The Proliferative and Apoptotic Behaviors of Ameloblastoma

2001

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The first part of the present thesis will be published, the second part is in press and the third part is in submission.

1. **Sandra, F., Mitsuyasu, T., Nakamura, N., Shiratsuchi, Y., Ohishi, M. (2001)** Immunohistochemical evaluation of PCNA and Ki-67 in ameloblastoma. Oral Oncol 37: 52-57.
2. **Sandra, F., Nakamura, N., Takeuchi, H., Mitsuyasu, T., Shiratsuchi, Y., Ohishi, M.** Two relatively distinct patterns of Ameloblastoma: an anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (center). Histopathology (in press).
3. **Sandra, F., Takeuchi, H., Nakamura, N., Kanematsu, T., Hirata, M., Ohishi, M.** The role of MDM2 in the proliferative activity of ameloblastoma (in submission).

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PART 1

Immunohistochemical evaluation of PCNA and Ki-67 in ameloblastoma

ABSTRACT

Thirty two ameloblastoma tissues were immunohistochemically studied by using monoclonal anti-PCNA and anti-Ki-67 antibodies. Positive cells were evaluated and analyzed in relation to the WHO classification, cytological pattern of the outer layer cell, clinical appearance, tumor's location, radiographic appearance and patient's age. In regard to the cytological pattern of the outer layer cells, the basal cell type had significantly higher PCNA ($P<0.05$) and Ki-67 ($P<0.05$) labelling indices than the cuboidal cell type. The solid type had significantly higher PCNA and Ki-67 labelling indices than the cystic and the mixed type. The labelling index of younger patient was found to be the lowest, the middle age was in the middle and the older patient was the highest. These results indicated that the proliferating activities of

ameloblastomas are quite variable, and the evaluation of Ki-67 and PCNA seems to be good indicators to assess the proliferating activity of each type of ameloblastoma.

INTRODUCTION

Ameloblastoma accounts for approximately one percent of all oral tumors. Ameloblastoma grows slowly, but is locally invasive (Cawson *et al.*, 1987; Lucas, 1984). WHO classifies ameloblastoma into several types, such as the follicular, plexiform, acanthomatous, basal cell and unicystic ameloblastoma, etc. (Kramer *et al.*, 1992; Shafer *et al.*, 1983). The unicystic ameloblastoma was termed as distinctive entity (Leider *et al.*, 1985).

Identification of proliferating activity in tumors may be useful to predict the biological behavior of the lesion (Robbins *et al.*, 1987). There is no adequate report concerning the relation between proliferating activity and clinical aspect. Therefore, in order to know the biological behavior of ameloblastoma and to verify whether biological behavior of ameloblastoma has any relation with clinical aspect or not, we performed this study by using monoclonal anti-PCNA (Proliferating Cell Nuclear Antigen) antibody and monoclonal anti-Ki-67 antibody. Cytological pattern of the tumor seems to be related to the clinical behavior of the ameloblastoma (Mitsuyasu *et al.*, 1997;

Nakamura *et al.*, 1994). In the present study, we found a relation between the proliferating activity of ameloblastoma and the cytological pattern of the outer layer cells. The analysis of WHO classification and some clinical factors showed that proliferating activity of some specific types was higher than that of others. Evaluation of the proliferating activity of the ameloblastoma will be useful to understand the behavior of ameloblastoma.

MATERIALS AND METHODS

Clinical materials

Thirty two ameloblastomas derived from the patients treated at the First Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Kyushu University during the period 1986-1999 were used in this study. The patient's ages ranged from 12 to 81 years old (mean = 38.2 years old). There were 20 men and 12 women. Twenty seven ameloblastomas occurred in the mandible (84.4%) and 5 in the maxilla (15.6%). All tumors were of primary

cases. The clinical appearances of excised specimens were categorized into the solid type (15 cases), mixed type (8 cases) and cystic type (9 cases). After the resection, these tumor tissues were fixed for 24-48 hours in 4 % formaldehyde freshly prepared from paraformaldehyde, processed and embedded in paraffin. Tissue blocks were sliced in 4 μm thickness and the tissue sections were mounted on 3-aminopropyltriethoxy-silane coated glass slides. Tissue sections were then stained with hematoxylin and eosin. According to the World Health Organization International, Histological Typing of Odontogenic Tumors 2nd edition (Kramer *et al.*, 1992), these ameloblastomas were classified into 9 follicular ameloblastomas, 9 plexiform ameloblastomas, 5 unicystic ameloblastomas, 3 basal cell ameloblastomas, 3 acanthomatous ameloblastomas and 3 desmoplastic ameloblastomas. Based on the X-ray and CT-Scan film, the tumors were classified into soap bubble (5 cases), multilocular with soap bubble (5 cases), multilocular (5 cases), and unilocular (17 cases). In relation to the location of the tumor, ameloblastomas were classified into anterior-premolar region of mandible (7 cases), molar region of mandible (8 cases), ramus region of mandible (9 cases), wide

area/bilateral of mandible (4 cases) and maxilla (4 cases). Patient's age was divided into three groups; ⩽20 years old (10 cases), 21-40 years old (10 cases), and ⩾41 years old (12 cases). Tissue sections with adequate parenchymal tissues were selected and used for further experiment.

Immunohistochemistry

For immunohistochemistry, all tissue sections were deparaffinized and autoclaved at 121°C for 10 min in solution of freshly prepared 10 mM citrate buffer pH 6.0 for antigen retrieval (Mitsuyasu *et al.*, 1997). After cooling to room temperature, tissue sections were rinsed in phosphate buffered saline (PBS) (5 min × 3), and incubated with 0.3 % hydrogen peroxide for 20 min to block endogenous peroxidase activity. Tissue sections were then washed in PBS, and 10 % normal goat serum was applied for 20 min to reduce nonspecific antibody binding. PCNA staining was performed with mouse monoclonal anti-PCNA antibody (PC10, Dako, Glostrup, Denmark). Tissue sections were incubated with PC10 at dilution of 1:1500 for 30 min in the moist chamber at room temperature. For Ki-67 staining, mouse monoclonal

anti-Ki-67 antibody (MM1, Novocastra, Newcastle, UK) was diluted to 1:100 and was applied to tissue sections overnight in the moist chamber at 4°C. After incubation with primary antibody, tissue sections were washed in PBS and treated with biotin-streptavidin method (LSAB2 Kit Peroxidase, Dako, Carpinteria, CA, USA). The peroxidase activity was visualized by immersing tissue sections in 3,3'- diaminobenzidine hydrochloride (DAB, Histofine, Nichirei, Tokyo, Japan), resulting in a brown reaction product. Tissue sections were finally counterstained with methyl green and mounted. Some tissue sections of oral mucosae were stained also as positive controls. For negative control, PBS was applied to substitute for the primary antibody.

Evaluation

Outer layer cells and inner layer cells of ameloblastomas were analyzed by using ×20 objective. In each tissue section, several fields were chosen randomly and the fields complied with the requirements, were selected further. The requirements were that the well-preserved fields, which were stained by both anti-PCNA and anti-Ki-67 antibodies and that the outer layer

of the field contained more than 200 cells. Clear brown nuclei were regarded as positive cells and the positive cells were counted by 2 persons without the knowledge of ameloblastoma using an eye-piece graticule and ×40 objective. At least 1200 outer layer cells per specimen were counted and analyzed in relation to WHO classification, cytological pattern of the outer layer cell, clinical appearance, tumor's location, radiographic appearance and patient's age. In the cytological pattern of the outer layer cell, ameloblastomas were classified into basal cell type, columnar cell type and cuboidal cell type. The percentage of labelling index (number of positive cells / total cells × 100) was calculated for each field. Both PCNA and Ki-67 were evaluated in the same way.

Statistical analysis

The labelling indices of PCNA and Ki-67 were counted; the mean and the standard deviation (SD) were calculated. The Mann-Whitney Test was used and P values of <0.05 were considered statistically significant. Data of PCNA and Ki-67 labelling indices were statistically compared by using

paired t-test, and P values of <0.05 were considered significant.

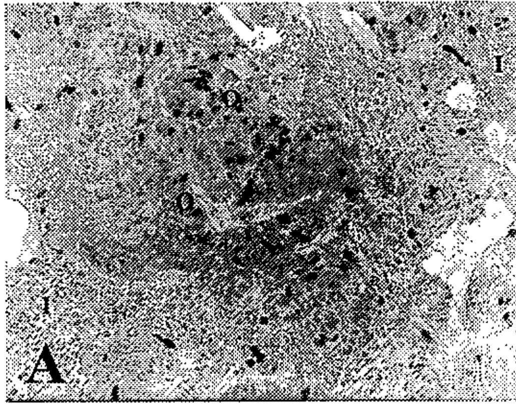
RESULTS

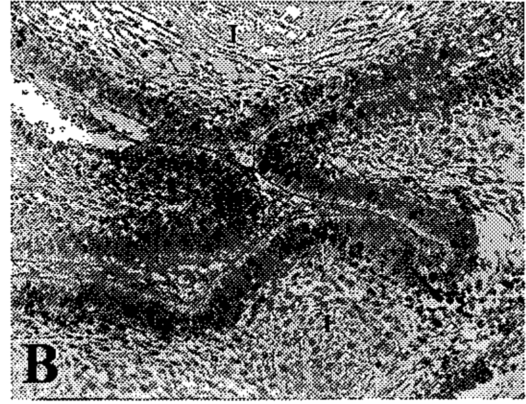
Both PCNA and Ki-67 were positively stained in all tissue sections (Fig. 1a,1b,1c,1d) mainly in the nuclei of the outer layer cells of ameloblastoma, and only a few positive cells were seen in the inner layer. Because most of the tissue sections didn't contain positive inner layer cells and outer layer cells are known to reflect the growth activity of ameloblastoma (Mitsuyasu *et al.*, 1997), we counted only the outer layer cells. The mean of PCNA labelling index was 14.43±5.41 (mean±SD) and the mean of Ki-67 labelling index was 3.82±1.98.

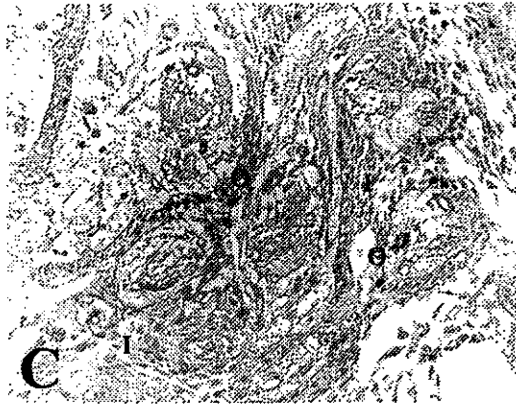
WHO classification

The basal cell ameloblastoma expressed the highest labelling index for both PCNA and Ki-67 (Table 1,2) and the lowest labelling index was

expressed by the unicystic ameloblastoma for PCNA (Table 1) and the desmoplastic ameloblastoma for Ki-67 (Table 2). The labelling index of the basal cell ameloblastoma was significantly higher than that of the desmoplastic ameloblastoma for both PCNA and Ki-67 ($P<0.05$) and the labelling index of the basal cell ameloblastoma was also significantly higher than that of the unicystic ameloblastoma for both PCNA and Ki-67 ($P<0.05$).







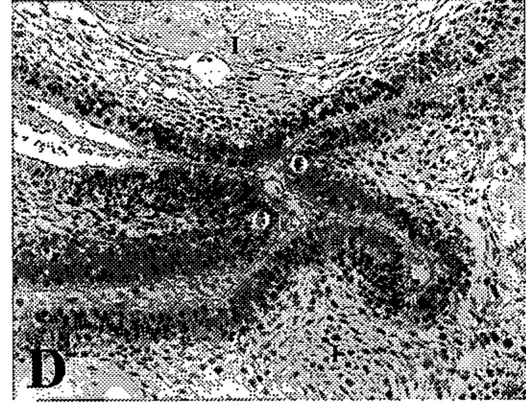


Fig.1. Ki-67 expression of basal cell (a), columnar cell (b), cuboidal cell types (c), and (d) PCNA expression of columnar cell type (cytological pattern of the outer layer cells). I = Inner Layer Cell, O = Outer Layer Cell (×200).

Table 1. PCNA labelling index in relation with WHO classification

Histological type	Number of cases	PCNA labelling index	
		Mean±SD (%)	Range (%)
Basal cell ameloblastoma	3	19.58±4.53	14.70–23.68
Follicular ameloblastoma	9	17.52±4.39	6.89–23.78
Plexiform ameloblastoma	9	16.19±3.76	11.50–22.35
Acanthomatous ameloblastoma	3	16.02±4.21	12.35–22.10
Desmoplastic ameloblastoma	3	12.61±5.79	5.20–19.74
Unicystic ameloblastoma	5	12.03±6.15	4.85–21.97

Cytological pattern of the outer layer cells of ameloblastoma

In this classification, the basal cell type (Fig. 1a) had the highest labelling indices for both PCNA and Ki-67, followed by the columnar cell type (Fig. 1b,1d), and the cuboidal cell type (Fig. 1c), respectively (Table 3,4).

Mixed type was found in 16 tissue sections. In the mixed type, basal cells had the highest PCNA and Ki-67 labelling indices (data not shown).

Table 2. Ki-67 labelling index in relation with WHO classification

Histological type	Number of cases	Ki-67 labelling index	
		Mean±SD (%)	Range (%)
Basal cell ameloblastoma	3	6.80±0.82	5.54–7.37
Follicular ameloblastoma	9	5.05±1.70	1.67–7.17
Plexiform ameloblastoma	9	3.70±1.76	1.10–6.86
Acanthomatous ameloblastoma	3	3.69±1.45	1.90–6.20
Unicystic ameloblastoma	5	2.85±1.95	0.70–6.90
Desmoplastic ameloblastoma	3	2.75±1.85	1.12–5.90

Table 3. PCNA labelling index in relation with cytological pattern of the outer layer cells of ameloblastoma

Cytological pattern	Number of cases	PCNA labelling index	
		Mean±SD (%)	Range (%)
Basal cell type	3	19.92±4.16	14.94–23.68
Columnar cell type	6	15.53±2.24	12.51–18.65
Mixed type	16	14.82±4.97	6.14-18.83
Cuboidal cell type	7	11.27±4.88	4.85–18.61

Table 4. Ki-67 labelling index in relation with cytological pattern of the outer layer cells of ameloblastoma

Cytological pattern	Number of cases	Ki-67 labelling index	
		Mean±SD (%)	Range (%)
Basal cell type	3	6.81±0.77	5.32–7.37
Columnar cell type	6	4.68±1.42	2.86–6.94
Mixed type	16	4.11±1.55	0.93-6.57
Cuboidal cell type	7	2.36±1.23	0.75–3.98

Clinical appearance, tumor's location, radiographic appearance and patient's age

In relation to the clinical appearance of ameloblastomas, the solid type had the highest labelling indices for both PCNA and Ki-67, followed by the mixed type. The cystic type was the lowest (Table 5,6). The solid type were significantly higher than the cystic type for both PCNA and Ki-67 ($P<0.05$).

Table 5. PCNA labelling index in relation with clinical appearance of ameloblastoma

Clinical appearance	Number of cases	PCNA labelling index	
		Mean±SD (%)	Range (%)
Solid type	15	16.69±4.34	12.16–22.10
Mixed type	8	14.84±3.17	11.86–18.3
Cystic type	9	12.15±5.68	6.10–18.90

Table 6. Ki-67 labelling index in relation with clinical appearance of ameloblastoma

Clinical appearance	Number of cases	Ki-67 labelling index	
		Mean±SD (%)	Range (%)
Solid type	15	5.08±1.47	2.60–7.37
Mixed type	8	4.24±1.91	1.75–6.82
Cystic type	9	2.88±1.68	0.75–4.20

In regard to the tumor location and the radiographic appearance, there were no significant differences between the types (data not shown).

Interestingly, in the present study, the younger patients (⩽20 years old) had the lowest PCNA (12.51±5.39) and Ki-67 (3.07±1.77) labelling indices, followed by the middle aged patients (21-40 years old) with PCNA (13.85±5.76) and Ki-67 (3.86±1.85). The older patients (⩾41 years old) had the highest PCNA (15.94±4.91) and Ki-67 (4.43±1.93) labelling indices.

Comparison between PCNA and Ki-67 labelling indices

By using paired t-test, there was a strong correlation between PCNA and Ki-67 labelling indices (P<0.05).

DISCUSSION

There are many reports that studied the proliferating activity of various tumours, including ameloblastoma, by applying PCNA and/or Ki-67 (Nakamura *et al.*, 1994; Brown *et al.*, 1990; Funaoka *et al.*, 1996; Kim *et al.*,

1994; Li *et al.*, 1995; Ong'uti *et al.*, 1997; Piatelli *et al.*, 1998; Takahashi *et al.*, 1998; Tsuji *et al.*, 1992). Li *et al.* (1995), Funaoka *et al.* (1996) and Ong'uti *et al.* (1997) reported that follicular ameloblastoma had higher PCNA and/or Ki-67 labelling indices than plexiform ameloblastoma, on the other hand, Kim *et al.* (1994), Piatelli *et al.* (1998) and Takahashi *et al.* (1998) reported that PCNA labelling index of plexiform ameloblastoma was higher than that of follicular ameloblastoma. In the present study, PCNA and Ki-67 labelling indices of follicular ameloblastoma were higher than that of plexiform ameloblastoma, but the difference was not statistically significant. This result suggested that the follicular type and the plexiform type have little difference in proliferating activity, and it's not meaningful.

Takahashi *et al.* (1998) reported that acanthomatous ameloblastoma showed no immunoreactivity for PCNA, but Kim *et al.* (1994) and Piatelli *et al.* (1998) explicitly reported high PCNA labelling indices of acanthomatous ameloblastoma. In the present results, acanthomatous ameloblastoma showed positive staining for both PCNA and Ki-67 and its labelling indices were higher than that of desmoplastic and unicystic ameloblastomas. But the inner

layers of epithelial component in acanthomatous ameloblastoma were negative and only outer layers were stained positively. This suggested that inner layer cells of acanthomatous ameloblastoma is in a mature stage and is not actively proliferating.

According to the cytological pattern of outer layer cells of ameloblastoma, the basal cell type is assumed to be the most immature cell type and retains the character of odontogenic stem cells (Mitsuyasu *et al.*, 1997). In fact, in the present study, the basal cell type had the highest PCNA and Ki-67 labelling indices, the cuboidal cell type had the lowest and the columnar cell type was in the middle (Table 3,4). Also in the mixed type, basal cells had the highest PCNA and Ki-67 labelling indices. It may be reasonable to assume that the basal cells are the most actively proliferating and accordingly, the most immature cells in the ameloblastoma.

Clinically, unicystic ameloblastoma is believed to be less aggressive and it is successfully treated by enucleation and curettage (Leider *et al.,* 1985; Gardner, 1984). In the present study, regarding the clinical appearance, PCNA and Ki-67 labelling indices of the cystic type, not only the unicystic,

but also the multicystic ameloblastoma, were lower than that of the solid type (Table 5,6).

Radiographic examination is important for the diagnosis and postoperative assessment of ameloblastoma (Lagundoye *et al.*, 1975). Based on the clinical data, Ueno *et al.* (1989) reported that the biological behavior of ameloblastoma is related to the radiographic appearance. But in the present study, we could not find any significant result in relation to the radiographic appearance. This result suggested that the proliferating activity of ameloblastoma could not be predicted using radiographic appearance only. Although there was no significant result of the proliferating activity of ameloblastoma in relation to the tumor's location, it's still an important factor in planning the treatment of ameloblastoma (Gardner, 1984; Gardner, 1996).

When the recurrence rate was compared between the patients aged <20 years and the patients aged ⩾20 years, the younger patients had better prognosis (Ueno *et al.*, 1989). Funaoka *et al.* (1996) reported that old patients had a higher incidence of PCNA-positive cells. Our result showed clearly this relation. PCNA and Ki-67 labelling indices of younger patient (⩽20 years

old) were the lowest, followed by the middle aged patient (21-40 years old) and the older patient (≥41 years old) showed the lowest indices of PCNA and Ki-67. By further analysis, 77.8 % of the younger patients showed the cystic type and this might be the reason why younger patient had the lowest PCNA and Ki-67 labelling indices. The patient's age might be related with the proliferating activity indirectly.

PCNA and Ki-67 labelling indices showed quite same results and there was a significant relation between them. It suggested both PCNA and Ki-67 can be used as proliferating markers, and it will be better to use both to confirm the result.

In conclusion, histological pattern, cytological pattern, clinical appearance and patient's age were related to the proliferating activity of ameloblastoma. On the contrary, we can't estimate the proliferating activity of ameloblastoma by the radiographic appearance only, and also the tumor's location had no correlation with ameloblastoma's proliferating activity. The basal cell type of cytological pattern was found to be the most actively proliferating cell type. This study will be useful to understand the behavior of

ameloblastoma, and the basal cell ameloblastoma should be considered as an entity of operative assessment.

PART 2

Two relatively distinct patterns of Ameloblastoma: An anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (center)

ABSTRACT

This study was performed to disclose the apoptotic behavior of ameloblastomas by analyzing the role of Bcl-2 family proteins in ameloblastomas and the location of terminally apoptotic cells in the ameloblastoma epithelial tissues. For immunohistochemistry, tissue sections of 32 patients were treated with antigen retrieval method. Primary antibodies against the apoptosis related proteins, Bcl-2, Bcl-X, Bax, and Bak were applied. Besides Immunohistochemistry, Western blotting and TUNEL were also performed. Most of the outer layer cells were predominantly stained by

the Bcl-2, while most of the inner layer cells were stained by antibodies against the apoptosis modulating proteins, Bax and Bak. Among the Bcl-2 family, Bcl-2 was the most ubiquitously expressed protein in ameloblastomas, while Bcl-X was expressed in the greatest concentrations. The major Bcl-X protein was Bcl-X_L. Some of the inner layer cells entered the terminal apoptotic stage, which were expressed by TUNEL. The acanthomatous areas overexpressed the apoptosis modulating proteins, especially Bak. Ameloblastoma has much more apoptosis inhibiting protein than the apoptosis modulating protein. Ameloblastoma has two relatively distinct patterns, an anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (center). And the acanthomatous area, which was stained strongly by Bak and contained numerous terminally apoptotic cells was suggested as a differentiated area.

INTRODUCTION

An ameloblastoma is a slow growing, non-metastatic, but highly recurrent tumor (Lucas, 1984). Ameloblastoma cells have various proliferating activities depending on the histological type and cytological pattern (Li *et al.*, 1995; Sandra *et al.*, 2001). Both apoptosis and the proliferating activity of the cell are implicated in the development of ameloblastoma (Kumamoto, 1997). Bcl-2 family proteins, Bcl-2, Bcl-X, Bax and Bak have been widely used to analyze the apoptotic behavior of various tumors and diseases (Pena *et al.*, 1999; Ravi *et al.*, 1999). It has been reported that these Bcl-2 family proteins are also expressed in changing patterns during critical stages of tooth morphogenesis (Krajewski *et al.*, 1998). Accordingly, it should be reasonable to study the expressions of these apoptosis related proteins in ameloblastoma, since the ameloblastoma is thought to arise from the epithelium of the odontogenic apparatus, or its remnant tissues (Kramer *et al.*, 1992).

In a previous investigation, we examined the expression of Bcl-2 in ameloblastoma (Mitsuyasu *et al.*, 1997). However, since other Bcl-2 family

proteins are expressed in different patterns and regulate cell life at different stages of cell differentiation (Krajewski *et al.*, 1994), it was considered to be important to analyze the expression pattern of these proteins in ameloblastoma. Accordingly, in the present study, we analyzed the apoptotic behavior of ameloblastoma using antibodies against Bcl-2, Bcl-X, Bax and Bak proteins in relation to the histological type and cytological pattern. Also, terminally apoptotic cells were studied by direct immunoperoxidase detection of degoxigenin-labeled genomic DNA.

MATERIALS AND METHODS

Tissue sample selection

We selected ameloblastoma tissue blocks from 32 patients, 20 males and 12 females, who had been operated on at our hospital. Fresh ameloblastoma tissues were fixed for 24-48 hours in 4% formaldehyde freshly prepared from paraformaldehyde in PBS at 4°C.

Twenty-seven ameloblastomas were taken from the mandible (84.4%) and five were from the maxilla (15.6%). The patients' age ranged from 12 to 81 years old (mean: 38.2 years old). When the tumors were classified according to the World Health Organization International, Histological Typing of Odontogenic Tumors, 2nd Ed. (Kramer *et al.*, 1992), there were 9 follicular, 9 plexiform, 5 unicystic, 3 basal cell, 3 acanthomatous and 3 desmoplastic ameloblastomas. Tissue sections, 4 μm thickness, were mounted on 3-aminopropyltriethoxy-silane coated glass slides.

Immunohistochemistry

For immunohistochemistry, an antigen retrieval method was employed. Tissue sections were deparaffinized and boiled at 121°C for 10 min in a solution of freshly prepared 10mM citrate buffer pH 6.0 by using an autoclave. After cooling to room temperature, the tissue sections were rinsed with phosphate buffered saline (PBS) (5 min x 3).

The tissue sections were incubated with 0.3% hydrogen peroxide for 20 min, to quench endogenous peroxidase, and 10% normal donkey serum

was applied for 20 min to diminish nonspecific antibody binding. The primary antibodies used were mouse monoclonal antibody to Bcl-2 (100/D5, novocastra, Newcastle, UK), diluted 1:50, rabbit polyclonal antibody to Bcl-X (Dako, Carpiteria, CA, USA), rabbit polyclonal antibody to Bax (Dako, Carpiteria, CA, USA) and goat polyclonal antibody to Bak (N-20, Santa Cruz Biotechnology, USA) all at 1:100 dilution. The tissue sections were incubated with primary antibodies overnight in a moist chamber at 4°C.

Normal oral mucosae were included as positive controls. Negative controls were provided by substituting PBS for the primary antibodies. In each step, samples were washed with PBS.

The tissue sections were labelled with a streptavidin-biotin method using DAKO-LSAB Kit (LSAB2 Kit Peroxidase, Dako, Carpiteria, CA, USA), excepting the tissue section for Bak, which was treated with 1:200 diluted, biotin-conjugated affinity-purified secondary antibody (Chemicon, Temecula, CA, USA). The tissue reactions were visualized with diaminobenzidine (DAB, Histofine, Nichirei, Tokyo, Japan). Finally, tissue sections were counterstained with hematoxylin and cover slipped.

TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick end-labelling index) method

The TUNEL assays were performed according to the protocol attached to the Apop Tag Plus in situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD). Briefly, tissue sections were deparaffinized. Twenty μg/ml proteinase K (Wako, Osaka, Japan) was applied for 15 min to digest protein in the tissue sections. Then, tissue sections were incubated in 3% hydrogen peroxide in PBS for 5 min and immersed in equilibration buffer for 15 seconds. Working strength TdT enzyme was applied to the tissue sections for 1 hour at 37°C in a humidified chamber. After applying antidegoxigenin-peroxidase for 30 min in a humidified chamber, tissue sections were immersed in DAB substrate working solution for 5 min. Finally, tissue sections were counter-stained with methyl green. Positive and negative controls were provided according to the protocol.

Western blotting

Fourteen fresh ameloblastoma tissues were obtained and immediately

frozen in the liquid nitrogen. The Hela cell line and the ameloblastoma tissues were treated with lysis buffer containing 50mM Hepes (pH: 7.2), 0.5M NaCl, 5mM EDTA, 20mM 2-Mercaptoethanol, Aprotinin (Sigma, Steinheim, Germany), Leupeptin (Sigma, St. Louis, MO, USA), Pepstatin A (Sigma, Steinheim, Germany) and *p*-Amidinophenyl methanesulfonyl Fluoride Hydrochloride (*p*-PMSF, Wako, Osaka, Japan), and shattered mechanically. 0.2% Triton X-100 and 0.1% Sodium Dodecylsulfate (SDS) were also added in to the mixture. The proteins were separated using a 15% SDS-polyacrylamide gel and transferred to a Polyvinylidene difluoride (PVDF) sheet. After blocking with 5% dried milk solution, the sheets were incubated with the following primary antibodies; 1:75 diluted mouse monoclonal antibody to Bcl-2 (100/D5, Novocastra, Newcastle, UK), 1:200 diluted rabbit polyclonal antibody to Bcl-X (Dako, Carpiteria, CA, USA), 1:500 diluted rabbit polyclonal antibody to Bax (Dako, Carpiteria, CA, USA) and 1:200 diluted goat polyclonal antibody to Bak (N-20, Santa Cruz Biotechnology, USA). Biotinylated sheep anti-mouse IgG (Amersham, Buckinghamshire, UK) or biotinylated donkey anti-rabbit IgG (Amersham, Buckinghamshire,

UK) or biotin-conjugated affinity-purified secondary antibody (Chemicon, Temecula, CA, USA) were applied as the secondary antibodies. The bound antibodies were visualized by the alkaline phosphatase reaction using Vectastain ABC Kit (Vector, Burlingame, CA, USA).

Evaluation and statistical analysis

The expressions of immunohistochemistry and TUNEL were analyzed as follows: at least 1200 cells were counted for each tissue section using a ×40 objective and an eye-piece graticule. The counted areas were randomly selected and counted by 2 persons.

The percentage of positive cells was evaluated in relation to WHO histological classification and cytological pattern. Data were analyzed using the Mann-Whitney U Tests and P values of <0.05 were considered to be statistically significant.

RESULTS

Immunohistochemistry

The numbers of positive and negative cases are shown in Table 1. Almost all tissue sections were stained by anti-Bcl-2 antibody. Bcl-2 was detected mainly in the outer layers of epithelial tissues in ameloblastomas, and only a few cells were positively stained in the inner layers (Fig.1a). Anti-apoptotic index of the outer layer cells was significantly higher than that of the inner layer cells ($P<0.05$) (Table 2).

Table 1. The positive and negative results of immunohistochemistry and TUNEL.

Staining	Bcl-2	Bcl-X	Bax	Bak	TUNEL
Positive	31	26	24	23	25
Negative	1	6	8	9	7

Table 2. The averages of anti-apoptotic (Bcl-2 & Bcl-X) and apoptotic (Bax, Bak, TUNEL) indices. OLC: Outer Layer Cell, ILC: Inner Layer Cell.

Antibodies	Average ± SD (%)	
	OLC	ILC
Bcl-2	41.95 ± 7.91	11.03 ± 1.34
Bcl-X	46.98 ± 8.73	33.85 ± 6.32
Bax	31.25 ± 6.15	39.32 ± 8.14
Bak	14.33 ±2.97	32.93 ± 6.17
TUNEL	0.28 ± 0.06	0.70 ± 0.14

Bcl-X and Bax were dispersed rather equally in the outer and inner layers (Fig.1b, Fig.1c, Table 2). Bak was more highly expressed in the inner layer cells (Fig.1d), and the apoptotic index of inner layers was significantly higher than that of the outer layers ($P<0.05$) (Table 2). Acanthomatous areas were densely stained by anti-Bak antibody (Fig.1d). Bcl-X had the highest anti-apoptotic index in the outer layer cells, while Bax had the highest apoptotic index in the inner layer cells.

There was no significant correlation between the apoptotic index and the histological typing of the ameloblastomas. The ratios of anti-apoptotic to apoptotic index in Table 3 showed that apoptosis was inhibited in the outer layer, but was dominant in the inner layer.

Table 3. The ratio of apoptotic inhibitor (Bcl-2, Bcl-X) and apoptotic modulator (Bax & Bak). OLC: Outer Layer Cell, ILC: Inner Layer Cell.

Ratio of Anti-apoptotic /Apoptotic Index	OLC	ILC
Bcl-2 / Bax	1.3	0.26
Bcl-2 / Bak	2.93	0.34
Bcl-X / Bax	1.46	0.78
Bcl-X / Bak	3.28	1.03

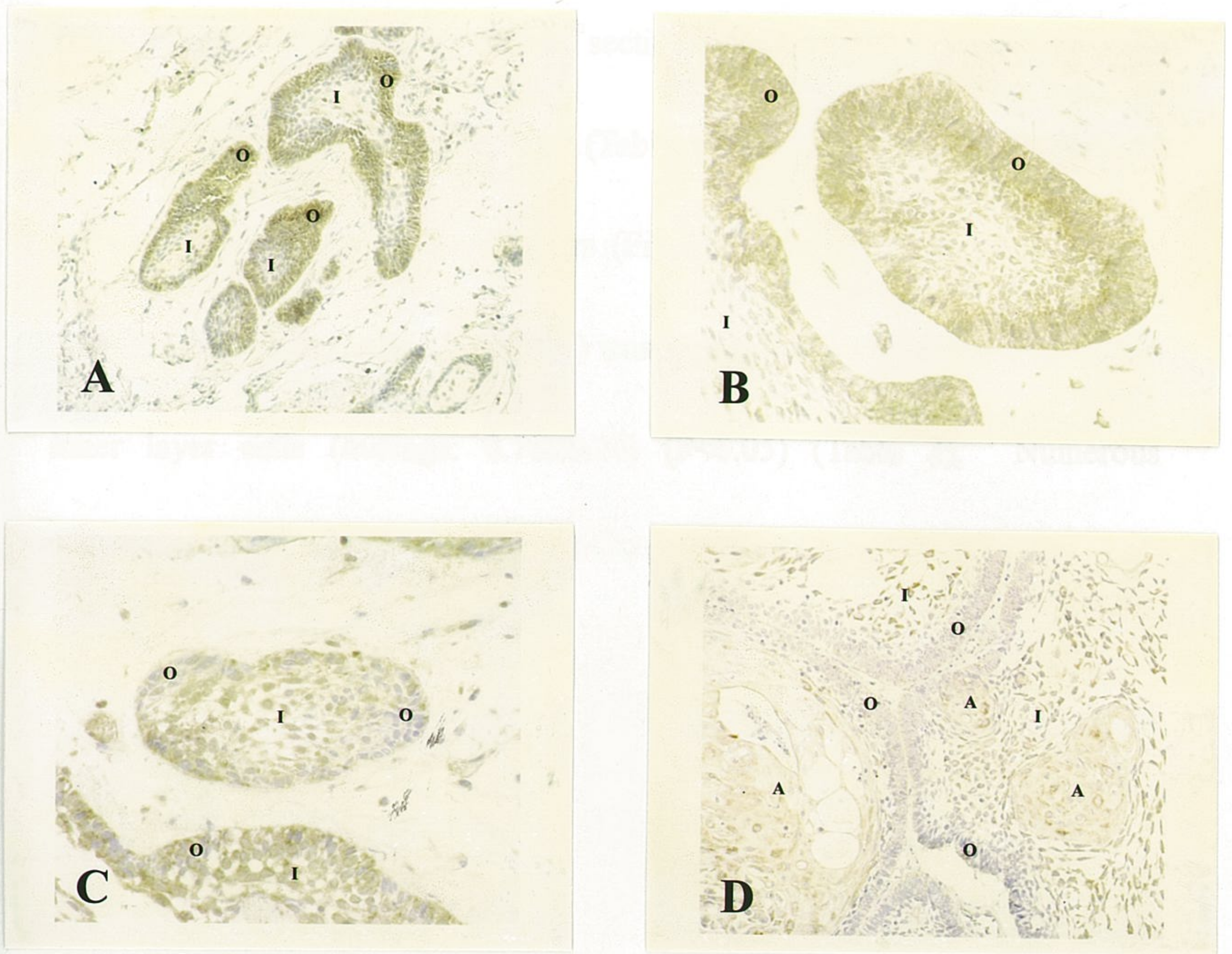


Figure 1. Immunohistochemical expression of ameloblastomas. Bcl-2 (a) (%150) and Bcl-X (b) (%300) were stained more in the outer layer cells; Bax (c) (%300) and Bak (d) (%150) were stained more in the inner layer cells. I: inner layer cells, O: outer layer cells, A: acanthomatous area.

TUNEL

Twenty five of 32 tissue sections were positively stained, but terminally apoptotic cells were few (Table 1,2). Terminally apoptotic cells were seen mostly in the inner layers (Fig.2a). The apoptotic index of the outer layer cells (average: 0.28±0.06) was significantly lower than that of the inner layer cells (average: 0.70±0.14) ($P<0.05$) (Table 2). Numerous terminally apoptotic cells were seen in the acanthomatous areas (Fig.2b).

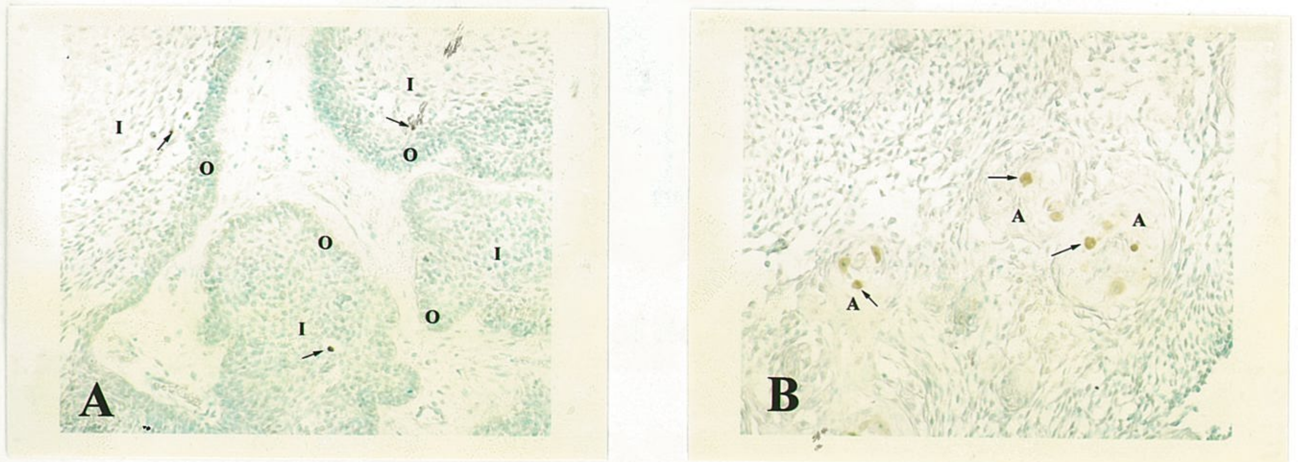


Figure 2. The distributions of terminally apoptotic cells (TUNEL) were mostly in the inner layers of parenchymal cells in ameloblastomas (a) and the acanthomatous areas (b) (arrow) (%150). I: inner layer cells, O: outer layer cells, A: acanthomatous area.

Western blotting

Both ameloblastoma tissue samples (Fig.3) and Hela cell line expressed positive bands with reasonable molecular weights for Bcl-2, Bcl-X, Bax, and Bak, in western blotting. Regarding the Bcl-X results, the 31 kDa band of Bcl-X_L was seen in all samples, while the 20 kDa band of Bcl-X_S was seen in only one sample.

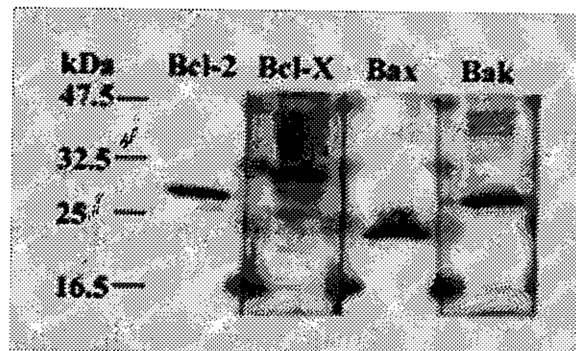


Figure 3. The western blotting result of Bcl-2, Bcl-X, Bax and Bak in tissue sample, and for Bcl-X results, only 31 kDa band of Bcl-X_L was seen.

DISCUSSION

Just as individual cells balance anabolic and catabolic reactions, multicellular organisms must equalize the rates of cell generation and cell death to maintain a constant size. An organism must also remove senescent, damaged, or abnormal cells that could interfere with organ function or develop into tumors (Carson *et al.*, 1993).

The family of Bcl-2 related proteins constitutes one of the biologically most relevant classes of apoptosis-regulatory gene products, acting at the effector stage of apoptosis (Kroemer, 1997). Bcl-2, a 26 kDa protein, was first identified in non-Hodgkin's follicular B cell lymphomas, encoded by the Bcl-2 oncogene in the molecular analysis of the t(14;18) chromosome translocation. The Bcl-2 gene has emerged as a critical regulator of programmed cell death in a variety of physiological and pathological contexts (Reed, 1994). Immunoreactivity for the Bcl-2 product is present mainly in cell populations, which are long lived, and/or with high proliferation ability

(Hockenberry *et al.*, 1991). In the present study, Bcl-2 was seen mainly in the outer layers of ameloblastoma. Among the Bcl-2 family proteins tested, Bcl-2 was most prevalently seen in the ameloblastoma tissues studied (only one case was negative), suggesting that most of the ameloblastoma tissues contained Bcl-2 protein.

Bcl-X, a Bcl-2 related gene that can function as a Bcl-2 independent regulator of programmed cell death (apoptosis), encodes two distinct protein products, Bcl-X_L and Bcl-X_S. Bcl-X_L, inhibits cell death (apoptosis), while Bcl-X_S inhibits the ability of Bcl-2 to enhance the survival of the growth factor-deprived cells (Boise *et al.*, 1993). Bcl-2 and Bcl-X differ in their ability to associate with at least one proapoptotic family member (Yang *et al.*, 1995), and are likely to have other alterations in cell cycle control pathways. In the present study, Bcl-X stained more strongly than Bcl-2 and its average anti-apoptotic index was higher than that of Bcl-2 (Table 2). In western blotting analysis, only the 31 kDa band of Bcl-X_L was detected in the ameloblastoma tissues, except for one sample in which the 20 kDa band of Bcl-X_S was seen. It suggested that the major protein of Bcl-X detected in this

study was the anti-apoptotic protein, Bcl-X_L.

Bax shows extensive amino acid homology with Bcl-2 and forms homodimers and heterodimers with Bcl-2 in vivo. The ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus. When Bax predominates, programmed cell death is accelerated and the death repressor activity of Bcl-2 is countered (Oltvai *et al.*, 1993). In the present study, the ratio of Bcl-2 to Bax and the ratio of Bcl-X to Bax were significantly higher for the outer layer cells than for the inner layer cells (Table 3), suggesting that the outer layer cells have the characteristic of apoptotic inhibition, while the inner layer cells were undergoing apoptosis.

The molecular mechanism by which Bak induces apoptosis is likely to involve the same pathway influenced by its relatives, Bcl-2, Bcl-X and Bax (Chittenden *et al.*, 1995). Bcl-2 and Bcl-X might suppress apoptosis by inhibiting the proapoptotic function of Bak and/or Bax (Chittenden *et al.*, 1995). In the present study, the ratio of Bcl-2 to Bak and that of Bcl-X to Bak were much higher in the outer layers than in the inner layers, indicating that Bak did not counter the Bcl-2 and Bcl-X in the outer layers. Expression of

Bak was much higher in the inner layers, especially, in the acanthomatous ameloblastoma.

Additionally we couldn't find any significant difference in anti-apoptotic and apoptotic indices between the ameloblastoma from maxilla and the ameloblastoma from mandible.

Our present results indicate conclusively that ameloblastoma has much more apoptosis inhibiting protein than the apoptosis modulating protein, considering that Bcl-2 was found in almost all samples and Bcl-X_L was the major protein of the Bcl-X, which had the highest labelling index. This suggested that the ameloblastoma might have a high survival activity and this also might be one of the reasons why ameloblastoma has a high recurrence rate. In conclusion, ameloblastoma has two relatively distinct patterns, an anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (center). The results are compatible with our previous report that the stem cells are maintained in the outer/peripheral layers of the tumor nest from which the proliferating cells are recruited (Sandra *et al.*, 2001; Mitsuyasu *et al.*, 1997). Acanthomatous area,

which was stained strongly by Bak and contained numerous terminally apoptotic cells, was suggested as a differentiated area.

PART 3

The role of MDM2 in the proliferative activity of ameloblastoma

ABSTRACT

Ameloblastoma is a unique tumor in oral and maxillofacial region with various levels of proliferative activity in each type. In order to determine whether p53 and MDM2 contribute to the proliferative activity of ameloblastoma, we performed the following study. The p53 tumor suppressor gene is the most commonly mutated gene in the human cancer and additionally, it is overexpressed in other lesions, such as ameloblastoma. The MDM2 protein is able to physically associate with p53 and block it's growth suppressive functions. In the present study, MDM2 was immunohistochemically overexpressed in ameloblastoma and the positive cell counting results showed different MDM2 labeling index for each type of

ameloblastoma based on both WHO classification and cytological pattern. Basal cell ameloblastoma, which has a high proliferative activity, had the highest MDM2 labeling index. We suggested that the MDM2 protein caused the high proliferative activity of ameloblastoma, particularly in basal cell ameloblastoma. MDM2 could be considered a target for ameloblastoma treatment.

INTRODUCTION

Ameloblastoma has a high recurrence rate and locally invasive (Lucas, 1984; Weiss *et al.*, 1985). It is a unique tumor, because each type of ameloblastomas has various levels of proliferative activity (Sandra *et al.*, 2001). All types of ameloblastoma have similarly high anti-apoptotic activity, suggesting that ameloblastomas have a high survival activity (Sandra *et al.*, in press).

The p53 protein, the most intensively studied protein in oncology, and a potent inhibitor of cell growth, is responsible for arresting the cell cycle at several distinct points, as well as in activating the apoptotic machinery leading to cell death in some circumstances (Levine, 1997; Ashcroft *et al.*, 1999; Lakin *et al.*, 1999). Activation of p53 can occur in response to a number of cellular stresses, including DNA damage, hypoxia and nucleotide deprivation (Lakin *et al.*, 1999). More than 2000 different p53 mutations have been discovered and more than half of all human tumours completely lack functional p53 as a result of the occurrence of a point mutation in one

allele coupled with a complete of the other allele (Lane, 1999).

MDM2 (Murine Double Minute 2), a negative regulator of p53, binds the amino terminal transactivation domain of p53. Overexpression of MDM2 protein has been detected in a number of diverse human malignancies, indicating that this oncogene plays a key role in human carcinogenesis (Momand *et al.*, 2000; Lozano *et al.*, 1998; Juven-Gershon *et al.*, 1999; Freedman *et al.*, 1999; Haines, 1997; Piette *et al.*, 1997; Yap *et al.*, 1999; Zhang *et al.*, 2000). It also has been suggested that MDM2 levels are associated with a poor prognosis of several human cancers (Zhang *et al.*, 2000).

In order to determine whether p53 and its negative regulator, MDM2, contribute to the proliferative diversity observed in ameloblastomas, we performed the following study. Several reports mentioned the immunohistochemical result with p53 could not be trusted (Wynford-Thomas, 1992; Battifora, 1994; Hall *et al.*, 1994), therefore we also performed western blotting for confirmation. To check the possibility of the existence of p53 mutation, we evaluated the present of p53 mutation in ameloblastoma tissues.

MATERIALS AND METHODS

Tissue sample selection

The ameloblastoma tissue blocks from 34 patients (20 males and 14 females), that had been obtained surgically at these facilities were selected. The resected tissues were fixed for 24-48 hours in 4% formaldehyde freshly prepared from paraformaldehyde in PBS at 4°C. Twenty-eight ameloblastomas were taken from the mandible (82.4%) and 6 were taken from the maxilla (17.6%). Based on the World Health Organization International, Histological Typing of Odontogenic Tumors, 2nd Ed. (Kramer *et al.*, 1992), 9 were follicular, 9 were plexiform, 5 were unicystic, 4 were acanthomatous, 4 were desmoplastic and 3 were basal ameloblastomas. Based on the cytological pattern, 3 were basal cell type, 7 were columnar cell type, 16 were mixed type and 8 were cuboidal cell type. Tissue sections sliced in 4 μm thickness were mounted on 3-aminopropyltriethoxy-silane coated glass slides. The next step was proceeded to the immunohistochemistry.

For western blotting, fresh ameloblastoma tissues of 14 patients (8 males and 6 females) were frozen immediately in the liquid nitrogen after resection. Ten ameloblastomas were taken from the mandible (71.4%) and 4 ameloblastomas were from the maxilla (28.6%). Based on the WHO classification, 3 were follicular, 3 were plexiform, 2 were unicystic, 2 were acanthomatous, 2 were basal and 2 were desmoplastic ameloblastomas. Based on the cytological pattern, 2 were basal cell type, 4 were columnar cell type, 5 were mixed typed and 3 were cuboidal cell type.

Immunohistochemistry

All tissue sections were deparaffinized and preheated for antigen retrieval. After cooling to room temperature, tissue sections were rinsed in phosphate buffered saline (PBS) (5 min × 3), and incubated with 0.3 % hydrogen peroxide for 20 min. Tissue sections were then rinsed in PBS, and 10 % normal goat serum was applied for 20 min. Mouse monoclonal anti-p53 antibody (diluted 1:50) (DO-7, Novocastra, Newcastle, UK) and mouse monoclonal anti-MDM2 antibody (diluted 1:100) (1B10, Novocastra,

Newcastle, UK) were applied overnight in a moist chamber at 4°C. Tissue sections of oral squamous cell carcinoma were stained as positive controls. Negative controls were provided by substituting PBS for the first antibodies. In each step, samples were washed with PBS.

The tissue sections were labeled with a streptavidin-biotin method using DAKO-LSAB Kit (LSAB2 Kit Peroxidase, Dako, Carpiteria, CA, USA) and visualized with diaminobenzidine (DAB, Histofine, Nichirei, Tokyo, Japan). Finally, tissue sections were counterstained with hematoxylin and coverslipped. One thousand cells were counted for each tissue section using ×40 objective and an eyepiece graticule. The counted areas were randomly selected and counted by 2 people. The average and standard deviation (SD) were calculated. Index = positive cells/1000 × 100%. The results were related to WHO histological classification and cytological pattern of outer layer cells. The Mann-Whitney Test was done and P values of <0.05 were considered statistically significant.

Western blotting

Fresh ameloblastoma tissues from 14 cases and the 431 cell line were treated with lysis buffer containing 50 mM Hepes/NaOH (pH 7.2), 0.5 M NaCl, 5 mM EDTA, 20 mM 2-Mercaptoethanol, Aprotinin (Sigma, Steinheim, Germany), Leupeptin (Sigma, St. Louis, MO, USA), Pepstatin A (Sigma, Steinheim, Germany) and *p*-Amidinophenyl methanesulfonyl Fluoride Hydrochloride (*p*-PMSF, Wako, Osaka, Japan), and shattered mechanically. 0.2% Triton X-100 and 0.1% Sodium Dodecylsulfate (SDS) were also added in to the mixture. The proteins were separated using a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% dried milk solution, the sheets were incubated with the following first antibodies: mouse monoclonal anti-p53 antibody (diluted 1:500) (DO7, Novocastra, Newcastle, UK) and mouse monoclonal anti-MDM2 antibody (diluted 1:50) (IF2, Oncogene, Cambridge, MA, USA). Biotinylated sheep anti-mouse IgG (diluted 1:500) (Amersham, Buckinghamshire, UK) was applied as the secondary antibodies. The bound antibodies were visualized by the alkaline phosphatase reaction using

Vectastain ABC Kit (Vector, Burlingame, CA, USA).

ELISA

The ELISA was performed according to the protocol attached to the p53 Mutant Selective Quantitative ELISA (Oncogene, Cambridge, MA). Briefly, the whole cell extract from A431 cells was done using swelling buffer (20 mM Tris/HCl, 5mM EDTA, 1 mM *p*-Amidinophenyl methanesulfonyl Fluoride Hydrochloride (*p*-PMSF, Wako, Osaka, Japan), pH 8). Fourteen fresh ameloblastoma tissues obtained from the operating room were immediately frozen in the liquid nitrogen and extracted using the same composition lysis buffer in the western blotting. The samples (cell lines and ameloblastoma extracts) and the p53 standards were applied into the coated wells and incubated at 4°C. Then the p53 reporter antibody was added into each well and incubated at room temperature for 2 hours. The peroxidase conjugate was then added into each well and incubated for 1 hour. Thirty minutes after applying the mixture of substrate A and B, the absorbance of each well was read on a spectrophotometric plate reader at a wavelength of

405 nm. In all steps, the wash buffer was used to rinse each well.

RESULTS

Immunohistochemistry

P53 (Fig. 1a) and MDM2 (Fig. 1b, 1c, 1d) stained within the nuclei of cells. Twenty-six samples (77%) were positively stained by anti-p53 antibody and 30 samples (88%) were positively stained by anti-MDM2 antibody. The average p53 labeling index was 2.61±1.74 and the average of MDM2 labeling index was 6.92±1.72. There was no significant difference in p53 labeling indices of each type of ameloblastomas, based on the WHO classification and cytological pattern (data not shown).

Based on the WHO classification, the MDM2 labeling index of basal cell ameloblastoma was seen to be the highest, followed by the MDM2 labeling indices of follicular ameloblastoma, plexiform ameloblastoma, acanthomatous ameloblastoma, desmoplastic ameloblastoma and unicystic

ameloblastoma respectively (Table 1). The MDM2 labeling index of basal cell ameloblastoma was significantly ($p<0.05$) higher than those of acanthomatous, desmoplastic and unicystic ameloblastomas.

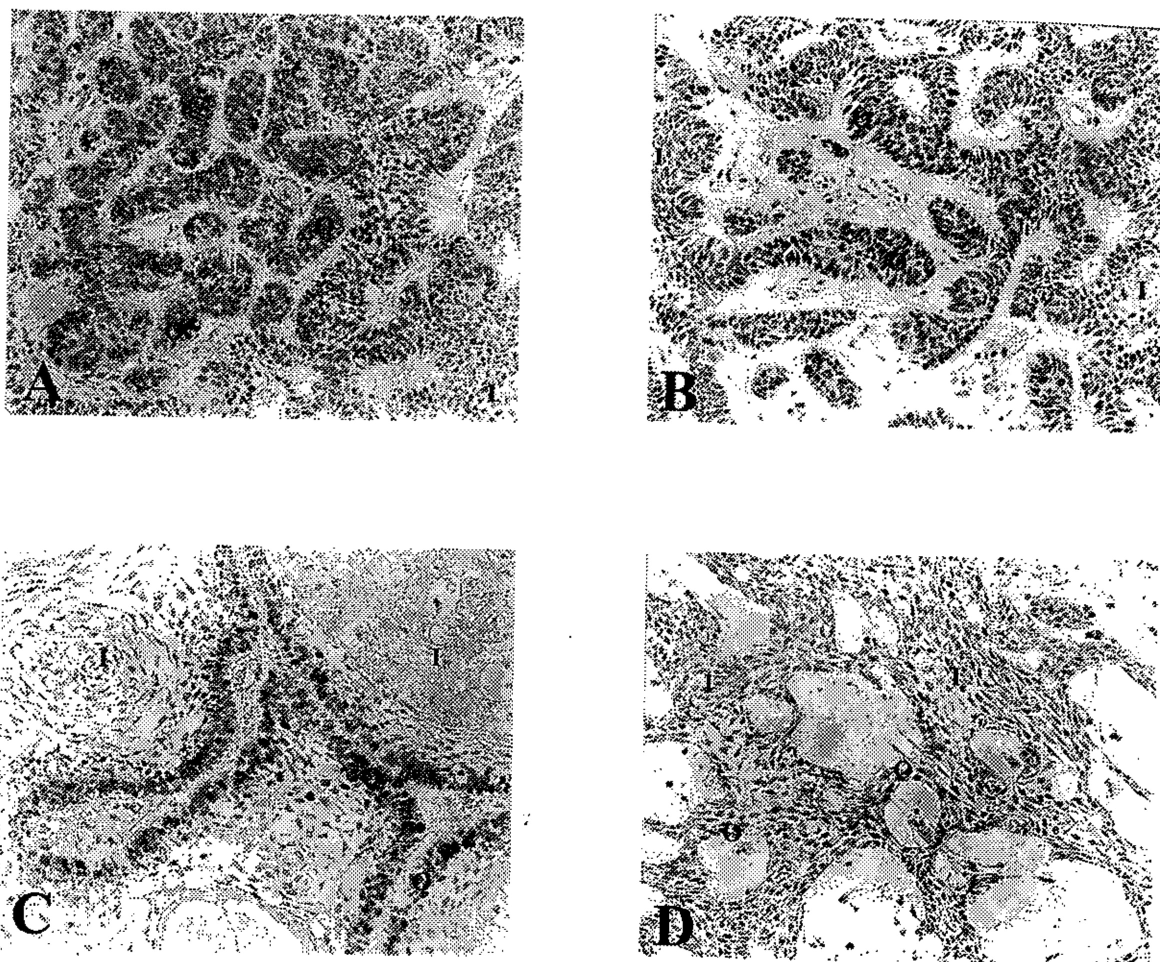


Fig. 1. P53 expression in the basal cell type (a) and MDM2 expressions in the basal cell (b), columnar cell (c) and cuboidal cell (d) type (cytological pattern of outer layer) of ameloblastoma. I: inner layer, O: outer layer. (%150).

Table 1. MDM2 labeling index in the relation with WHO classification

Histological type	Number of cases	MDM2 labeling index	
		Mean±SD (%)	Range (%)
Basal cell ameloblastoma	3	12.60±1.82	9.14-14.55
Follicular ameloblastoma	7	7.29±1.69	2.55–10.86
Plexiform ameloblastoma	7	6.32±2.24	2.06–10.14
Acanthomatous ameloblastoma	4	5.03±1.97	1.56–7.75
Desmoplastic ameloblastoma	5	3.31±1.88	0.79–6.78
Unicystic ameloblastoma	4	2.15±1.73	1.30–5.58

Based on the cytological pattern, the MDM2 labeling index of basal cell type was the highest, followed by the MDM2 labeling indices of columnar, mixed and cuboidal cell type respectively (Table 2). MDM2 labeling index of basal cell type was significantly ($p<0.05$) higher than that of cuboidal cell type.

Table 2. MDM2 labeling index in relation with cytological pattern of the outer layer cells of ameloblastoma.

Cytological pattern	Number of cases	MDM2 labeling index	
		Mean±SD (%)	Range (%)
Basal cell type	3	12.95±1.47	9.43–14.55
Columnar cell type	8	7.16±1.39	2.49–10.96
Mixed type	12	6.13±1.87	3.13-9.88
Cuboidal cell type	7	5.78±1.21	1.30–7.28

Western blotting

Twelve cases (86%) showed the weak band of p53 and all cases showed the MDM2 band. The thick bands of A431 cell line were shown for both p53 and MDM2 as the positive controls (Fig. 2).

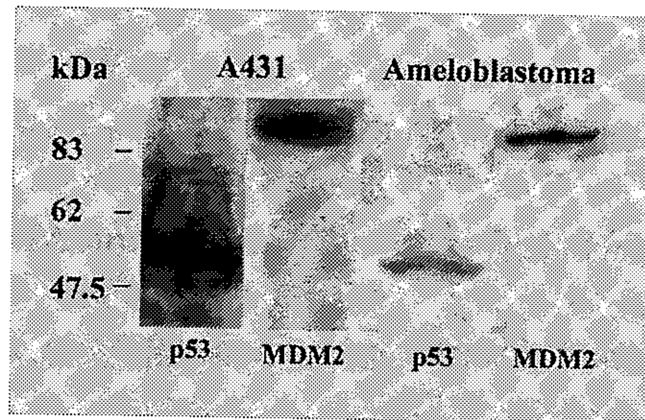


Fig. 2. Western blotting expressions of p53 and MDM2 in the A431 cell line and ameloblastoma.

ELISA

Only 2 cases were positive for p53 mutations (14%). Furthermore the amount of p53 in these cases were very low (0.33 ng/ml and 0.25 ng/ml), compared to the amount of p53 present in the A431 cell extract (3.48 ng/ml).

DISCUSSION

Several papers reported on the expression of p53 and/or MDM2 in malignant tumors (Agarwal *et al.*, 1999; Matsumura *et al.*, 1996; Yoneda *et al.*, 1998; Yao *et al.*, 1999) and benign tumors (Carvalhais *et al.*, 1999; Slootweg, 1995) of oral and maxillofacial region. Carvalhais *et al.* (1999) and Slootweg (1995) reported that p53 and MDM2 were specific markers to identify the proliferative activity and tumor aggressiveness and that p53 and MDM2 may be related to the odontogenic lesions.

Slootweg *et al.* (1995) reported a similar distribution and level of p53 and Ki-67, and that p53 expression was related to the proliferation in odontogenic epithelial lesions. Using the proliferating markers, PCNA and Ki-67 (Sandra *et al.*, 2001), our group previously reported that each type of ameloblastomas had differences in proliferative activity, however, in the present study, we couldn't find a significant difference in the p53 labeling index in each type of ameloblastoma, whether based on the WHO classification or the cytological pattern.

Since the results of immunohistochemistry of p53 were doubt (Wynford-Thomas, 1992; Battifora, 1994; Hall *et al.*, 1994), even though the anti-p53 antibody used was specific, we confirmed its expression by western blotting. To test whether the stained cells contained p53 mutations, we performed the p53 mutant selective test. With this test, we found that in most of the stained cells, p53 was wild type. These results suggest that the presence or absence of p53 mutations was not the underlying cause of differential proliferative activities of ameloblastomas.

MDM2, which was reported to be an inhibitor of tumor suppressor function of p53 (Momand *et al.*, 2000; Lozano *et al.*, 1998; Juven-Gershon *et al.*, 1999; Freedman *et al.*, 1999; Haines, 1997; Piette *et al.*, 1997; Yap *et al.*, 1999; Zhang *et al.*, 2000), was examined in the present study. In addition to the p53 protein, MDM2 protein interacts with many additional proteins, such as pRB and E1F2, important factors involved in the G1/S phase transition of the cell cycle (Momand *et al.*, 2000; Lozano *et al.*, 1998; Juven-Gershon *et al.*, 1999; Freedman *et al.*, 1999; Yap *et al.*, 1999; Zhang *et al.*, 2000). Agarwal, *et al.* (1999) and Matsumura *et al.* (1996) reported that oral premalignant and

malignant tumors differed in their MDM2 expression levels. In the present study, we found that MDM2 labeling indices were related to the WHO classification and the cytological pattern, and that most of the MDM2 positive cells were seen in the outer layer, similar to the PCNA and Ki-67 expression patterns (Sandra *et al.*, 2001). The basal cell ameloblastoma that showed the highest MDM2 labeling index was suggested to be more progressive than others.

Several methods to block the MDM2 expression and activate wild type p53 were reported, such as blocking the interaction between MDM2 and p53, lowering the levels of MDM2 and blocking the nuclear-cytoplasmic shuttling of MDM2 (Momand *et al.*, 2000; Freedman *et al.*, 1999; Zhang *et al.*, 2000). These gene therapies could be suggested as one possible alternative for the treatment of ameloblastoma in which MDM2 was found to be overexpressed.

CONCLUSION

1. Histological pattern, cytological pattern, clinical appearance and patient's age were related to the proliferating activity of ameloblastoma.
2. The radiographic appearance and the tumor's location had no correlation with ameloblastoma's proliferating activity.
3. The basal cell type of cytological pattern was found to be the most actively proliferating and the most immature cell type.
4. Most of the ameloblastoma tissues contained Bcl-2 protein.
5. The major protein of Bcl-X detected in this study was the anti-apoptotic protein, Bcl-X_L.
6. The outer layer cells have the characteristic of apoptotic inhibition, while the inner layer cells were undergoing apoptosis
7. Acanthomatous area, which was stained strongly by Bak and contained numerous terminally apoptotic cells, was suggested to enter the differentiated stage.
8. p53 was detected in the ameloblastoma tissue and most of the p53 stained cells was the p53 wild type.

9. MDM2 labeling indices were related to the WHO classification and the cytological pattern, and that most of the MDM2 positive cells were seen in the outer layer.

10. The basal cell ameloblastoma that showed the highest MDM2 labeling index was suggested to be more progressive than others.

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