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ORIGINAL ARTICLE

p63 Expression and its Relation to Epithelial Cells Proliferation in Dentigerous Cyst, Odontogenic Keratocyst, and Ameloblastoma

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Abstract

The current controversy about the classification of odontogenic keratocyst reflects the shortage in the understanding of the odontogenic cysts and tumors. The aim of the present study was to investigate p63 immunoexpression and its relation to the proliferation of the epithelial lining in dentigerous cyst (DC), odontogenic keratocyst (OKC), and follicular type of ameloblastoma (AB). The study involved 36 samples, which are DC ($n = 12$), OKC ($n = 9$), and AB ($n = 15$). p63 protein expression was evaluated by immunohistochemistry. The results on Ki-67 expression were obtained from our previous studies and correlated with p63 expressions. p63 was expressed differently in the studied lesions with various distribution in different study samples. Statistical analysis using Kruskal-Wallis test showed a significant difference in the expression of p63 protein among DC, OKC, and AB ($p = 0.048$). Subsequently, Mann-Whitney U test revealed the expression of p63 protein was significantly higher in OKC than DC ($p = 0.018$). Interestingly, Spearman's correlation analysis showed a positive correlation between the expression of p63 and Ki-67 in the odontogenic epithelium of DC ($\sigma = 0.757$, $P = 0.004$) and OKC ($\sigma = 0.741$, $P = 0.022$). While no such a positive correlation was found between the two studied markers in AB group ($\sigma = 0.006$, $P = 0.983$). In conclusion, the present results indicated various expression and correlation of p63 with the proliferation of odontogenic epithelial cells in DC, OKC, and AB. This diversity could reflect a different role and pathways of ΔNp63 in odontogenic tumor than that in odontogenic cyst. These together will help in better understanding the pathogenesis and biological behavior of odontogenic cysts and tumors.

Keywords p63 · Ki-67 · Odontogenic · Dentigerous · Keratocyst · Ameloblastoma

Introduction

Dentigerous cysts (DC) are developmental odontogenic lesions [1], arising from the crown of unerupted teeth. Enucleation of the cyst and extraction of the associated tooth is the current standard treatment for DC [2]. Odontogenic keratocyst (OKC) is another odontogenic cystic lesion which has aggressive behavior and high recurrence rate [3]. The last WHO classification in the year 2017 reclassified OKC from odontogenic tumor to be an odontogenic cyst, declaring that the overall evidence was not sufficiently supporting OKC as a neoplasm [1]. This reclassification was criticized denying the OKC behavior as a benign but aggressive tumor [3]. Indeed, considering OKC as a cyst in the last WHO classification was criticized from a clinical and surgical points of view, but acknowledged from a pathological point of view. The treatment of OKC ranges from as simple as marsupialization to radical resection with subsequent bone graft reconstruction [4, 5]. Ameloblastoma (AB) is a benign odontogenic tumor, has slow-growing and locally invasive property involving mainly the mandible and less commonly the maxilla. Because of its high recurrence rate, the radical surgical excision is the current standard of care for AB including en bloc resection with 1–2 cm free bone margins with immediate bone reconstruction [6].

Studying the proliferation rate in any lesion is important to determine its biological behavior. Ki-67 protein is extensively used as a proliferation marker for evaluating cellular division in odontogenic cysts and tumors [7, 8]. When Ki-67 protein is

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expressed in a cell, it indicates that this cell is in a proliferation state. This is due to the fact that Ki-67 antigen is expressed in all phases of the cell cycle, except in G0 phase [9].

p63 is a protein that has important functions in tumorigenesis. Actually, p63 and p73 are other members of the p53 family which discovered after a long time thought of p53 to be unique in their structures and functions [10]. Currently, p63 believed to encode at least 10 different isoforms, five of them have the transactivating domain and other five lacking this domain. These isoforms exert reversed biological properties. Basically, TAp63 forms are capable of transactivating p53 target genes, while ΔNp63 forms behave in a dominant negative mode towards p53 and TAp63 forms [10, 11].

Consistent with the growth and regenerative requirements of normal proliferating cells, the epithelial cells found to predominantly express ΔNp63 [10]. p63 is restricted mainly to the basal and parabasal layers of normal oral mucosa. The p63 protein plays an important role in epithelial proliferation and development of limb and craniofacial structures. p63 knockout-mice display defects in tissues that have stratified epithelium, like skin, esophagus, and oral cavity. These animals did not develop hair follicles, salivary, lacrimal and mammary glands, and teeth [12, 13]. As DC, OKC, and AB are odontogenic epithelial lesions, p63 could have a role in the pathogenesis and progression of these odontogenic lesions.

Studies of p63 in odontogenic lesions help in understanding the pathogenesis and biological behavior of odontogenic cysts and tumors. Moreover, we could reach a specific target for a non-surgical approach in the management of these lesions. The present study aimed at evaluating the immunoexpression of p63 in the epithelial lining of DC, OKC, and AB. Furthermore, possible correlation between p63 expression and epithelial cells proliferation was also analyzed.

Materials and Methods

A total of 36 cases were included in the current study. The samples were formalin fixed paraffin embedded (FFPE) tissues that were retrieved from Oral Pathology Laboratory of Tongji Hospital. While the clinical data regarding sex, age, and location were obtained from the medical records of the patients. The samples were categorized into three groups, which were DC ($n = 12$), OKC ($n = 9$), and follicular type of AB ($n = 15$). The diagnosis of the pathological lesions was based on clinical, radiological and histopathological findings. No real tissue commensurate exists to act as a control group in this study, as odontogenic lesions include reactive tissues and tumors that replacing the natural jaw bones. A consent form was obtained from patients and the study was approved by the research ethics committee of Tongji Medical College in accordance with the Declaration of Helsinki.

Immunostaining of the current study is done using the method of standard streptavidin-biotin peroxidase complex. All reagents used in this study are from Wuhan Boster Biological Technology Company, Ltd. Samples were cut, dewaxed and rehydrated. Antigen retrieval was performed using citrate solution (0.01 M) in a microwave and the endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Then, samples were treated with goat serum for 50 min at room temperature and incubated at 4 °C overnight with the 1:100 diluted primary antibodies. The primary antibodies were polyclonal rabbit anti-human p63 antigen (BA1326, dilution 1:100, which mainly recognize ΔNp63 isoforms) and polyclonal rabbit anti-human Ki-67 antigen (BA1326, dilution 1:100). After that, sections were treated with 10 μg/ ml of biotinylated secondary antibody (goat anti rabbit IgG, BA1003) at room temperature for 2 h. Then, the sections were stained with streptavidin-biotin-peroxidase complex (20 μg/ml). Finally, the sections were developed by 3,3′-diaminobenzidine substrate and counterstained with Mayer's hematoxylin.

Positive staining was evaluated semiquantitatively using a standard light microscope. The percentage of positive cells was counted in at least 10 representative high power fields (×400). Immunohistochemical reactivity for p63 was detected in the epithelial cells of all studied samples and the positivity was recommended when it was only nuclear. Noteworthy, some cytoplasmic p63 staining were present in the present study. Original data on Ki-67 was obtained from our previous studies [7, 8], recommending only cases that have available FFPE block for the subsequent p63 study. The semi quantitative scoring for p63 and Ki-67 immunostaining was as follows: The scoring was strong when >40% positivity rate was present in the odontogenic epithelium; mild for a positivity rate of 21–40%; weak for a positivity rate of ≤20%; and absent, when there was no identified staining in the nucleus of the odontogenic epithelium or when the staining was questionable.

Data were analyzed using Kruskal-Wallis test followed by post hoc Mann-Whitney U test. Correlation of p63 with Ki-67 proteins were analyzed with Spearman's rank correlation coefficient test. Differences were deemed statistically significant if ($P < 0.05$). All statistical analyses were performed using SPSS version 19.0 software (SPSS Inc., Armonk, NY, USA).

Results

The study composed of 36 samples. Of them, 16 (44.4%) were females, and 20 (55.6%) were males. The maxilla was the location of 12 (33.3%) of the cases while 24 (66.7%) of the cases were located in the mandible. The mean age was 40.11 with SD of ±17.567.

The study involved the odontogenic epithelium of DC, OKC, and AB. p63 location was mainly nuclear (Fig. 1), but some histopathological fields showed true cytoplasmic positivity for p63 (Fig. 2). For reasons mentioned in the discussion section, only nuclear staining was calculated and assumed as a positive result in the current study. In DC samples, positively stained cells were distributed throughout the cystic epithelial layers (Fig. 1). p63 expression was absent in 58.3%, weak in 25%, and strong in 16.7% of odontogenic epithelial cells (Table 1). In OKC samples, p63 was found throughout all the epithelial layers, but mainly concentrated in the upper layers of the epithelium (Fig. 1). p63

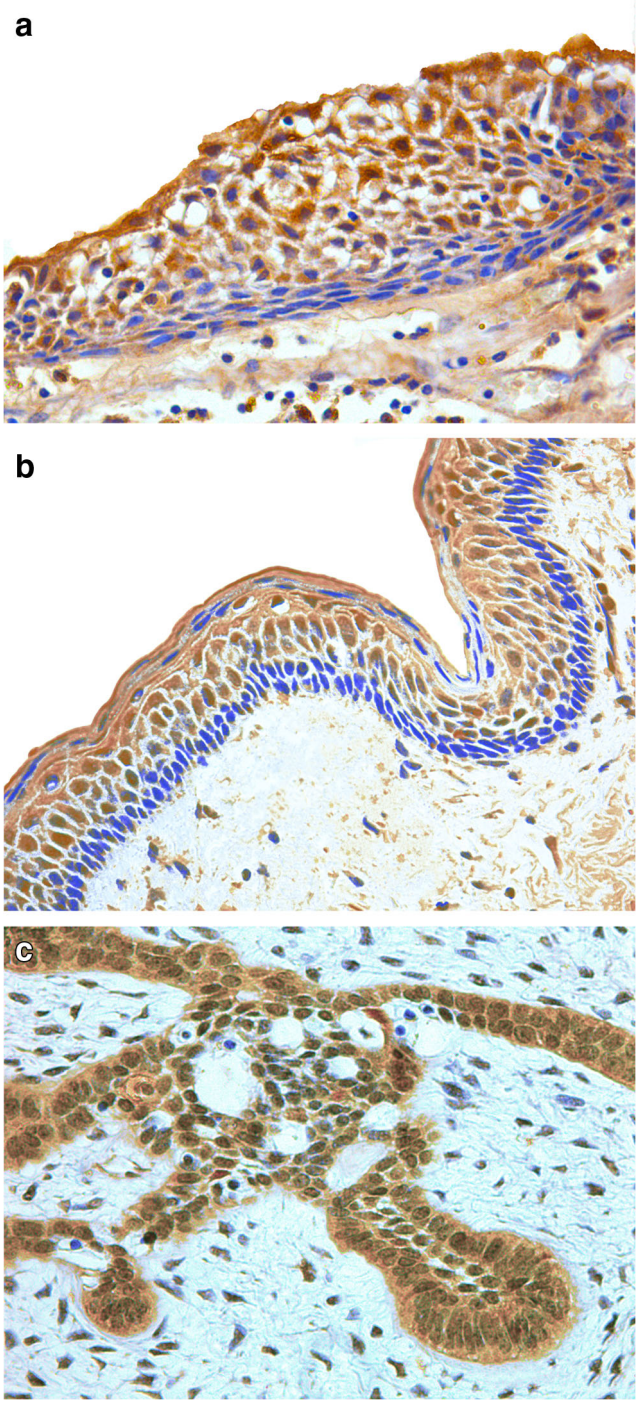

Fig. 1 Photomicrograph showing nuclear immunohistochemical staining of p63 proteins in dentigerous cyst (DC), odontogenic keratocyst (OKC), and ameloblastoma (AB) (magnification, ×400). p63 expression is in all layers of DC (a), mainly through upper epithelial layers of OKC (b), and in both central stellate reticulum like cells and peripheral columnar cells of AB (c)

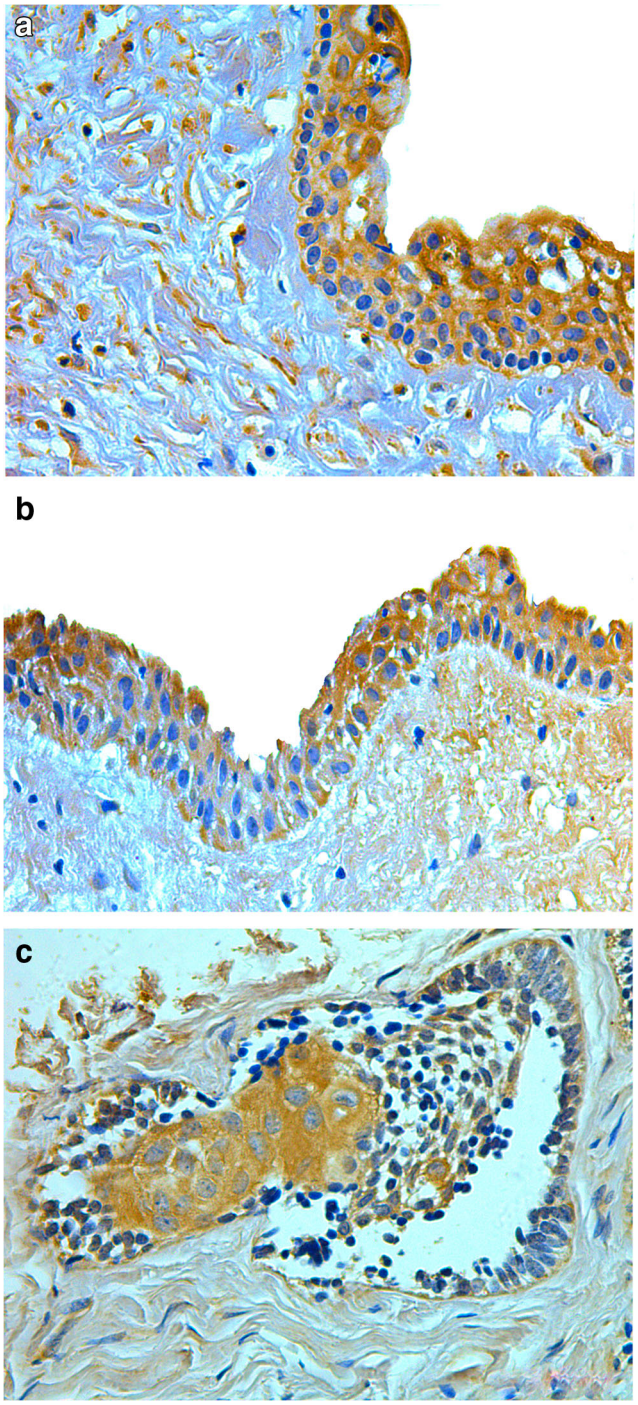

Fig. 2 Photomicrograph showing cytoplasmic immunohistochemical staining of p63 proteins in dentigerous cyst (DC) (a), odontogenic keratocyst (OKC) (b), and ameloblastoma (AB) (c) (magnification, ×400)

Table 1 Semi-quantitative analysis of p63 and Ki-67 protein expression in the studied samples

Clinical variants	No. of cases	Immunohistochemical reactivity of p63				Immunohistochemical reactivity of Ki-67			
		Absent	Weak	Mild	Strong	Absent	Weak	Mild	Strong
Dentigerous cyst	12	7 (58.3%)	3 (25.0%)	0 (0%)	2 (16.7%)	5 (41.7%)	0 (0%)	6 (50%)	1 (8.3%)
Odontogenic keratocyst	9	1 (11.1%)	2 (22.2%)	1 (11.1%)	5 (55.6%)	0 (0%)	2 (22.2%)	0 (0%)	7 (77.8%)
Ameloblastoma	15	5 (33.3%)	4 (26.7%)	2 (13.3%)	4 (26.7%)	1 (6.7%)	5 (33.3%)	3 (20%)	6 (40%)

expression was absent in 11.1%, weak in 22.2%, mild in 11.1%, and strong in 55.6% of the total OKC cases (Table 1). The expression of positive p63 stained cells in AB was in both the central stellate reticulum like cells and the peripheral columnar cells (Fig. 1). It was absent in 33.3%, weak in 26.7%, mild in 13.3%, and strong in 26.7% of the total AB cases (Table 1). Statistical analysis using Kruskal-Wallis test revealed significant differences in the expression of p63 protein among DC, OKC, and AB ($p = 0.048$). Subsequently, Mann-Whitney U test showed that the expression of p63 protein was significantly higher in OKC than DC ($p = 0.018$) (Table 2).

As mentioned in our previous reports [7, 8], Ki-67 protein expression was nuclear in the odontogenic epithelial cells of DC, OKC, and AB (Fig. 3). In the DC cases, positive cells were distributed throughout the cystic wall. Ki-67 expression was absent in 41.7%, mild in 50%, and strong in 8.3% of odontogenic epithelium (Table 1). In OKC sections, positive cells were located throughout the cystic wall of OKC. However, more positive staining rate was found in the upper epithelial layers than that of the basal epithelium (Fig. 3). All OKC samples showed positive Ki-67 staining. It was weak in 22.2% and strong in 77.8% of odontogenic epithelial cells of OKC samples (Table 1). Positive Ki-67 stained cells in AB were distributed through both the central stellate reticulum like cells and the peripheral columnar cells (Fig. 3). Ki-67 expression was absent in one case (6.7%), weak in 33.3%, mild in 20%, and strong in 40% of odontogenic epithelial cells in AB samples (Table 1). Kruskal-Wallis test revealed significant differences in the expression of Ki-67 protein among DC, OKC, and AB ($p = 0.022$). Subsequently, Mann-Whitney U test showed that the expression of Ki-67 protein was significantly higher in OKC than DC ($p = 0.007$) (Table 2).

Spearman's correlation analysis showed a positive correlation between the expression of p63 and Ki-67 in the odontogenic epithelium of DC ($\sigma = 0.757$, $P = 0.004$) and OKC ($\sigma = 0.741$, $P = 0.022$). While no such correlation was found between the two studied markers in AB group ($\sigma = 0.006$, $P = 0.983$).

Discussion

Studying of p63 expression in different odontogenic lesions will add knowledge for better understanding of the biological behavior of those lesions. p63 expression has been detected in the epithelium of tooth germ and dental follicle [14, 15]. While p63 expression in tooth germ are similar to those in human keratinocytes [14, 16], the high expression of p63 in the dental follicle of completely impacted teeth than the partially impacted one supported the prophylactic removal of impacted teeth to avoid the development of pathologies [15]. Investigators concluded that p63 plays a crucial role in the proliferation and differentiation of normal odontogenic epithelial cells. Yet, p63 could have oncogenic effect in odontogenic tumor [17, 18]. This suggested different functions of p63 in developing and neoplastic odontogenic tissues [14]. For its essential role in epithelial development, several studies have investigated the expression of p63 in the epithelium of odontogenic lesions [13, 14, 19–29].

Table 2 Statistical analysis of IHC expression of p63 and Ki-67 in odontogenic lesions

Cases	p63				Ki-67			
	Mean rank	Kruskal-Wallis test	Mann-Whitney U test		Mean rank	Kruskal-Wallis test	Mann-Whitney U test	
Dentigerous cyst	13.75	$\chi^2 = 6.078$ $P = .048$	DCs,OKCs	$Z = -2.365$ $P = .018$	12.92	$\chi^2 = 7.660$ $P = .022$	DCs,OKCs	$Z = -2.721$ $P = .007$
Odontogenic keratocyst	24.83		DCs, ABs	$Z = -1.323$ $P = .186$	25.17		DCs, ABs	$Z = -1.544$ $P = .123$
Ameloblastoma	18.50		OKCs, ABs	$Z = -1.496$ $P = .135$	18.97		OKCs, ABs	$Z = -1.552$ $P = .121$

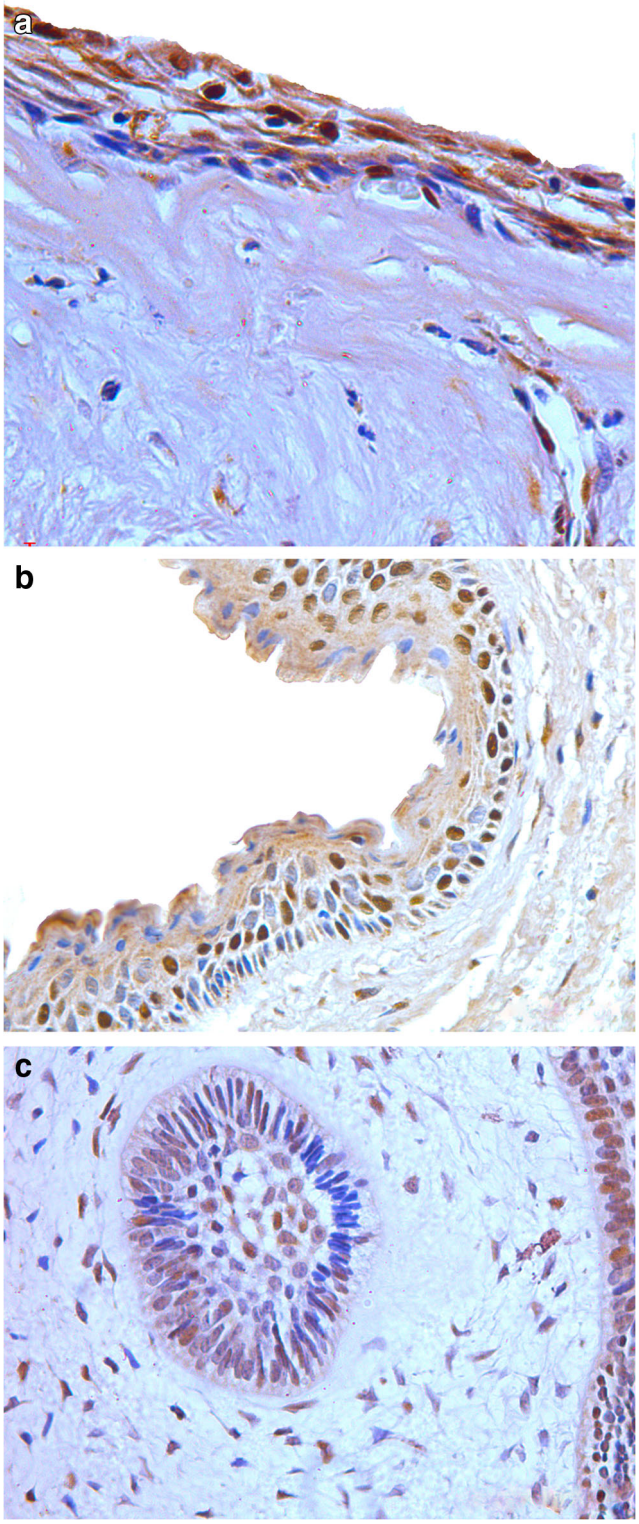

Fig. 3 Photomicrograph showing immunohistochemical nuclear staining of Ki-67 proteins in dentigerous cyst (DC), odontogenic keratocyst (OKC), and ameloblastoma (AB) (magnification, ×400). p63 expression is in all layers of DC (a), mostly through upper epithelial layers of OKC (b), and in both central stellate reticulum like cells and peripheral columnar cells of AB (c)

p63 have different isoforms, they are mainly of two subclasses, which either contain a p53-homologous transactivation domain, (TAp63) or lack this domain (ΔNp63). Alternative splicing generates different C-termini, for a total of at least 10 p63 isoforms, five in each main subclass (TA p63 α, β, γ, δ, ε and ΔNp63 α, β, γ, δ, ε) [11]. The TAp63 forms are capable of transactivating p53 target genes that favor cell differentiation and inducing apoptosis, while ΔNp63 forms behave in opposite way as a dominant negative fashion towards p53 and TAp63. Nonetheless, several evidences indicate that ΔNp63 isoforms are bona fide transcription factors which functions beyond the dominant negative action to p53 [17]. Although ΔNp63 could differently function in normal developing tissue [14], ΔNp63 isoform acts as enhancer for cellular proliferation in human tumor, suggesting that it could play an oncogenic role. In addition, ΔNp63 was found to induce oncogenesis through its regulation of metabolic reprogramming in tumors [18]. The target p63 in the present study was the ΔNp63 isoform.

Noteworthy, we identified several cytoplasmic staining of p63. Likewise, several studies showed cytoplasmic staining of p63 in different pathological epithelial cells [30–33]. The cytoplasmic expression of p63 was regarded as an adverse prognostic factor in patients with adenocarcinoma of the lung [31], breast epithelia [32], and in prostate cancer [33]. Excluding its false positive expression, a more large study is recommended to investigate the ectopic p63 expression in odontogenic cysts and tumors. Ectopic p63 expression could influence the biological behavior of odontogenic cysts and tumors. All the available data are discussing only the nuclear staining as it should be the main location of p63 protein expression in odontogenic lesions. In addition to the limited sample size of the study, another reason behind recommending only p63 nuclear staining in our study is to correlate the p63 expression with the cellular proliferation and compare it with the previous studies that exclusively recommended p63 nuclear staining.

In the current report, the higher expression of p63 in OKC could lead to more mitotic activity of epithelial cells in OKC than DC. Together with antagonizing the action of p53, ΔNp63 might contribute to tumor genesis by conferring a proliferative potential on tumor cells through the transactivation of target genes necessary for cell division [34]. Along with its effect on cell proliferation, another interpretation of ΔNp63 expression in odontogenic cysts and tumors is suggesting a possible impact of ΔNp63 in the existence of the odontogenic lesions. Both explanations could eventually lead to more aggressive behavior and recurrence rate of odontogenic epithelium in OKC and AB than DC. It has been clear that p63 is engaged in a broad spectrum of biological activities, including cell proliferation, development, differentiation, survival, senescence, and apoptosis [17]. Lo Muzio et al. found a higher expression of p63 in benign odontogenic, locally aggressive tumors with a high risk of

recurrence than benign odontogenic, non-aggressive tumors with a low risk of recurrence [21]. Furthermore, high expression of p63 was found in cyst type with more aggressive and invasive phenotype like OKC than other cysts like DC and radicular cyst [19].

Comparable to our results, previous studies found higher expression of p63 in OKC than DC [13, 19, 24], and in AB than DC [28], and non-different p63 expression in OKC, AB [26]. Contrary, other studies did not find significant differences in the expression of p63 among DC, OKC, and AB [25].

In accordance with other studies finding [13, 25], we recognized that positive staining of p63 was equally distributed through odontogenic epithelium of DC. However, other contradictory studies demonstrated p63 immunostaining was mainly in the basal and parabasal layers of DC [19, 24, 28], in addition to some extra positive staining in the intermediate layers [19].

Our study found p63 positive staining was more in the upper layers in OKC. This result could explain the findings of the previous studies that located higher mitotic activity in the upper layers of OKC [7, 35]. The more expression of ΔNp63 in the upper layers of OKC could influence the epithelial cell cycle leading to more proliferation rate in these layers of OKC. In agreement with our study, de Brito et al. found predominant p63 expression in the upper epithelial layers of nonsyndromic, both primary and recurrent OKC. However the p63 positive expression was in both basal and upper layers of syndrmoatic OKC samples [27]. Several researches found the p63 expression is through all epithelial layers of OKC [13, 22, 24]. Foschini et al. found p63 expression in all layers of recurrent OKC, but just in basal and parabasal layers of non-recurrent OKC and OKC associated with Gorlin-Goltz syndrome [20]. Contrary to our results, other previous studies found p63 positive staining in the basal and parabasal layers, while the superficial layer was negative [19, 25, 26, 29]. The discrepancy in the distribution of positive p63 stained cells in DC and OKC in the previous studies could be attributed to the different types of primary antibodies used, nonspecific recognition of the exact isoform of p63, and heterogeneity of the studied lesions. We found p63 was expressed in both peripheral central cells of AB. Consistently, several previous reports showed non-significant difference in the expression of p63 between peripheral columnar and central stellate reticular like cells of AB [14, 21, 25, 26, 28].

Ki-67 expression in DC, OKC, and AB was discussed in our previous reports [7, 8]. The present study showed a positive correlation between p63 and epithelial cellular proliferation in DC and OKC. Furthermore, the distribution of both markers have the same location in these lesions. However, statistical correlation was absent in AB samples. The difference in the correlation of p63 and proliferation index between odontogenic cyst and tumor could reflect different function and pathway of p63 in odontogenic cysts and tumors. Specifically, it could indicate a role of p63 other than just enhancing odontogenic epithelial proliferation in AB. Indeed, the ratio between TAp63 and ΔNp63 in addition to their interactions with other members of p53 family, plays a role in tumor formation and progression [17]. This results come in concurrence with the last WHO classification in 2017, which re-classified OKC from tumor, in the 2005 classification, to odontogenic cyst. Away from the proliferative capacity which is on par in both OKC and AB in the current study, a different intrinsic growth mechanisms and associations of different factors could recognize odontogenic cysts from the tumors. The last WHO reclassification was criticized denying the OKC behavior as a benign but aggressive tumor [3]. Singh suggested that OKC should be classified into two types, which are cystic and neoplastic depending on radiographic features, extent of tissue destruction, and histopathology [36]. It is clear that the last WHO classification regarding OKC as a cyst was criticized from a clinical and surgical points of view, but at the same time was acknowledged from a pathological point of view.

The importance of ΔNp63 isoform in the oncogenesis and aggressiveness of a tumor is well understood [14, 34]. Nonetheless, different studies indicated that cervical squamous cell cancer progression is associated with the reduction of ΔNp63α expression, concluding that ΔNp63α may be regarded as a marker for cervical squamous cancer differentiation [30, 37]. Correspondingly, Zhu et al. concluded that increased expression of ΔNp63 in various cancers may reflect an unsuccessful cell compensatory mechanism to prevent a high rate of tumor cell proliferation [38]. In our opinion, this controversy could be attributed to the different types of tumor and p63 isoforms detected in variable studies. While at least ten p63 isoforms are exist till now, most of the available studies do not recognize the exact isoform of p63.

Till now, no medical treatment for odontogenic cysts and tumors is available. Basically, the current management of odontogenic cysts and tumors is surgical removal which in turn leaves a considerable surgical defect and could need subsequent surgical reconstruction. Recently, several studies targeted ΔNp63 and result in the regression of oncogenesis [39, 40]. Napoli et al. demonstrated that ΔNp63 can be targeted by HDAC inhibitors that effectively reduce the levels of ΔNp63 through different pathways both in vitro and in vivo, concluding that the inhibition of ΔNp63 could result in a more efficacious strategy than reactivate mutant p53 proteins in human tumors [40]. In the current study, the high expression of ΔNp63 in odontogenic cysts and tumor could suggest the experimental analysis of anti ΔNp63 in the non-surgical management of odontogenic lesions.

Conclusion

The present study demonstrates the expression of ΔNp63 in odontogenic epithelium of DC, OKC, and AB with higher expression rate in OKC and AB than DC. Furthermore,

ΔNp63 is correlated with proliferative capacity of the odontogenic epithelium in DC and OKC, but not in AB. This diversity could reflect a different role and pathways of ΔNp63 in odontogenic tumor than that in odontogenic cyst. These together will help in better understanding the pathogenesis and biological behavior of odontogenic cysts and tumors.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval Ethical approval obtained for retrospective studies.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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