Original Article

The relation of pericoronal third molar follicle dimension and bcl-2/ki-67 expression: An immunohistochemical study

Seyed Mohammad Razavi[1], Daryush Hasheminia[2], Mojde Mehdizade[3], Bijan Movahedian[2], Forouz Keshani[1]

[1]Torabinejad Dental Research Center and Department of Oral and Maxillofacial Pathology, [2]Dental Implant Research Center and Department of Oral and Maxillofacial Surgery, [3]Torabinejad Dental Research Center and Department of Oral and Maxillofacial Radiology, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran

ABSTRACT

Background: Most common impacted teeth are the mandibular third molars and decision about extraction of them is usually controversial. The presence of pericoronal pathologic changes is an acceptable reason for removal of impacted teeth. Differences in the proliferation rate and apoptosis of odontogenic epithelial cells may influence on the formation of odontogenic epithelial lesions. Therefore, the aim of the present study is to evaluate the immunohistochemical expression of the bcl-2 apoptosis-inhibiting protein and the cell-cycle-related ki-67 antigen in pericoronal follicle of impacted third molars with ≥2.5 mm and <2.5 mm radiolucency.

Materials and Methods: This was a cross-sectional study that 20 follicles with <2.5 mm radiolucency and 20 follicles with ≥2.5 mm radiolucency were selected by a professional radiologist in digital panoramic radiographs and then referred to a surgeon. Formalin fixed paraffin-embedded tissues were immunohistochemical analyzed for immunoreactivity of bcl-2 protein and ki-67 antigen. The data was analyzed using logistic regression, Spearman correlation coefficient and t-test and Mann-Whitney. $P<0.05$ was considered significant.

Results: The findings showed correlation between size of the third molar's follicles and expression of bcl-2 protein ($P < 0.001$, $r = 0.556$) but there was no correlation between size of third molar's follicles and staining with ki-67 antigen ($P = 0.546$, $r = 0.098$). The follicles with radiolucency ≥2.5 mm showed increased immunoreactivity for bcl-2 protein.

Conclusion: The results of study suggest that impacted third molars with radiolucency ≥2.5 mm may be associated with deregulation of cell death, indicated with increased expression of the anti-apoptotic protein bcl-2, while cell proliferation (ki-67) does not seem to play a significant role.

Key Words: Apoptosis, bcl-2 antiapoptotic protein, immunohistochemistry, ki-67 antigen, third molar

INTRODUCTION

An impacted tooth is a tooth which could not be erupted partially or completely because of a physical barrier such as another tooth, bone or soft tissue.[1]

Access this article online
Website: www.drj.ir

Dental follicle is the developmental sac that surrounded the crowns of unerupted teeth and composed of fibrous tissue, nests of odontogenic epithelium and reduced enamel epithelium (REE).[2,3]

The most common impacted teeth are the mandibular third molars.[4] Removal of third molars is one of the most frequently performed procedures by oral and maxillofacial surgeons.[5] Although, extraction of impacted third molars is controversial in dentistry, usually, the presence of pericoronal pathologic changes is an acceptable reason for removal of impacted teeth.[5] Rakprasitkul[6] suggested that impacted third molar teeth should be removed before they develop

pathological changes specially in patients over 20 years. In the other study Cabbar *et al.*[7] asserted that it is better to remove asymptomatic impacted third molar teeth for prevention and reduction the complexity and difficulty of subsequent surgical procedures. But still many authors have questioned whether or not they should be removed routinely.

Occasionally dental follicle adjacent to impacted teeth will be remained and lead to the development of cysts and tumors for example dentigerous cysts, odontogenic keratocyst and ameloblastoma.[2,8] One study showed that radiographic signs may not be used as a reliable marker to ensure that no lesion is around the third molars.[5] But according to Edamatsu's study,[9] pericoronal radiolucency ≥2.5 mm around the crown of teeth is suggestive pathology. Previous studies have also suggested that pericoronal radiolucency ≥2.5 mm on a panoramic radiograph may state an abnormality.[9-12]

Differences in the proliferation rate of odontogenic epithelial cells may play an important role in the formation of epithelial tumors and odontogenic cysts.[7]

Ki-67 is a non-histon protein that has been widely used in cellular proliferation. it is expressed in all stages of the cell cycle except G0.[6,7] Cabbar *et al.*[7] reported that ki-67 overexpression is a reliable marker for differantiation between normal epithelial cells from cysts and tumors. In two studies, Razavi and Colleagues[13,14] have used ki-67 forcomparison of odontogenic keratocyst and ameloblastoma and adenomatoid odontogenic tumor. In these studies, ki-67 expression was used to evaluate proliferative activity of odontogenic epithelium.

Apoptosis, defined as physiological cell death, plays an important role in homeostasis, embryogenesis, and certain pathologic events.[6,7,9] The bcl-2 proto-oncogene expresses a 26 kD a protein that inhibits apoptosis. Piattelli *et al.*[15] reported that overexpression of bcl-2 is an important stage in carcinogenesis.

Seifi *et al.*[16] compared follicular cyst and odontogenic keratocyst with bcl-2 expression, also this marker has been used to evaluate biological behavior of ameloblastoma and odontogenic keratocyst in many studies.

Therefore, bcl-2 expression level was used for checking the role of apoptosis in odontogenic cysts pathogensis in the present study.

Regarding to the controversies in pathologic changes in pericoronal follicles of impacted third molars, the aim of the present study is to determine expression of ki-67 antigen and bcl-2 protein in odontogenic epithelial cells of pericoronal tissue of impacted third molar teeth and to investigate the relationship between this pattern and occurrence of odontogenic tumors and cysts.

MATERIALS AND METHODS

Using simple random sampling method, 20 patients with follicles radiolucency <2.5 mm and 20 patients with follicles radiolucency ≥2.5 mm were included in this cross-sectional study from patients referred to Isfahan's clinics for extraction of impacted third molars in 2011. Exclusion criteria consisted of cases with inflammation, incomplete extracted follicle or follicles with imperfect radiography.

Radiographic analysis was conducted using digital panoramic radiographs. The professional radiologist imported the radiographies to Digora system (Soredex, Finland) and then calibration was done based on a certain magnification.The density of images was visually regulated by Sigma 1 and Sigma 2 options and to evaluate pericoronal spaces, the sharpness option was used. The pericoronal spaces were measured from the mesial, distal, and occlusal surface, and the largest width was recorded.

Thereafter following removal of the impacted third molar by oral and maxillofacial surgeon, a remnant of pericoronal tissue was curetted from bony socket. Tissue samples were measured in formalin 10%. Samples were embedded in paraffin wax, sectioning and H & E staining. Tissue samples were examined under light microscope (Olympus BX41TF, Tokyo, Japan). In H and E staining, samples without odontogenic epithelium were excluded.

In immunohistochemical staining, sections were mounted on poly-L-lysine-coated slides. After deparaffinization and rehydration, the sections were incubated in 0.01M citrate buffer in a microwave oven for antigen retrieval. The slides were then washed in phosphate-buffered saline (PBS) and incubated in 0.5% H_2O_2 in methanol to block endogenous peroxidase activity. A mouse anti-human bcl-2 monoclonal antibody (Clone: Bcl-2-100, Zymed, South San Francisco, CA, USA) with a 1/50 dilution and the monoclonal antibody to the cell proliferation-associated antigen ki-67 (Mib-1, BioGenex Laboratory, San Ramon, CA, USA) diluted 1:100, was applied as

primary antibody and placed in a humidified chamber at 4°C diluted. Immune complexes were subsequently treated with post-primary block and then detected by Novolink polymer (Novocastra7111, Germany) for 30 min, both incubated for 30min at room temperature. Rabbit-mouse antibody link solution (Zymed ABL008, South San Francisco, CA, USA) was used as secondary antibody to enhance the sensitivity of the procedure. After rinsing with PBS, the immunoreactivity was visualized by diaminobenzidine (DABO, DAKO, Denmark). Sections were finally counterstained with hematoxylin, cleared and mounted with P.V mount. Negative controls consisted of phosphate buffered saline instead of primary antibody and samples of lymphoma and breast cancer were used as the bcl-2 and ki-67 positive controls. Slides were blindly viewed independently by two oral pathologists without knowledge of the clinical and radiographic features. In cases of disagreement a consensus diagnosis was recorded after a joint review.

Scoring

The specimens, magnified ×400, were examined using light microscope. The percentage of epithelial cells, demonstrating cytoplasmic positivity for bcl-2 protein and nuclear positivity for ki-67 antigen, was estimated in the full length of the odontogenic epithelium and classified: (+1) 1-24% positive cells; (+2) 25-49% positive; (+3) 50-74% positive; (+4) 75-100% positive.

Also intensity of staining with bcl-2 protein and ki-67 antigen evaluated based on the following criteria: (-)cells with no staining; (+1) the possibility of stained epithelial cells recognition with ×40 magnification; (+2) the possibility of stained epithelial cells recognition with ×100 magnification; (+3) the possibility of stained epithelial cells recognition with ×400 magnification [Figures 1 and 2].[17]

Finally the SID (staining-intensity distribution) score of the proportion of stained cells for each field was multiplied by the score of the staining intensity in that field to provide an SID score.[18]

The data was analyzed using SPSS 11.5 and logistic regression, Spearman correlation coefficient and two-tailed independent student's *t*-test and Mann-Whitney were used to compare data between ki-67 and bcl-2.

RESULTS

Samples without epithelium were removed after evaluation. The study cases included 20 third molar with radiolucency more than 2.5 mm and 20 molar follicles with radiolucency less than 2.5 mm. Patients were 15-33 years old (average 21.7, with SD = 4.3). From 40 patients, 28 samples were female and 12 were male.

IHC examination was performed for evaluation ki-67 and bcl-2 expression patterns.

Spearman correlation coefficient showed correlation between size of third molar's follicles and expression of bcl-2 protein ($P < 0.001$, $r = 0.556$) but there were no correlation between size of third molar's follicles and staining with ki-67 antigen ($P = 0.546$, $r = 0.098$). So the follicles with radiolucency ≥2.5 mm showed increased immunoreactivity for bcl-2 protein.

Table 1 shows descriptive indicators expression of bcl-2 and ki-67 markers in two groups of dental follicle size greater than 2.5 mm and less than 2.5 mm radiolucency [Table 1].

Mean and standard deviation of SID index for ki-67 and bcl-2 markers were determined in two groups of impacted third molar teeth [Table 2 and Figure 3].

It can be seen in Figure 3, that the mean of SID index for both bcl-2 and ki-67 markers in tooth follicles with radiolucency greater than 2.5 mm was more than the tooth follicles with radiolucency of less than 2.5 mm. The mean difference of bcl-2 marker in two dental follicles groups is more than the mean difference of ki-67 in these two groups.

Table 1: Descriptive indicators of expression of bcl-2 and ki-67 in two groups of dental follicle with radiolucency greater than 2.5 mm and less than 2.5 mm

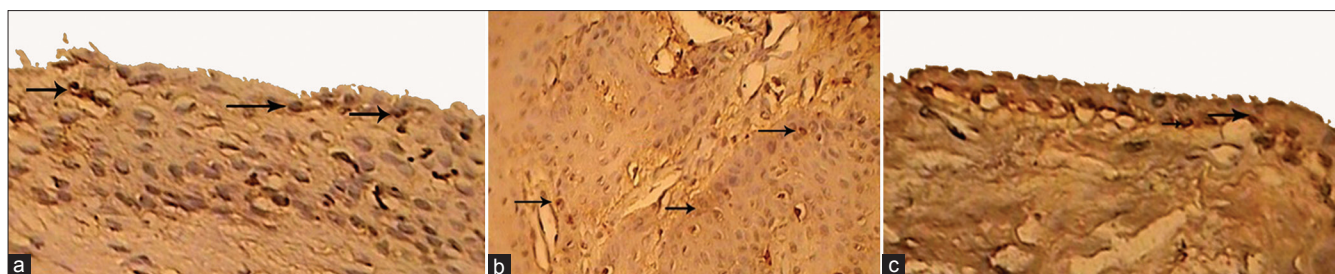| Size	bcl-2	ki-67
<2.5		
Mean	3.05	2.45
Standard deviation	2.72	1.63
Minimum	1	1
Maximum	12	6
≥2.5		
Mean	6.4	3.05
Standard deviation	3.2	2.41
Minimum	1	1
Maximum	12	9
Total		
Mean	4.72	2.75
Standard deviation	3.38	2.06
Minimum	1	1
Maximum	12	9

Figure 1: Various intensity stain of odontogenic epithelium in dental follicle with bcl-2 (×400). int=1 (a), int=2 (b), int=3 (c)
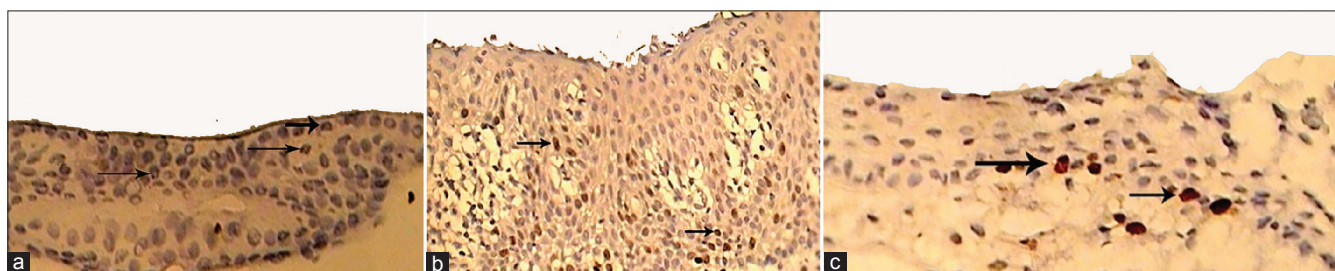

Figure 2: Various intensity stain of odontogenic epithelium in dental follicle with ki-67 (×400), int=1 (a), int=2 (b), int=3 (c)
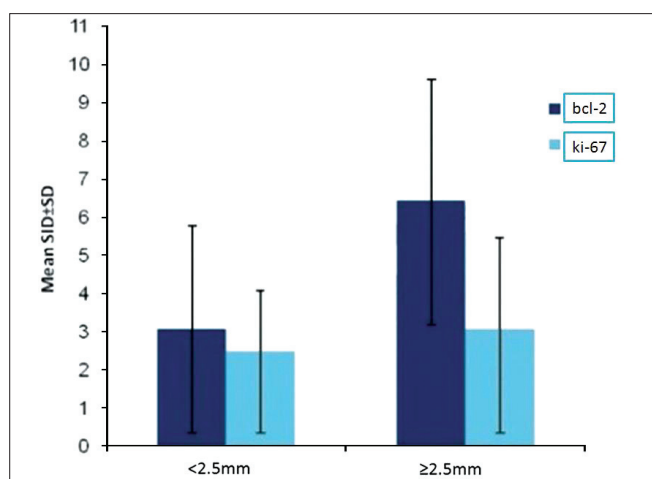

Figure 3: Mean and standard deviation of bcl-2 and ki-67 markers in two groups of dental follicle size with radiolucency greater than 2.5 mm and less than 2.5 mm
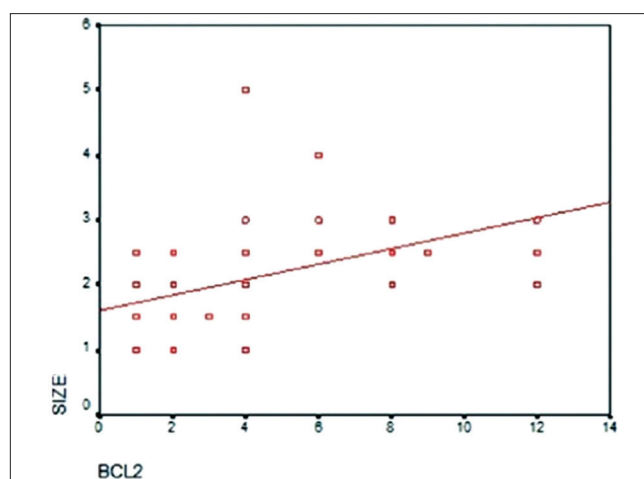

Figure 4: Correlation between radiographic size of third molar's follicles and expression of bcl-2 protein

Scatter graph were also used to show correlation of bcl-2 protein expression and follicle size [Figure 4]. This figure shows that the cases of dental follicles with a size of radiolucency greater than 2.5 mm have larger amounts of bcl-2 expression. Also high levels of bcl-2 SID index can be suspected in less than 2.5 mm dental follicles.

Mann-Whitney test showed no signifi cant difference between sex of patients and SID index for bcl-2 ($P > 0.05$) and ki-67 ($P = 0.539$).

According to logistic regression analysis, except for bcl-2 ($P = 0.006$), other variables such as age, sex and ki-67 in radiographs showed no significant relationship with follicle size.

DISCUSSION

Since the mismanagement of unerupted third molars may lead to variety of serious consequences, the present study was conducted to determine the pathologic latency of pericoronal tissues of asymptomatic unerupted third molar. Also this study aimed to assess the relation between follicle size and the rate of cell proliferation and cell apoptosis.

Although many studies have shown that dental follicles of impacted third molar teeth with

Table 2: Mean±SD of bcl-2 and ki-67 expression in two groups of dental follicle with radiolucency greater than 2.5 mm and less than 2.5 mm

	Mean±SD	
	<2.5	≥2.5
bcl-2	3.05±2.72	6.4±3.20
ki-67	2.45±1.63	3.05±2.41

radiolucency ≥2.5 mm may have much more probability of being pathologic, a few little studies have evaluated proliferation and apoptosis markers in pericoronal tissue of impacted third molar teeth.[4-7]

Most of studies suggested that pericoronal radiolucency with the size of more than 2.5 mm was pathologic.[4-7,19,20] Also in this study, the odontogenic epithelium of pericoronal radiolucency ≥2.5 mm showed more expression of bcl-2 than pericoronal radiolucency <2/5 mm. It appears that inhibiting apoptosis levels was a more probable reason to accumulate cells and then being pathologic.

Radiographic annotation of a pericoronal space as normal or pathologic is difficult. Thus, several researchers have concluded that radiographs alone are insufficient to diagnose pathological changes. Therefore, histological diagnosis seems necessary.[19,21]

In contrary to Cabbar's[7] and Gurgel's[22] studies regarding the evaluation of cell proliferation in odontogenic epithelium of asymptomatic impacted third molar follicle there was no correlation between expression of ki-67 antigen and dental follicle's radiolucency size in the present study.

Mateus *et al.*[23] reported the biologic behavior of odontogenic keratocyst associated with proliferative and apoptotic markers. Edamatsu *et al.*[9] also found that proliferative and apoptotic markers probably have a role in pathogenesis of dentigerous cyst.

The present study supports this hypothesis that apoptosis inhibition and cell accumulation in odontogenic epithelium of impacted third molar follicles with radiolucency ≥2.5 may lead to cystic lesions and odontogenic tumors. Consistently Seifi *et al.*[16] has shown an increase in expression of bcl-2 marker in aggressive type of odontogenic keratocyst. However, Razavi *et al.*[14] concluded the aggressive behavior and recurrence of odontogenic keratocyst and ameloblastoma is related to proliferative activity of cells.

Regarding to Piattelli *et al.*[15] and Jahanshahi *et al.*[17] studiesand the present investigation, the expression of bcl-2 is related to increased possibility of creation cysts and tumors.

In the Venta *et al.*[24] study, impacted third molars had little changes at age 38 years and these changes have been reported more in age range of 20-32 years than 32-38 years old. Also according to Rakprasitkul *et al.*[6] study,more pathological changes were shown in younger people, but there is not any correlation between increasing expression of apoptotic and proliferation markers and age in this study.[6]

According to Saravana and Subhashraj[4] study,the prevalence of cystic changes in dental follicle was twice as common as in men than woman, but the present study found no relation between sex and increasing in proliferative and apoptotic markers in hyperplastic follicles. Results of Adelsperger *et al.*[5] and Glosser *et al.*[19] studies were also consistent with this study.

There is consensus among researchers and clinicians regarding the extraction of impacted third molar teeth with considerable size radiolucency (≥2.5 mm). Also in the present study it was shown that there is a great tendency towards pathological changes in the follicles of impacted third molar teeth with radiolucency ≥2.5 mm. As a result the removal of these impacted teeth should be considered. There is a low probability of pathological changes in impacted third molar teeth with radiolucency <2.5 mm, therefore removal of these teeth is also recommended in order to avoid complications (e.g. problems resulting from surgery, etc.) unless there are strong clinical reasons not to extract them.

CONCLUSION

In conclusion, the immunohistochemical findings in the odontogenic epithelium of pericoronal dental follicles investigated in the present study suggest that impacted third molar with radiolucency ≥2.5 may be associated with deregulation of cell death, indicating increased expression of the antiapoptotic protein (bcl-2), while cell proliferation (ki-67) does not seem to play a significant role.

Because of increased bcl-2 protein, we recommend carrying out careful histologic examinations and further immunohistochemical investigations. We conclude that the prophylactic removal of impacted third molars should be considered as a choice to prevent complication.

ACKNOWLEDGMENT

REFERENCES

1. Neville B, Damm D, Allen C, Bouquot J. Oral and maxillofacial pathology. 2nd ed. Philadelphia: WB Saunders Co; 2009. p. 74-5.
2. da Silva Baumgart C, da Silva Lauxen I, Filho MS, de Quadros OF. Epidermal growth factor receptor distribution in pericoronal follicles: Relationship with the odontogenic cysts and tumors. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103:240-5.
3. Kulkarni D, Kshirsagar K. The unerupted/impacted teeth: A critical appraisal of their pathologic potential - A clinical study. JIDA 2011;5:483-5.
4. Saravana GH, Subhashraj K. Cystic changes in dental follicle associated with radiographically normal impacted mandibular third molar. Br J Oral Maxillofac Surg 2008;46:552-3.
5. Adelsperger J, Campbell JH, Coates DB, Summerlin DJ, Tomich CE. Early soft tissue pathosis associated with impacted third molars without pericoronal radiolucency. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2000;89:402-6.
6. Rakprasitkul S. Pathologic changes in the pericoronal tissues of unerupted third molars. Quintessence Int 2001;32:633-8.
7. Cabbar F, Guler N, Comunoglu N, Sencift K, Cologlu S. Determination of potential cellular proliferation in the odontogenic epithelia of the dental follicle of the asymptomatic impacted third molars. J Oral Maxillofac Surg 2008;66:2004-11.
8. Nickolaychuk B, Mc Nicol A, Gilchrist J, Birek C. Evidence for a role of mitogen-activated protein kinases in proliferating and differentiating odontogenic epithelia of inflammatory and developmental cysts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;93:720-9.
9. Edamatsu M, Kumamoto H, Ooya K, Echigo S. Apoptosis-related factors in the epithelial components of dental follicles and dentigerous cysts associated with impacted third molars of the mandible. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005;99:17-23.
10. de Paula AM, Carvalhais JN, Domingues MG, Barreto DC, Mesquita RA. Cell proliferation markers in the odontogenic keratocyst: Effect of inflammation. J Oral Pathol Med 2000;29:477-82.
11. Li TJ, Browne RM, Matthews JB. Expression of epidermal growth factor receptors by odontogenic jaw cysts. Virchows Arch A Pathol Anat Histopathol 1993;423:137-44.
12. Aitasalo K, Lehtinen R, Oksala E. An orthopantomographic study of prevalence of impacted teeth. Int J Oral Surg 1972;1:117-20.
13. Razavi SM, Tabatabaie SH, Hoseini AT, Hoseini ET, Khabazian A. A comparative immunohistochemical study of Ki-67 and Bcl-2 expression in solid ameloblastoma and adenomatoid odontogenic tumor. Dent Res J (Isfahan) 2012;9:192-7.
14. Razavi M, Tabatabae H. Immunohistochemical study of the incidence of bcl-2, Ki-67 and apoptosis using the TUNEL method in the odontogenic keratocyst comparison with the Ameloblastoma. J Isfahan Dent Sch 2010;5:139-47.
15. Piattelli A, Fioroni M, Rubini C. Differentiation of odontogenic keratocysts from other odontogenic cysts by the expression of bcl-2 immunoreactivity. Oral Oncol 1998;34:404-7.
16. Seifi S, Mahjoub E, Sadegh M. BCL2 expression in follicular cyst and odontogenic keratocyst. J Islamic Dent Assoc Iran 2008;28:164-70.
17. Jahanshahi G, Talebi A, Shirvani A. Expression of bcl-2 in the epithelial lining of odontogenic keratocysts. J Dent Tehran Univ Med Sci Tehran, Iran 2006;3:30-5.
18. Florez-Moreno G, Henao-Ruiz M, Santa-Saenz D, Castaneda-Pelaez D, Tobon-Arroyave S. Cytomorphometric and immunohistochemical comparision between central and peripheral giant cell lesions of the jaws. Oral SurgOral Med Oral Pathol Oral Radiol Oral Endod 2008;105:625-32.
19. Glosser JW, Campbell JH. Pathologic change in soft tissues associated with radiographically 'normal' third molar impactions. Br J Oral Maxillofac Surg 1999;37:259-60.
20. Conklin WW, Stafne EC. A study of odontogenic epithelium in the dental follicle. J Am Dent Assoc 1949;39:143-8.
21. Meer S, Galpin JS, Altini M, Coleman H, Ali H. Proliferating cell nuclear antigen and Ki-67 immunoreactivity in ameloblastomas. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;95:213-21.
22. Gurgel CA, Ramos EA, Azevedo RA, Sarmento VA, da Silva Carvalho AM, dos Santos JN. Expression of ki-67, p53 and p63 protein in keratocyst odontogenic tumors: An immunohistochemical study. J Mol Histol 2008;39:311-6.
23. Mateus GC, Lanza GH, de Moura PH, Marigo Hde A, Horta MC. Cell proliferation and apoptosis in keratocystic odontogenic tumors.Med Oral Patol Oral Cir Bucal 2008;13:E697-702.
24. Venta I, Ylipaavalniemi P, Turtola L. Clinical outcome of third molars in adults followed during 18 years. J Oral Maxillofac Surg 2004;62:182-5.