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Differential expression of apoptosis-related proteins in various cellular components of ameloblastomas

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Abstract. To evaluate the expression patterns of apoptosis-related proteins, including Fas, Fas-ligand (FasL), caspase-3 and Bcl-2, in various cellular components of ameloblastomas, 39 cases of ameloblastoma were examined using immunohistochemistry. The staining intensity of the antigens in the 4 types of tumour cellular component, peripheral basal cells of tumour nests, central stellate reticulum-like cells, and foci of squamous and granular cells, was scored using a semi-quantitative scale, and comparisons were made by statistical analysis. Expression of Fas, FasL and caspase-3 was detected in the majority of cases, with a similar pattern of strong staining in the foci of squamous metaplasia and granular cells usually situated in the central area of tumour islands. In contrast, expression of Bcl-2 was predominantly seen in the peripheral basal cell layer. There were significant differences in the staining intensity of Fas, caspase-3 and Bcl-2 among the 4 types of tumour cell. The differential expression of apoptosis-related proteins in various cellular components of ameloblastomas, with pro-apoptotic proteins, Fas, FasL and caspase-3 being closely associated with squamous metaplasia and granular transformation of the tumour cells, suggests that Fas/FasL-induced apoptotic cell death may play a role in the disposal of terminally differentiated or degenerative tumour cells in ameloblastomas.

Key words: ameloblastoma; apoptosis; immunohistochemistry.

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The ameloblastoma is a benign but locally aggressive neoplasm derived from odontogenic epithelium. Histologically, there are 2 main patterns, follicular and plexiform, in which various cellular changes such as squamous metaplasia and granular transformation are commonly seen, and when these are extensive, the terms acanthomatous type and granular cell type are applied, respectively. Cyst formation within the epithelial islands of the follicular type is common. Other rare histological variants, including basal cell ameloblastoma, desmoplastic ameloblastoma and keratoameloblastoma, have also been described[5]. Despite numerous studies, correlation between these histological patterns and tumour behaviour has not been consistently established. The mechanisms of oncogenesis, cytodifferentiation and tumour progression of ameloblastoma are still largely unknown[2,12,18].

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In multicellular organisms, the ability to regulate cell death to the same extent to cell growth and differentiation is vital. Programmed cell death mainly proceeds by apoptosis, a tightly controlled process to remove unwanted cells efficiently with characteristic cytoplasmic and nuclear condensation and DNA fragmentation. Apoptosis is also a fundamental process in tumour cell kinetics. It is believed that tumour cell growth results from a disturbance in the balance between the rate of proliferation and cell death, and therefore mutations affecting genes that are either inducers or repressors of apoptosis may be common occurrences during the development of neoplasms[13]. Among the various apoptotic pathways, the Fas/FasL system plays a key role. FasL, a cell-surface molecule belonging to the tumour necrosis factor family, binds to its receptor Fas, a member of the tumour necrosis factor receptor family. This triggers a series of intracellular events leading to the activation of caspases that execute the apoptosis process by cleaving various intracellular substrates[17,20,23]. Fas is a type-I membrane protein that is ubiquitously expressed in a variety of normal cells, including activated T and B cells, hepatocytes[20], and oral[4,16] and colonic[24] epithelial cells, whereas FasL is expressed predominantly in activated T cells. In the immune system, Fas and FasL are involved in down-regulation of immune reactions as well as in T-cell-mediated cytotoxicity[17]. Altered expression of Fas and/or FasL has been detected in many neoplasms, implying that Fas/FasL-induced apoptosis may play a role in the development and progression of some tumours[1,21,24].

Recent studies have detected apoptotic cells and several apoptosis-related proteins, such as Bcl-2 family gene products, Fas, Fas-ligand (FasL) and caspase-3, in developing tooth germs, odontogenic keratocysts and ameloblastomas[4,6–9,14,16,19], suggesting that apoptosis may play important roles in normal odontogenesis as well as in the pathogenesis of odontogenic lesions. The aim of this study was to evaluate the expression pattern of apoptosis-related proteins in different histological subtypes and various cellular components of ameloblastomas.

Materials and methods

Thirty-nine cases of classic intraosseous ameloblastoma were selected from the files of the Department of Oral Pathology, School of Stomatology, Peking University during the period 2000–2003. Sixteen patients were male and 23 were female. The age at diagnosis ranged from 11 to 73 years with a mean of 36 years. Thirty-three tumours occurred in the mandible and 6 in the maxilla. There were 21 primary and 18 recurrent lesions. Of the 18 patients with recurrent lesions, 16 were treated initially by enucleation or curettage and the other 2 by block resection. The duration ranged from 10 days to 20 years with a median of 1 year. On reviewing the histology, the present series consisted of 27 follicular (foci of squamous cells and granular cells were observed in 21 and 10 cases, respectively) and 12 plexiform (2 tumours contained occasional squamous cell clusters and 1 showed extensive granular transformation) ameloblastomas. All specimens had been routinely fixed in 10% formalin, processed and embedded in paraffin.

Serial sections 4-μm thick were cut and immunocytochemical staining was performed using a standard streptavidin–biotin–peroxidase complex method. The primary antibodies used were as follows: Fas (polyclonal, 1:400; Santa Cruz Biotechnology, Inc., CA, USA), FasL (polyclonal, 1:100; Santa Cruz), caspase-3 (polyclonal, 1:200; Boster Biological Co. Ltd, Wuhan, PR China), Bcl-2 (clone Bcl-2-100, ready to use; Zymed, CA, USA) and Ki67 (clone 7B11, ready to use; Zymed). To enhance immunostaining, sections were microwave-heated in citrate buffer (pH 6.0) for Bcl-2 and Ki67. Staining was revealed using LAB-SA kits (Zymed) and 3,3′-diaminobenzidine. Immunoreactivity of Fas, FasL, caspase-3 and Bcl-2 for each case was recorded and percentages of positive cases in different groups, i.e. follicular type, plexiform type, primary tumour and recurrent tumour, were calculated. The staining intensity in various cellular components of the tumor, i.e. peripheral columnar basal cells, central stellate reticulum-like cells, foci of squamous and granular cells, was assessed separately using a semi-quantitative scale. Briefly, using an eyepiece graticule, at least 1000 peripheral cells, 1000 central cells, 500 squamous cells and 500 granular cells were counted for each case under the microscope in randomly selected areas. The results were expressed as: (+) positive (more than 25% of tumour cells were positive), (±) weak positive (less than 25% of tumour cells were positive) and (−) negative (no positive staining were observed). Statistical analysis was performed using SAS software (version 8.01). Chi-square test was used to analyse the differences in percentage of positive cases between the 2 histological subtypes as well as between the primary and recurrent tumours. The differences in staining intensity among the 4 types of ameloblastoma cellular components were also analysed by Chi-square test.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL) was performed in 9 follicular and 2 plexiform ameloblastomas, using an *in situ* apoptosis detection kit (Boster Biological Co.). Briefly, the 4-μm paraffin sections were deparaffinized and treated with 20 μg/ml proteinase K solution for 15 min. After immersing in hydrogen peroxide to block the endogenous peroxidase, the sections were incubated with TdT together with digoxygenin-labelled dUTP in a moist chamber at 37 °C for

Table 1. The expression of apoptosis-related proteins in the 2 histological subtypes of ameloblastoma

	Fas	FasL	Caspase-3	Bcl-2
Follicular type (n = 27)	26 (96.3)	23 (85.2)	17 (63.0)	13 (48.1)
Plexiform type (n = 12)	9 (75.0)	11 (91.7)	7 (58.3)	7 (58.3)
Total (n = 39)	35 (89.7)	34 (87.2)	24 (61.5)	20 (51.3)
P value*	0.078	1.000	1.000	0.731

Values are number of cases (percentage) showing positive reaction.
*Chi-square test.

Table 2. The expression of apoptosis-related proteins in primary and recurrent ameloblastomas

	Fas	FasL	Caspase-3	Bcl-2
Primary (n = 21)	18 (85.7)	19 (90.5)	15 (71.4)	11 (52.4)
Recurrent (n = 18)	17 (94.4)	15 (83.3)	9 (50.0)	9 (50.0)
Total (n = 39)	35 (89.7)	34 (87.2)	24 (61.5)	20 (51.3)
P value*	0.609	0.647	0.203	1.000

Values are number of cases (percentage) showing positive reaction.
*Chi-square test.

2 h. Biotin-conjugated anti-digoxygenin antibody was applied to the sections at 37 °C for 30 min, and then reacted with streptavidin–peroxidase complex at 37 °C for 60 min. The reaction products were visualized by diaminobenzidine solution.

Results

Immunohistochemical staining for Fas, FasL, caspase-3 and Bcl-2 antigens was noted in 35 (89.7%), 34 (87.2%), 24 (61.5%) and 20 (51.3%) cases of ameloblastoma, respectively. By statistical analysis, the difference in the percentage of positive cases between the 2 histological subtypes of ameloblastoma and between primary and recurrent tumours showed no significance ($P > 0.05$; Tables 1 and 2). In addition, statistical analysis failed to reveal any association between percentage

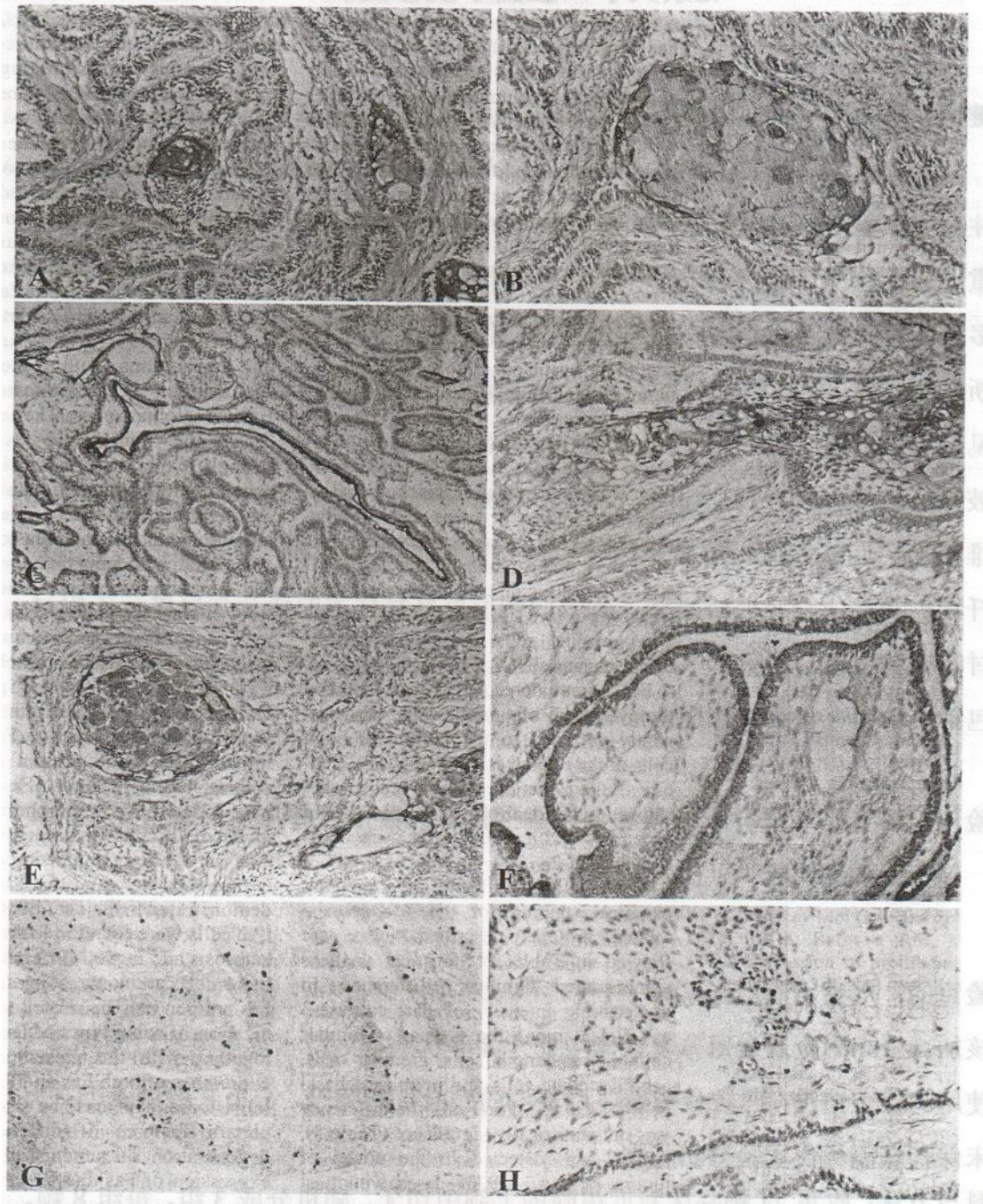

Fig. 1. Immunohistochemical reactivity of Fas is mainly detected in the foci of squamous cells (A, streptavidin–biotin, original magnification ×100) and granular cells (B, streptavidin–biotin, original magnification ×150), and in the tumour cells lining the cystic areas in follicular ameloblastomas (C, streptavidin–biotin, original magnification ×60). Immunostaining of FasL shows a similar pattern with strong staining being in the foci of squamous cells (D, streptavidin–biotin, original magnification ×100) and granular cells (E, streptavidin–biotin, original magnification ×80). Bcl-2 immunoreactivity is predominantly seen in the peripheral basal cells of the tumour islands (F, streptavidin–biotin, original magnification ×180). Ki67 positively stained nuclei are also mainly situated in the peripheral basal cells (G, streptavidin–biotin, original magnification ×150). Apoptotic cells detected by TUNEL method are mainly seen in the central squamous or cystic areas of tumour islands (H, TUNEL, original magnification ×200)

Table 3. The expression of apoptosis-related proteins in the 4 types of tumour cells in ameloblastomas

	Fas			FasL			Caspase-3			Bcl-2		
	−	±	+	−	±	+	−	±	+	−	±	+
Squamous cell (n = 23)	0 (.0)	0 (.0)	23 (100.0)	7 (30.4)	6 (26.1)	10 (43.5)	10 (43.5)	5 (21.7)	8 (34.8)	23 (100.0)	0 (.0)	0 (.0)
Granular cell (n = 11)	0 (.0)	4 (36.4)	7 (63.6)	2 (18.2)	6 (54.5)	3 (27.3)	4 (36.4)	6 (54.5)	1 (9.1)	11 (100.0)	0 (.0)	0 (.0)
Central cell (n = 39)	19 (48.7)	13 (33.3)	7 (17.9)	10 (25.6)	28 (71.8)	1 (2.6)	23 (59.0)	15 (38.5)	1 (2.6)	28 (71.8)	11 (28.2)	0 (.0)
Basal cell (n = 39)	25 (64.1)	14 (35.9)	0 (.0)	11 (28.2)	28 (71.8)	0 (.0)	27 (69.2)	12 (30.8)	0 (.0)	19 (48.7)	5 (12.8)	15 (38.5)
χ^2*	4.909			3.739			8.228			23.666		
P value*	<0.0001			0.0532			0.0041			<0.0001		

Values are number of cases (percentage) showing the intensity of staining: positive (+), weak positive (±), negative (−).
* Chi-square test.

of positive cases and the age/sex of the patients or site/duration of the tumours.

Fas antigen was expressed mainly in the cytoplasm of tumour cells. In most of the follicular and some of the plexiform ameloblastomas, foci of squamous cells or granular cells were observed in the centre of some tumour islands, and they showed consistent strong or moderate staining for Fas (Fig. 1A and B). The loosely connected, stellate reticulum-like central cells and cuboidal or columnar cells of the outer layers were mostly negative or only weakly positive. Occasional weak or moderate staining could be detected in the tightly arranged, central fusiform cells with no distinct stellate reticulum-like appearance. In addition, Fas reactivity also appeared to be associated with tumour areas exhibiting cystic change. In 17 (63.0%) follicular tumours, Fas staining was detected in the epithelial cells adjacent to the cystic cavity formed within the tumour island (Fig. 1C).

Immunoreactivity for FasL and caspase-3 was detected mainly in the cytoplasm of tumour cells. The intensities were weaker than that of Fas, but positive cells appeared to be more diffusely distributed. With stronger staining in the squamous and granular cells (Fig. 1D and E), FasL and caspase-3 were also weakly positive in some inner stellate reticulum-like cells and outer cuboidal or columnar cells.

Bcl-2 immunoreactivity was found in the cytoplasm of tumour cells, predominantly confined to the peripheral basal cells of the tumour islands (Fig. 1F). Central stellate reticulum-like cells only showed occasional positivity, and foci of squamous and granular cells were negative for Bcl-2.

The detailed results of immunohistochemical staining for the 4 apoptosis-related proteins described above are showed in Table 3. Statistical analysis demonstrated significant differences in the staining intensity of Fas, caspase-3 and Bcl-2 among the 4 types of tumour cells, i.e. squamous cells, granular cells, central stellate cells and peripheral basal cells ($P < 0.05$). For FasL, the difference was not statistically significant (Table 3).

Ki67 was detected in the nuclei of tumour cells in 38 (97%) cases, with most of the positive cells being peripheral basal cells (Fig. 1G). TUNEL staining was performed in 11 cases of ameloblastoma. Positive cells, although generally very few, were detected in 10 cases. These positive cells were almost exclusively found in the foci of squamous and granular cells or around the cystic areas (Fig. 1H). Interestingly, numerous TUNEL-positive cells were found in one case of plexiform ameloblastoma showing extensive granular transformation.

Discussion

In the present study, 35 (89.7%) cases of ameloblastoma expressed Fas, and strong reactivity was detected in the central areas of the tumour nests exhibiting squamous metaplasia or granular transformation. Some tightly arranged fusiform cells without an apparent stellate reticulum-like appearance in the centre of the tumour islands also showed moderate reactivity. Similarly, FasL was expressed in 34 (87.2%) ameloblastomas. Although the staining pattern of FasL tended to be more diffusing than that of Fas, the most intensive staining was also detected in the foci of squamous and granular cells. Cystic degeneration within the epithelial component is a common feature of follicular ameloblastomas. The tumour cells lining the microcystic cavities also strongly expressed Fas, which has not been mentioned in previous studies, suggesting that the cystic change within the tumour islands of ameloblastoma may be associated with apoptotic processes. In the immunohistochemical study of Kumamoto et al.[7] keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas were found to be strongly positive for Fas, whereas basal cell ameloblastoma showed weak or negative Fas staining. In contrast to the present results, the authors found that Fas, FasL and caspase-3 reactivity in peripheral columnar or cuboidal cells was more intense than that in central polyhedral cells; this requires further study. Muraki et al.[16] demonstrated in their study that Fas-positive cells were found in leukoplakia with keratosis and in well-differentiated squamous cell carcinoma, suggesting that the Fas antigen may be related to the keratinization of some types of lesions. Taken together with the present results, the expression of both Fas and FasL in ameloblastomas appears to be associated with certain features of differentiation or degeneration of tumour cells. The co-expression of Fas and FasL implies that an autocrine mechanism of the Fas/FasL pathway may exist. As for the 2 histological types, intensity for Fas was higher in the follicular group than in the plexiform group, possibly due to the fact that more follicular than plexiform ameloblastomas exhibited squamous metaplasia and granular transformation.

Caspases comprise a structurally related group of cysteine proteases among which caspase-3 is the most downstream enzyme in the apoptosis-inducing protease pathway and is probably the most clearly associated with cell death[22]. Recently, expression of caspase-3 has been proven to correlate with the clinical outcome of several neoplasms[3,25]. Twenty-four (61.5%) ameloblastomas in the present series expressed caspase-3. The staining pattern was quite similar to that of Fas and FasL, i.e. the more intensive staining in the foci of squamous and granular cells, but a relatively lower positive percentage and staining intensity were identified. It has been reported that inhibitors such as Bcl-2 protein in the apoptosis pathway may influence the expression of caspases[17]. The consistent strong staining for Fas and relatively weaker staining for caspase-3 may also suggest that Fas is capable of inducing alternative caspase-independent cell-death pathways in ameloblastomas as has been discussed elsewhere[10].

The Bcl-2 gene was the first described member of a family of genes that has been found to be important in controlling programmed cell death[15]. It is expressed in a wide spectrum of both benign and malignant cell populations, particularly in progenitor cells. Bcl-2 enhancement of cell survival is due to its ability to repress apoptosis in these cells[13]. In the present study, Bcl-2 was positive in 20 (51.3%) ameloblastomas, showing reactivity mainly in the peripheral basal cells of the tumour islands. Most Ki67-positive cells were also found in these areas. A similar staining pattern has been described in several previous studies[8,11,19]. These results suggest that apoptosis is inhibited in the proliferating or undifferentiated tumour cells in ameloblastomas.

The apoptotic cells detected by TUNEL in the present study were scanty, which may be due to the limitation of the method. Apoptosis is a dynamic process and thus all static methods used to measure it are likely to underestimate its frequency[13]. The TUNEL-positive apoptotic cells detected in the present series were almost exclusively located in the foci of squamous and granular cells, as similarly reported previously[6,19]. In one unique plexiform ameloblastoma with extensive granular transformation, numerous granular cells were TUNEL positive. Apoptotic cell death in granular cell ameloblastomas has previously been examined by immnohistochemistry using anti-single-stranded DNA (ssDNA) antibody. The authors suggested that cytoplasmic granularity might be caused by increased apoptotic cell death, and subsequent phagocytosis by neighbouring neoplastic cells[9]. Our results appear to indicate that apoptosis in ameloblastomas may be closely associated with the frequently occurring process of squamous metaplasia and granular transformation of the tumour cells.

SANDRA et al[19]. found that in ameloblastomas most of the outer basal layer cells were predominantly stained by Bcl-2, while most of the inner layer cells were stained by antibodies against the apoptosis-modulating proteins, such as bax and bak. The authors suggested that ameloblastoma had 2 relatively distinct patterns, an anti-apoptotic proliferating site in the peripheral layer and a pro-apoptotic site in the central layer of the tumour islands. The present results lend support for this hypothesis. While expression of pro-apoptotic proteins, such as Fas, FasL and caspase-3, was mainly detected in the foci of squamous and granular cells in the centre of tumour islands, anti-apoptotic protein, Bcl-2, and the proliferating cell marker Ki67 were principally expressed in the peripheral basal cells. Thus, the present study demonstrated the differential expression of apoptosis-related proteins in various cellular components of ameloblastomas, with pro-apoptotic proteins and apoptotic cells being closely associated with squamous metaplasia and granular transformation of the tumour cells. These results suggest that Fas/FasL-induced apoptotic cell death may function in the disposal of terminally differentiated (squamous metaplasia) or degenerative tumour cells (granular transformation and cystic changes) in ameloblastomas.

From a clinical point of view, it would be interesting to determine whether there are differences in expression of these apoptosis-related proteins between tumours arising for the first time and those that are recurrent. These results failed to detect any significant difference between the primary and recurrent groups, indicating that recurrence of ameloblastomas may not be associated with apoptotic activities. While the findings disclosed here do not entirely explain why ameloblastomas tend to exhibit aggressive behaviour, they do seem to indicate which cellular components in particular account for progression of this neoplasm.

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