10</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Ameloblastoma</td>
<td>10</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Follicular pattern</td>
<td>7</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Plexiform pattern</td>
<td>3</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Malignant ameloblasta</td>
<td>2</td>
<td>1 (50%)</td>
</tr>
</tbody>
</table>

Table 2. Expression of p21, p53, and Ki-67 in ameloblastoma and malignant ameloblastoma.
Table 3. Relationship of p21^WAF1/CIP1, p53, and Ki-67.

<table>
<thead>
<tr>
<th></th>
<th>p21 protein</th>
<th>Ki-67-LI (%)</th>
<th>p53 protein</th>
<th>Ki-67-LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.7 ± 2.4</td>
<td>+</td>
<td>2.7 ± 2.4</td>
</tr>
<tr>
<td>Ameloblastoma</td>
<td>-</td>
<td>4.2 ± 3.6</td>
<td>-</td>
<td>4.5 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20.9</td>
<td>+</td>
<td>3.7 ± 2.9</td>
</tr>
<tr>
<td>Malignant ameloblastoma</td>
<td>+</td>
<td>3.6</td>
<td>-</td>
<td>12.2 ± 12.2</td>
</tr>
</tbody>
</table>

Abbreviation: LI = labelling index.

half-life. Therefore, it has been suggested that mutant-type p53 protein was detected by immunohistochemistry. Girod et al suggested that mutation of p53 was related to the carcinogenesis and the malignant transformation of oral precancerous lesions. In the present study, the mean p53-LI of malignant ameloblastomas was significantly higher than that of normal mucosa and other ameloblastomas.

These findings suggest that p53 protein could be associated with oncogenesis and malignant transformation in ameloblastoma. It has previously been reported that expression of the p53 protein was correlated with Ki-67 antigen reactivity in head and neck tumours. Kumamoto et al reported that Ki-67 antigen reactivity in p53 protein-positive ameloblastomas tended to be higher than in negative ameloblastomas. In the present study, however, there was no significant difference between Ki-67 antigen reactivity in p53 protein-positive and -negative ameloblastomas. Therefore, overexpression of the p53 protein in ameloblastomas could be associated with oncogenesis and malignant transformation rather than cellular proliferative activity.

Although p21^WAF1/CIP1 was first identified as a p53-inducible gene, expression of p21^WAF1/CIP1 is also known to be induced by a p53-independent pathway. Some authors reported that its protein was related to tumour differentiation. Although p21 protein over expression appears to vary in human malignancies, there are few reports of p21^WAF1/CIP1 expression in ameloblastoma.

In the present study, immunoactivity of p21^WAF1/CIP1 was detected in all normal oral mucosas, although no expression of p21^WAF1/CIP1 was observed in the 10 ameloblastomas. These findings suggest that loss of p21^WAF1/CIP1 expression may be associated with oncogenesis in ameloblastoma. The correlation between p21^WAF1/CIP1 and p53 has not been clarified in malignant ameloblastoma and ameloblastoma.

In conclusion, although the present study shows that p53 protein was overexpressed in malignant ameloblastoma and ameloblastoma, p21^WAF1/CIP1 was not detected in ameloblastoma. Overexpression of the p53 protein could be associated with oncogenesis and malignant transformation in ameloblastoma. Loss of p21^WAF1/CIP1 expression may be associated with oncogenesis. However, the correlation between p21^WAF1/CIP1 and p53 has not been clarified in malignant ameloblastoma and ameloblastoma. The sample size of this study is very small and further reliable data are necessary by investigation of more cases and examination at DNA level.

References