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# Role of Apoptotic Biomarkers in Ameloblastoma and Dental Follicle

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**Objectives** Ameloblastoma is an odontogenic neoplasm with locally aggressive behavior. Fas and FasL play important roles in apoptotic pathways. The aim of this study was to determine the possible role of expression of apoptotic pathways (Fas and FasL) in human ameloblastoma and the relationship of apoptosis with the clinical biological characteristics of ameloblastoma.

**Methods** In this descriptive retrospective study, we investigated the anti-Fas and anti-FasL antibody expression in 11 dental follicles and 56 ameloblastoma specimens (35 conventional, 15 unicystic and 6 ameloblastic carcinoma samples) by immunohistochemical (IHC) staining and polymerase chain reaction (PCR). The percentage of positive cells was calculated by using the Mann-Whitney U test and Kruskal-Wallis test.

**Results** The rate of expression of markers was significantly lower in dental follicles than all subtypes of ameloblastoma (P=0.01 for Fas, and P=0.0001 for FasL). The FasL proportional score was significantly higher in conventional ameloblastoma than in unicystic ameloblastoma and ameloblastic carcinoma (P=0.003). There was no significant relationship between the type of ameloblastoma and expression of Fas.

**Conclusion** This study shows that the process of apoptosis in ameloblastomais a sign of behavioral change in odontogenic epithelial cells especially in conventional ameloblastoma and that the apoptotic factors may not play an effective role in the malignancy of ameloblastoma.

**Keywords** Fas Receptor; Fas ligand protein; Apoptosis; Ameloblastoma

## Introduction

The odontogenic epithelium is responsible for tooth development in physiological conditions. It can also give rise to odontogenic cysts and tumors.[1, 2] Ameloblastoma is one of the most commonly encountered benign odontogenic epithelial tumors. It is clinically characterized as a benign but locally invasive tumor with a high recurrence rate.[3] Ameloblastic carcinoma is a type of ameloblastoma with cytological features of malignancy at the primary or metastatic site.[4] Apoptosis, also known as programed or physiological cell death, plays diverse roles in embryogenesis and normal homeostasis as well as in oncogenesis.[5,6] Mutations affecting genes, viruses, radiation, growth factors, heat shock proteins, and Fas antigen [also known as Fas, FasR, apoptosis antigen 1 (APO-1 or APT), cluster of differentiation 95 (CD95) or tumor necrosis factor receptor superfamily member 6 (TNFRSF6)] play important roles in apoptosis.[7, 8, 9] Among the various apoptotic pathways, the Fas/FasL system plays a key role. The Fas ligand (FasL or CD95L), a cell-surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas. This triggers a series of intracellular events leading to the activation of caspases that execute the apoptosis process by cleaving various intracellular substrates.[10, 11]

Altered expression of Fas and/or FasL has been detected in many neoplasms, which implies that Fas/FasL-induced apoptosis may play a role in the development and progression of some tumors.[12, 13] Down-regulation of Fas and up-regulation of FasL were observed in neoplastic ameloblastoma as matched with benign ameloblastoma, signifying the release from death of cells attacked by the immune cells. Basal cell ameloblastomahas shown low expression and desmoplastic ameloblastoma has shown high expression of Fas and caspase-3.[14, 15] The expression of FasL and Fas are closely associated with squamous metaplasia and granular transformation of the tumor cells, suggesting that apoptosis induced by FasL may play a role in the terminally differentiated or degenerative ameloblastoma cells.[16]

Evidence shows that apoptotic pathways may play an important role in odontogenesis or cytodifferentiation of odontogenic epithelium. Considering the high incidence rate of ameloblastoma, it seems that prompt differentiation between subtypes of ameloblastoma based on their growth rate, invasion and malignant transformation potential is warranted. In the present study, the immunohistochemical (IHC) expression of Fas and FasL was examined in dental follicles, conventional ameloblastoma, unicystic ameloblastoma and ameloblastic carcinoma, and the correlation between them was analyzed.

## Methods and Materials

This study was performed in the Oral and Maxillofacial

Pathology Department of School of Dentistry, Shahid Beheshti University of Medical Sciences. This descriptive retrospective study was conducted on 11 dental follicles and 56 ameloblastoma specimens (35 conventional, 15 unicystic and 6 ameloblastic carcinoma samples). Formalin-fixed paraffin-embedded specimens of 31 males and 25 females with a mean age of 34.2 years (range 12-72 years) were evaluated. Four specimens were from the maxilla (7.1%) and 52 specimens were from the mandible (92.9%); 35specimens were conventional ameloblastoma, 15 specimens were unicystic ameloblastoma and 6 specimens were ameloblastic carcinoma.

*IHC staining:*

Serial sections (4-µm thick) were made from the tissue blocks and processed for IHC examination. For immunohistochemical staining, we used the labeled streptavidin-biotin method with anti-Fas and FasL antibodies. The tissue sections were deparaffinized and dehydrated with gradient alcohol (96%); then, the tissue sections were immersed in buffering sodium citrate (pH=6) for antigen retrieval and were heated in a microwave. The Fas-L marker was heated for 15 minutes (five minutes with 800W, 10 minutes with 600W[600 watt]) and the Fas marker was heated for 30 minutes (five minutes with 800W 10 minutes with 600W and 15 minutes with 350W). After cooling at room temperature, the specimens were rinsed thoroughly with phosphate buffered saline (PBS, pH= 7.6) for five minutes, immersed in hydrogen peroxide for 10 minutes and finally rinsed with distilled water and PBS.

After this protocol, the sections were stained with primary antibodies. The applied antibodies were Fasmonoclonal antibody (1:50 dilution, CloneGM 30,1 mL, Novocastra, UK) and Fas-L monoclonal antibody (1:50 dilution, Clone 5D1, 1mL, Novocastra, UK). The Fas and Fas-L specimens were incubated at room temperature for 90 and 60 minutes, respectively. Next, the sections were incubated with secondary antibodies for 30 minutes. We used diaminobenzeneaschromogen and hematoxylin for background staining. For negative control studies of the antibodies, the serial sections were treated with PBS and normal IgG instead of the secondary antibodies and for the positive control, the colon epithelium was used.

*Microscopic analysis:*

The IHC staining was membranous/ cytoplasmic. For evaluation of Fas and Fas-L expressions, we used the total score (total score=intensity score+ proportional score). In this study, the intensity score was referred to the intensity of cell staining (0= no staining, 1= weak staining, 2= moderate staining, and 3= severe staining). The proportional score was referred to the proportion of positively stained cells (0= no staining, 1≤ 1%, 2=1%-10%, 3=10%-33.3%, 4=33.3%-66.6 and 5=Up to 66.6%) based on the Dako catalogue. All specimens were analyzed by two maxillofacial pathologists at different times.

*Polymerase chain reaction (PCR):*

For dental follicle and different types of ameloblastoma specimens, DNA extraction was performed and primers (Fas, FasL) (CinnaGen company, Iran) were prepared and processed as follows (Figure 1):

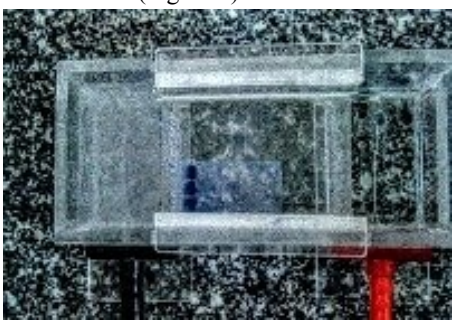

**Figure 1- Electrophoresis of extracted DNA**

Fas F:5'-CTACCTAAGAGCTATCTTACCGTTC-3'
Fas R:5'-GGCTGTCCATGTTGTGGCTGC-3'

*Data analysis:*

The total score was calculated for ameloblastoma specimens and dental follicles. The Mann-Whitney U test and the Kruskal-Wallis test were used for data analysis via the SPSS version 15.0 P values <0.05 were considered statistically significant.

## Results

Based on the proportional score, Fas staining was negative for dental follicles and mildly positive for ameloblastoma (Table 1, Figures 2 and 3).

**Table 1-** Fas staining based on the proportional score in ameloblastoma and dental follicle

| Proportional score | 0 | 1 | 2 | Total |
|---|---|---|---|---|
| Ameloblastoma | 31(55.4%) | 20(35.7%) | 5(8.9%) | 56 (100%) |
| Dental follicle | 11 (100%) | 0(0%) | 0 (0%) | 11(100%) |
| Total | 42(62.7%) | 20(29.9%) | 5(7.5%) | 67 (100%) |

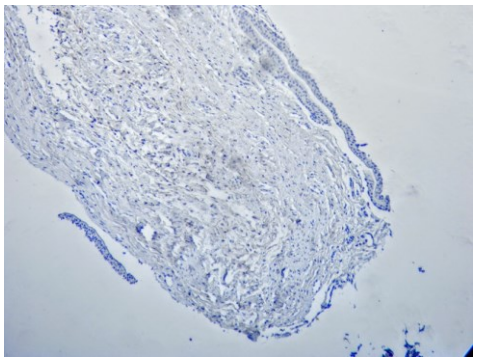

**Figure 2- Low expression of Fas in dental follicle at x100 magnification (IHC staining)**

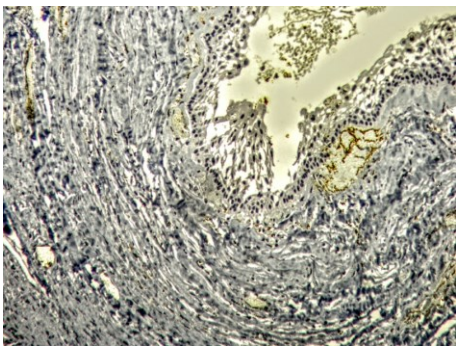

**Figure 3- Low expression of Fas in ameloblastoma at x100 magnification (IHC staining)**

In general, 54.5% of dental follicle samples and 96.3% of ameloblastoma samples were highly stained for FasL markers based on the proportional score (Table 2, Figure 4). Based on the intensity score, Fas staining was negative in dental follicle samples and positive in 44.6% of ameloblastoma samples (Table 3).

Overall, 62.5% of ameloblastoma samples were highly stained for FasL marker and 81.8% of dental follicle samples were mildly stained for this marker based on the intensity scores (Table 4).

In comparison between dental follicle and types of ameloblastoma, the intensity score for FasL was lower in dental follicle than in ameloblastoma samples (P=0.0001). Rate of expressions based on the total score was lower in dental follicle than in ameloblastoma, and these differences were statistically significant (P=0.01 for Fas and P=0.0001 for FasL). The FasL proportional score was higher in conventional ameloblastoma than in unicystic and ameloblastic carcinoma (Table 5), and this difference was statistically significant (P=0.003). There was no significant relationship between the types of ameloblastoma and expression of Fas marker (P=0.28, Table 6).

**Table 2-** FasL staining based on the proportional score in ameloblastoma and dental follicle

| | 0 | 1 | 2 | 3 | 4 | 5 | Total |
|---|---|---|---|---|---|---|---|
| Ameloblastoma | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 2(3.6%) | 54(96.4%) | 56(100%) |
| Dental follicle | 1(9.1%) | 1(9.1%) | 1(9.1%) | 1(9.1%) | 1(9.1%) | 6(54.5%) | 11(100%) |
| Total | 1(1.5%) | 1(1.5%) | 1(1.5%) | 1(1.5%) | 3(5.4%) | 60(89.6%) | 67(100%) |

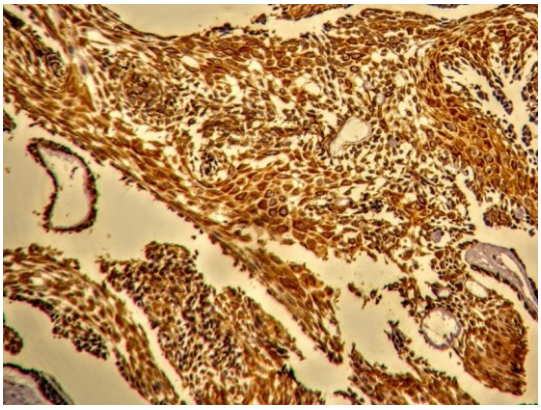

**Figure 4- High expression of FasL in conventional ameloblastomaat x100magnification (IHC staining)**

**Table 3-** Fas staining based on the intensity score in ameloblastoma and dental follicle

| | Without staining | Mild | Moderate | Severe | Total |
|---|---|---|---|---|---|
| Ameloblastoma | 31(55.4%) | 6(10.7%) | 5(8.9%) | 14(25%) | 56(100%) |
| Dental follicle | 11(100%) | 0 (0%) | 0 (0%) | 0 (0%) | 11(100%) |
| Total | 42(62.7%) | 6(9%) | 5(7.5%) | 14(20.9%) | 67(100%) |

**Table 4-** Fas staining based on the intensity score in ameloblastoma and dental follicle

| | Negative | Mild | Moderate | Severe | Total |
|---|---|---|---|---|---|
| Ameloblastoma | 0 (0%) | 3(5.4%) | 18(32.1%) | 35(62.5%) | 56(100%) |
| Dental follicle | 1(9.1%) | 9(81.8%) | 1(9.1%) | 0 (0%) | 11(100%) |
| Total | 1(1.5%) | 12(17.9%) | 19(28.4%) | 35(52.2%) | 67(100%) |

**Table 5-** FasL staining based on the proportional score in subtypes of ameloblastoma

| Proportional score | 4 | 5 | Total |
|---|---|---|---|
| Conventional ameloblastoma | 0(0%) | 35(100%) | 35(100%) |
| Unicystic ameloblastoma | 1(6.7%) | 14(93.3%) | 15(100%) |
| Ameloblastic carcinoma | 1(16.7%) | 5(83.3%) | 6(100%) |
| Total | 2(3.6%) | 54(96.4%) | 56(100%) |

**Table 6-**Fas staining based on the proportional score in subtypes of ameloblastoma

| Proportional score | 0 | 1 | 2 | Total |
|---|---|---|---|---|
| Conventional ameloblastoma | 20(57.1%) | 13(37.1%) | 2(5.8%) | 35(100%) |
| Unicystic ameloblastoma | 7(46.7%) | 6(40%) | 2(13.3%) | 15(100%) |
| Ameloblastic carcinoma | 4(66.6%) | 1(16.7%) | 1(16.7%) | 6(100%) |
| Total | 31(55.4%) | 20(35.7%) | 5(8.9%) | 56(100%) |

The comparison between different subtypes of ameloblastoma showed that in ameloblastic carcinoma, the proportional score of Fas was similar to that of dental follicle (P=0.001); this score was 83.3% for FasL, which was lower than that of conventional ameloblastoma (100%) and unicystic ameloblastoma (93.3%).

The purpose of the PCR method was to detect single-nucleotide polymorphisms at -670 of Fas gene promoter.

Due to the use of paraffin samples in PCR, the PCR steps were repeated but no results were obtained.

## Discussion

Ameloblastoma is among the most commonly encountered benign odontogenic epithelial tumors. It is clinically characterized as a benign but locally invasive tumor with a high recurrence rate.[3] Based on its clinical and radiographic features, ameloblastoma is classified into three general subtypes of multicystic (75-86%), unicystic (13-21%) and peripheral (1-4%).[17] Apoptosis, also known as programed or physiological cell death, may be induced by mutations affecting genes, viruses, radiation, growth factors, heat shock proteins, and Fas antigen.[5-9]

The Fas/FasL system was first thought to play a role in the immune system[18]; however, the expression of Fas and FasL has been detected in a variety of tissues, suggesting that the Fas/FasL system is implicated in apoptotic cell death during the physiological cell turnover.[12] In the current study, immune reactivity of Fas and FasL was detected in ameloblastoma subtypes and in dental follicle.

Mutations have been reported in the Fas gene and single nucleotide pleomorphism in the promoter region of the Fas gene. Mutations have also been reported in some tumors.[19] Also, tumor suppressor and anti-apoptotic pathways have been implicated in ameloblastoma pathogenesis. IHC studies have shown the expression of p53 and Mouse Double Minute 2 in the majority of ameloblastoma cases.[20] A recent study found some proteins related to apoptosis such as Bcl-2, Fas, FasL and caspase 3, which play major roles in proliferation of cells in dental follicle, odontogenic keratocyst, and ameloblastoma.[21]

Recently, down-regulation of Fas and up-regulation of FasL were detected in many human neoplasms, suggesting that neoplastic cells might defend against T-lymphocytes or other effector cells of the immune system.[22] In the current study, Fas expression was slightly lower than Fas-L expression in ameloblastoma samples. None of the dental follicle samples expressed Fas marker, but FasL marker was positive in dental follicle samples. However, a previous study demonstrated the expression of both Fas and its ligand in dental follicles and suggested a relationship between the Fas/FasL system and tooth development.[23, 24] This difference between the two studies may be due to IHC staining methods. Another study (Jain et al), similar to the present study, showed lower expression of Fas in ameloblastoma samples. [25] The expression of Fas and FasL in various patterns in tooth buds and benign and malignant ameloblastoma has also been reported.[26] But, compared with tooth buds, level of expression of Fas was lower in ameloblastoma; whereas, FasL had similar expression in tooth buds and ameloblastoma[23, 24] similar to our findings. A previous study detected the expression of Bcl-2 and Bcl-x in ameloblastoma.[11] It has been proven that Bcl-2 and Bcl-x proteins inhibit the Fas-mediated apoptotic pathway.[19] These findings suggest that the Fas/ FasL system might be suppressed by the Bcl-2 family of proteins in ameloblastoma.[21, 27] For the comparison of results of PCR, we searched MeSH, PubMed and Google Scholar but no similar research was found. In our study, due to the use of paraffin samples in PCR, the PCR steps were repeated but no results were obtained.

The statistical analysis in the current study showed that the expression of Fas and FasL markers in ameloblastoma was significantly higher than that in dental follicle samples, but their expressions in subtypes of ameloblastoma were the same, except for Fas-L marker, which showed significantly higher expression in conventional ameloblastoma versus the unicystic ameloblastoma and ameloblastic carcinoma. This finding shows that the Fas-FasL system does not affect the behavior of ameloblastoma subtypes. The comparison between different subtypes of ameloblastoma revealed that in ameloblastic carcinoma, the proportional score of Fas is similar to that of the dental follicle, and this score for FasL was 83.3% which was lower than that of conventional ameloblastoma (100%) and unicystic ameloblastoma (93.3%).

## Conclusion

This study indicated that the process of apoptosis in ameloblastoma is a sign of behavioral changes in odontogenic epithelial cells especially in conventional ameloblastoma and the apoptotic factors may not play an effective role in the malignancy of ameloblastoma.

## Conflict of Interest

No Conflict of Interest Declared■

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