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ORIGINAL ARTICLE

Co-expression of Ki-67 and p53 protein in ameloblastoma and keratocystic odontogenic tumor

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Abstract

Objectives. The purpose of this study was to evaluate the cell proliferation and p53 protein expression in ameloblastomas (ABs), keratocystic odontogenic tumor (KCOT) and dentigerous cyst (DC). ***Method.*** The immunohistochemistry were carried out for Ki-67 and p53 protein expression by using MIB-1 clone and DO-7 clone, respectively, in ABs ($n = 23$), KCOT ($n = 32$), DC ($n = 30$), normal oral mucosa (NOM) ($n = 12$) and fetal oral mucosa (FOM) ($n = 10$). ***Results.*** Both the Ki-67 LI Labeling index (LI) and p53 LI was significantly higher in ABs than KCOT, DC, NOM and FOM. The Ki-67 LI and p53 LI was significantly higher in KCOT as compared to DC. Ki-67 LI and p53 LI was observed in descending order in ABs, KOCT, FOM, NOM and DC. There was significant correlation between Ki-67 expression and p53 expression in ABs, KCOT, DC and NOM. The densely stained p53 positive cells were noted higher in ABs than KCOT. The very few densely p53 positive cells were noted in DC, NOM and FOM. ***Conclusion.*** The results suggest that the p53 protein expression does not necessarily imply an association with malignant disease and/or p53 gene mutation, but a tendency to be expressed in an increasing quantitative and qualitative manner, as the biologic behavior of odontogenic cyst or tumors becomes more aggressive. p53 over-expression may promote cell proliferation in odontogenic lesions. Thus, it can be stipulated that Ki-67 and p53 protein expression can be used as a prognostic marker in odontogenic lesions.

Key Words: *Ameloblastomas, keratocystic odontogenic tumor (odontogenic keratocyst), dentigerous cyst, Ki-67 labellng index, p53 labelling index*

Introduction

Odontogenic tumors are remarkable among oral lesions because of their clinic and histological heterogenecity. This diversity reflects in the complex development of dental structures, because odontogenic tumors derive from aberrations in odontogenesis. Ameloblastoma (ABs) is a rare neoplasm that accounts for ~ 1% of all other oral tumors [1]. The ABs deserves special attention, not only because of its particular complex biologic behavior, exhibiting great infiltrative potential, high recurrence rate (even in patients treated with radical therapy) and capacity to metastasize [2,3], but also due to the relatively high frequency that it is diagnosed among odontogenic tumors. Recently, ABs have been classified from a clinical point of view in four distinct types: solid/multicystic ameloblastoma (SMA), unicystic ameloblastoma (UA), peripheral ameloblastoma and desmoplastic ameloblastoma [1]. Odontogenic keratocysts represents 11.2% of all odontogenic developmental cysts and are thought to arise from derivatives of embryologic dental lamina or its remnants and extensions of basal cells from the overlying epithelium [4]. The World Health Organization in 2005 [1] based on behavior, histology and genetics, reclassified the odontogenic keratocysts as keratocystic odontogenic tumor (KCOT). Dentigerous cysts (DC) are the most common developmental odontogenic cysts making up to 16.6% of all jaw cysts [4].

Several studies [2,5–12] have investigated cellular proliferative activity in ABs, KCOT and DC, but only

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one study [13] has been carried out to study the correlation between cell proliferation and p53 protein expression in odontogenic lesions. The proliferative activity can be assessed by measuring the proportion of cells committed to cell cycle that is 'growth fraction', so as to assess the intrinsic growth potential of the lesion, which is easily assessed by the Ki-67 Labeling Index (LI). Ki-67 antigen is present in all the active parts of cell cycle—G1, S, G2, M phase and absent in the G0 phase. Its expression increases with the cell cycle progression and reaches its peak during the G2 and M phases, and is rapidly degraded after mitosis. The half-life of Ki-67 is 60–90 min and it has been suggested that Ki-67 staining is more accurate than the counting of mitosis or proliferating cell nuclear antigen (PCNA) staining [14]. p53 protein is a product of the tumor suppressor *p53* gene. *p53* gene functions in G1 arrest to allow the repair of DNA damage and to prevent the cell from entering the S phase of the cell cycle or alternatively to guide the damaged cells to apoptosis [15]. A low concentration of wild type p53 protein is usually found in cells because of its relatively short half-life, ~ 20 min. Its concentration increases as its half-life is extended, which may occur due to TP53 gene mutation, association of wild type p53 with other proteins or disruption of its degradation pathway [16]. Cruz et al. [17] and Pillai et al. [18] have demonstrated over-expression of p53 protein is found in lesions without *p53* gene mutation or even in normal tissue. Thus, to evaluate correlation between Ki-67 and p53 protein expression in ABs, KCOT and DC, the immunohistochemistry for Ki-67 and p53 antigens was employed by using the MIB-1 clone and DO-7 clone (detects both wild and mutant types of p53 protein).

Materials and methods

The present study was carried out at the Department of Oral and Maxillofacial Pathology and Microbiology, Sharad Pawar Dental College and Hospital, Datta Meghe Institute of Medical Sciences, University, Sawangi (M), Wardha, Maharashtra, India. The study protocol was approved by our Institutional Ethical Committee. The study included histopathologically diagnosed 23 cases of ABs, 32 cases of KCOT, 30 cases of DC, 12 normal oral mucosa (NOM) and 10 fetal oral mucosa (FOM). The 23 cases of ABs consisted of 14 SMA and nine UA. SMA constituted of histological sub-types of five cases of follicular ameloblastoma and three cases each of acanthomatous, plexiform and basal cell ameloblastomas. Immunohistochemistry was performed on tissues fixed in 10% neutral buffered formalin, paraffin embedded tissue. The sections were cut serially to 5 μm for immunohistochemistry to evaluate expression of Ki-67 and p53 antigens.

Procedure

Immunohistochemical method for detection of Ki-67 and p-53 antigen. For immunohistochemistry, Peroxidase Detection System (Streptavidin-Biotin Detection System HRP-DAB; Product Code: RE7110K, Novocastra kit) was employed. Endogenous peroxidase activity was blocked by treating hydrated sections with 3% H_2O_2 in methanol for 30 min. The slides were heated in a microwave oven for 10 min in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval and bench cooled for 20 min and again the same cycle was repeated. To prevent non-specific reactions, sections were incubated with 10% serum for 10 min. Prediluted Ki-67 antibody (clone MIB-1; Product code: N1633, Dako, Denmark) and p53 antibody (clone DO-7; Product code: N1581, Dako, Denmark) were incubated at room temperature in a humidifying chamber for 60 min and then at 4°C overnight. Known hyperplastic lymph node and squamous cell carcinoma tissue showing good Ki-67 and p53 expression were used as a positive control, respectively. One section from each positive control was used as the negative control by incubating with non-immune serum instead of primary antibody. This was followed by incubation with secondary biotinylated antibody and streptavidin-peroxidase reagent at room temperature in a humidifying chamber for 30 min. Freshly prepared substrate/chromogen solution of 3, 3' Diaminobenzidine (mixing 5 μl of concentrated DAB in 50 ml of substrate buffer) was used to visualize the antigen–antibody reaction. Finally, the sections were counterstained in Mayer's hematoxylin.

Assessment of immunohistochemically stained sections. The cells were considered positive for Ki-67 and p53 antigen if there was an intra-nuclear DAB staining (brown color). All the stained nuclei were scored positive regardless of their intensity of staining. Cells that lacked a clear nucleus were excluded. Minimum of 1000 cells were counted in each section. Tissue sections were scanned at ×100 magnification for most heavily labeled Ki-67 and p53 positive cells in the epithelial linings. The cell counts were made on captured image at ×400 magnification with conventional light microscope in 10 randomly selected fields. The counting was done by two observers and the mean was taken as a final count. The number of positively stained nuclei was expressed as a percentage of the total number counted. The index of positivity, i.e. Labeling index (LI) was obtained for Ki-67 and p53 protein expression for all the groups.

$$\text{Ki} - 67/\text{p53 (LI)} = \text{Number of IHC Positive Cells}$$
$$(\text{Ki} - 67/\text{p53}) \times 100/\text{Total number of cells observed}$$

The staining intensity in p53 positive cases was further categorized as dense or weak as follows:

Dense = densely stained nuclei, easily seen at low magnification (objective 10×) and Weak = faint nuclei staining that could only be detected by using higher magnification (objective 40×).

Statistical analysis

Group means for Ki-67 LI and p53 LI were derived for each group. The data was analyzed statistically using SPSS 16.0 version software for Windows, Mann-Whitney U-test, Kruskal-Wallis test and Pearson's rank correlation analysis test. One-way Analysis of Variance (ANOVA), independent student *T*-test and Multiple comparisons using post-hoc Bonferroni test were also carried out. The level of statistical significance is at $p < 0.05$.

Results and observation

Ki-67 LI

Ki-67 antigen was expressed in 100% of ABs, 100% of KCOT, 93.34% of DC, 100% of NOM and 100% of FOM. Ki-67 was expressed predominantly in peripheral cells rather than cells in the central area of the ameloblastic follicle, both in SMA and UA. Ki-67 was expressed predominantly in suprabasal cell layers, with uniform distribution in the epithelial lining of KCOT. In DC, Ki-67 positive cells were very few in the basal cell layer. In NOM and FOM, Ki-67 positive cells were mainly located in the basal cell layer (Figure 1).

In ABs, a non-significant difference of Ki-67 LI was noted between UA and SMA ($p = 0.845$). However it was found to be higher in SMA than in UA (Table I). Statistically significant variation of mean Ki-67 LI was noted amongst all groups ($p < 0.001$). Ki-67 LI was observed in descending order in ABs, KOCT, FOM, NOM and DC (Table II). Ki-67 LI was significantly higher in ABs than KCOT ($p < 0.001$), DC ($p < 0.001$), NOM ($p < 0.001$) and FOM ($p = 0.011$). Ki-67 LI was significantly higher in KCOT than DC ($p = 0.001$). Ki-67 LI was significantly higher in FOM than NOM ($p = 0.001$) and DC ($p < 0.001$).

p53 LI

p53 antigen was expressed in 100% of ABs, 100% of KCOT, 50% of DC, 100% of NOM and 100% of FOM. p53 protein was expressed with predominantly dense immunoreactivity in peripheral cells and cells in the central area of the ameloblastic follicle of SMA. However, the high number of weakly positive cells was noted in stellate reticulum like cells (suprabasal cells) of UA. p53 immunolabeling was dense and scattered in basal and suprabasal cell layers in KCOT, whereas very few densely stained cells were sporadically located in the basal cell layer in DC and NOM. The weakly stained cells were noted to be diffusely distributed in the complete epithelium of KCOTs. In DC and NOM, the weakly stained cells were noted mainly in the basal cell layer and very few in the suprabasal cell layers. In FOM, the weak immunoreactivity for p53 protein was noted predominantly in the suprabasal cell layers (Figure 2).

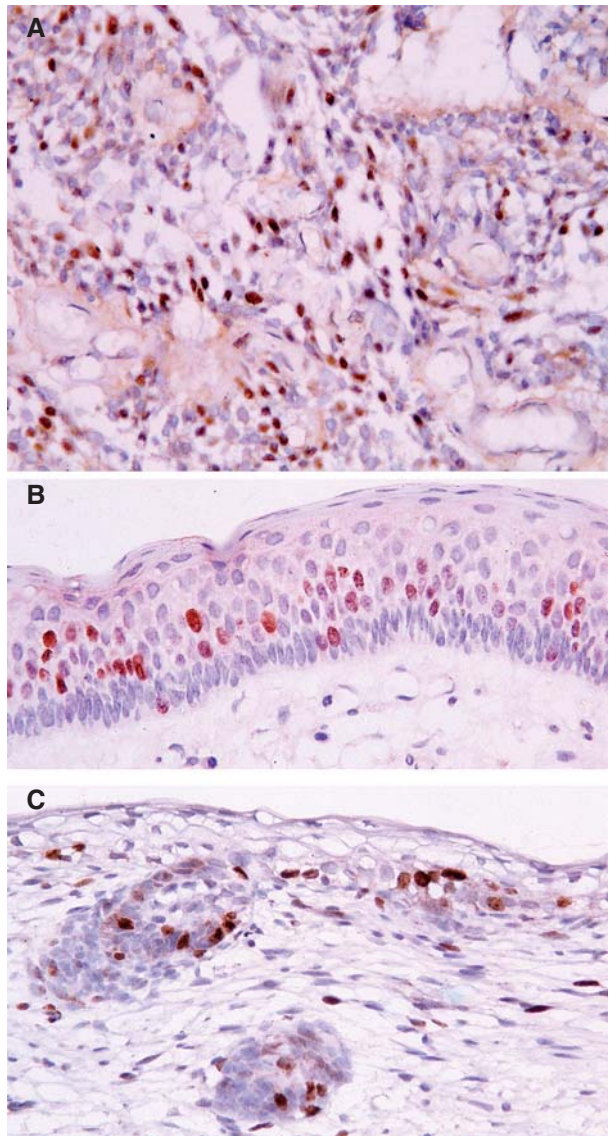

Figure 1. Photomicrograph of immunostaining for Ki-67. (A) In ameloblastomas, Ki-67 positive cells present mainly in basal cell layers of the tumor islands, with fewer positive cells within the stellate reticulum-like areas; (B) In KCOTs, the Ki-67 immunopositive cells are predominantly noted in the suprabasal cell layers of lining epithelium; (C) In FOMs, the Ki-67 immunopositive cells are predominantly found in basal cell layer (original magnification × 400).

In ABs, a non-significant difference of p53 LI was noted between UA and SMA ($p = 0.388$). However, it was found to be higher in UA than in SMA (Table I). p53 LI were observed in descending order in ABs, KOCT, FOM, NOM and DC. p53 LI was significantly higher in ABs than KCOT ($p < 0.001$), DC ($p < 0.001$), NOM ($p < 0.001$) and FOM ($p < 0.001$).

Table I. Ki67 LI and p53 LI in ameloblastomas.

Ameloblastomas		*n*	Ki-67 LI		p53 LI	
			M	SD	*M*	SD
SMA	Follicular	05	25.19	11.99	35.88	15.38
	Acanthomatous	03				
	Basal cell	03				
	Plexiform	03				
UA		09	21.65	12.40	39.20	21.68

SMA: Solid multicystic ameloblastomna; UA: Unicystic ameloblastoma; SD: Slandered deviation.

p53 LI was significantly higher in KCOT than NOM ($p = 0.021$) and DC ($p = 0.001$) (Table II).

Correlation between Ki-67 LI and p53 LI

A statistically significant positive correlation was noted between Ki-67 expression and p53 expression in ABs ($p = 0.000$), KCOT ($p = 0.001$), DC ($p = 0.000$) and NOM ($p = 0.014$).

Discussion

In ABs, the Ki-67 LI index was significantly higher than KCOT, DC, NOM and FOM. An interesting finding of the present study and previous studies [5,7,8,10,11] was that the proliferating markers were observed predominantly in peripheral cells of tumor islands of both UA and SMA, whereas the proliferating markers were observed predominantly in suprabasal cell layers of KCOT. This could explain the locally infiltrating growth of ABs and unique epithelial proliferation and differentiation of KCOT. Thus, the tendency of neoplastic islands to invade the adjacent normal tissue suggests the locally infiltrating aggressive biological behavior of ABs. Non-significant difference of Ki-67 LI was observed between UA and SMA, however it was higher in SMA than UA. Other previous studies [3,10,13] have noted this as either higher or the same, with non-significant difference of mean labeling indices of either PCNA or Ki-67 in SMA as compared to UA. The higher proliferative activity along with aggressive infiltrating nature of SMA as well as different morphology between UA and SMA facilitates more difficulty in surgical accessibility in SMA than UA [7,19]. This could suggest the recurrence and worse prognosis for SMA than UA. As for the SMA, the

Table II. Ki-67 LI and p53 LI in all the groups.

Groups	*n*	Ki-67 LI		p53 LI	
		M	SD	*M*	SD
Ameloblastomas	23	24.24	12.27	37.14	18.11
KCOT	32	12.92	4.23	13.56	4.54
DC	30	4.91	3.60	2.90	3.84
NOM	12	6.13	1.28	4.07	1.78
FOM	10	18.47	3.25	13.34	2.27

KCOT: Keratocystic Odontogenic tumor; DC: Dentigerous cyst; NOM: Normal oral mucosa; FOM: Fetal oral mucosa; SD: Standard deviation.

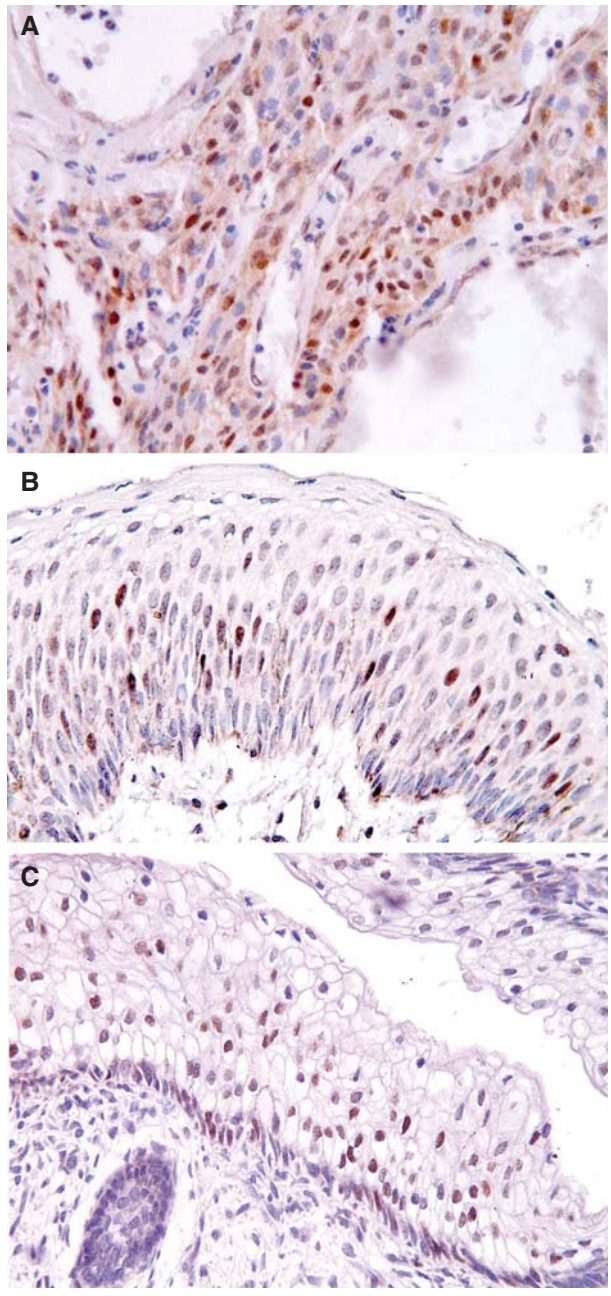

Figure 2. Photomicrograph of immunostaining for p53. (A) In ameloblastomas, p53 positive cells with dense immunoreactivity were predominantly noted in basal cell layer and p53 positive cells with weak immunoreactivity were predominantly noted in suprabasal cell layers; (B) In KCOTs, the p53 positive cells showing dense and weak staining are observed in the suprabasal cell layers and few in the basal cell layer of lining epithelium; (C) In FOMs, the p53 immunopositive cells showing weak staining are predominantly found in the suprabasal cell layers (original magnification × 400).

present study results were in accordance with other studies [8,10,11,13] which have found non-significant differences of proliferative activity among the different histological sub-types of SMA. This may suggest that the histological sub-type of SMA does not have any significant effect on prognosis. Future studies should be carried out by using a large number of cases of each histological sub-type of SMA.

As for KCOT, in the present study, the proliferative activity was observed similar to previous studies [6,10] which found that the proliferating cells were predominantly located in suprabasal cell layers. This unusual proliferation represents an epithelial disorganization and could suggest the epithelial dysplasia as similar to dysplasia of oral squamous epithelium in pre-malignant lesions of the oral mucosa [20]. In DC, few Ki-67 positive cells were predominantly noted in the basal cell layer as in accordance with other studies [6,10], which could suggest very low proliferative activity, which might be related to regular maintenance of 2–3 cell layer thickness of the epithelium. The Ki-67 expression was higher in FOM than NOM and KCOT. The higher proliferative activity in FOM could suggest that the fetal oral epithelium is dynamic with regard to its higher epithelial turn-over rate, subsequent differentiation process and regularly regenerates itself on a relatively rapid basis.

In this study, non-significant p53 LI was observed in histological sub-types of SMA [21]. The significant positive correlation was found between p53 protein expression and Ki-67 expression in ABs. Ki-67 reactivity and densely p53 positive cells were mainly located in peripheral cells of neoplastic islands of both ABs. These results might indicate that p53 abnormalities (i.e. over-expression) can play a crucial role in early neoplastic transformation during the development of ABs. This concept has been previously suggested in the early development of other neoplasm's [22]. Thus, the higher p53 protein expression in ABs could suggest the locally invasive biological behavior and its role in pathogenesis and development of ABs. An infrequent p53 gene mutation in limited number of neoplastic cells on ELISA and yeast functional assay was noted in ABs [23]. Kumamoto et al. [24] have noted *p53* gene mutation in 0% of cases (not detected in 10 cases) on exons 5–8 by using direct p53 sequencing. Allelic loss of tumor suppressor genes was found frequently in ameloblastomas and was not correlated with histological sub-type and prognosis [25]. *p53* gene status in the above studies implied that *p53* gene mutation might play a minor role and not be essential for neoplastic changes of odontogenic epithelium [24]. It can be hypothesized that the mutation of *p53* gene in ameloblastomas may be sporadic and cumulative. However, the over-expression of p53 protein in ameloblastoma was thought to effect the cellular proliferative activity and can be used as an important prognostic marker [21]. Non-significant difference of p53 LI was observed between UA and SMA; however it was higher in UA than SMA [26]. In UA, the high numbers of weak positive cells were noted in stellate reticulum-like cells (suprabasal cells). It has been suggested that detectable wild type of p53 proteins may reflect stabilization of the protein via interactions with other intracellular proteins [27] or transcriptional induction, rather than intragenetic mutations of the p53 locus [28]. The active and accumulating p53 wild type might contribute to the more benign course and low recurrence rate commonly reported for UA in comparison with SMA [3], through the relative suppression of transforming tumor cells and limitation of their growth potential.

In this study, the p53 LI was significantly higher in KCOT than DC and NOM. These findings corroborated with the previous studies [6,29]. The high reactivity of p53 protein in KCOT can be explained by various clinical factors peculiar to the KCOT such as locally aggressive behavior, high mitotic rate, rapid growth through the cancellous bone and tendency to recur [3,4,30,31]. The significant positive correlation was noted between Ki-67 and densely and weakly stained p53 expression in KCOT, which is in agreement with the previous studies [6,13,29]. This could indicate that the negative growth regulation of normal p53 protein is suppressed at least to some extent, resulting in increased proliferation. Li et al. [29] have failed to discover any *p53* gene mutation using single stranded conformations polymorphisms method in 10 KCOT. Agaram et al. [32] and Henley et al. [33] have succeeded in detecting loss of heterozygosity of *p53* using one polymorphic marker in KCOT at frequencies of 66% (two out of three cases) and 20% (three out of 15 cases), respectively. Recently, Malcic et al. [34] and Kuroyanagi et al. [35] confirmed mutations at codon 237 in two out of 12 cases in KCOT and *p53* mutations at exons 5–8 in 10 cases (31.3%) out of 32 KCOTs by using a direct sequencing method. Kuroyanagi et al. [35] did not find any remarkable associations of carrying *p53* mutations to clinico-pathological and immunohistochemical p53 protein expression in KCOTs. Overall, results of the present study were in accordance with Slootweg [13], who studied the immunohistochemical expression of p53 protein (using monoclonal antibody BP53-12) in odontogenic cysts and tumors. The over-expression of p53 protein in KCOT probably may not be solely due to *p53* gene mutation, but is thought to be associated with over-production and/or stabilization of normal/wild p53 protein product [28,36–39]. Hence, it can be hypothesized that a high proliferation activity may result in detectable concentrations of wild type p53 protein in odonotogenic lesions. Thus, p53 protein reactivity may explain its role in intrinsic growth potential of epithelium and biological locally aggressive behavior of KCOT.

Keratocystic Odontogenic Tumor represents a locally aggressive benign low-grade cystic neoplasm rather than developmental cyst [6].

In FOM, the weak immunoreactivity for p53 protein was noted predominantly in suprabasal cell layers. Insignificant positive correlation was found between Ki-67 expression and p53 protein expression. In spite of higher proliferative activity, particularly in basal cell layers as indicated by Ki-67 expression, the FOM maintains the regular thickness of the fetal epithelium. This suggests that the accumulation of wild type of p53 protein (accumulated due to rapidity of cell turn over a short interval of time) is related to the apoptosis of suprabasal cells. In DC, the significant correlation between Ki-67 LI and weakly staining p53 protein in basal cell layer could indicate the normal wild type p53 protein expression; related to apoptosis and cell proliferation.

In conclusion, the higher Ki-67 expression and p53 protein over-expression in ameloblastomas could explain the complex biological character and higher recurrence rate even in patients treated with radical therapy. p53 protein over-expression in the lining epithelium of sporadic KCOT and ameloblastomas does not necessarily imply an association with malignant disease or *p53* gene mutation, but a tendency to be expressed in increasing quantitative and qualitative manner, as the biologic behavior of KCOT and ameloblastomas becomes more aggressive. The p53 protein expression was significantly correlated with that of Ki-67 expression in ABs and KCOT suggested that altered functioning of p53 protein pathways. From a clinical point of view, odontogenic lesions with increased p53 protein over-expression generally have more locally aggressive biological behavior. Thus, it can be stipulated that Ki-67 and p53 protein expression can be used as a prognostic marker in odontogenic lesions.

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