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P63 and Ki-67 expression in radicular cyst

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ABSTRACT

Objectives: The aim of the current study was to identify the expression of P63 and its relation to odontogenic epithelial cell proliferation, severity of the inflammatory infiltrate and size of radicular cysts (RCs).
Methods: In this retrospective cross-sectional study, 30 cases of paraffin-embedded RCs were randomly selected from the archive. P63 and Ki-67 expression was assessed by immunohistochemistry.
Results: Epithelial P63 expression was absent in four (13.3%), weak in 10 (33.3%), and moderate in 16 (53.3%) cases. In the connective tissue wall of RC, P63 expression was absent in two (6.7%) cases, weak in 24 (80.0%) cases, and moderate in four (13.3%) cases. Ki-67 was found to be weakly expressed in 12 (40.0%) cases, moderately expressed in 13 (43.3%), and strongly expressed in five (16.7%) cases. No correlation was found between Ki-67 expression in odontogenic epithelium and P63 expression in the odontogenic epithelium (rho = 0.110, p = .563) or fibrous capsule (rho = 0.160, p = .399). Nevertheless, we found a positive correlation between Ki-67 expression in the odontogenic epithelium and the size of the RC (rho = 0.450, p = .013). The inflammatory infiltrate was negatively correlated with P63 expression in the odontogenic epithelium (rho = −0.428, p = .018), and with the size of cysts (rho = −0.728, p < .001).
Conclusions: There is a high expression of P63 throughout the odontogenic epithelium and connective tissue capsule of the RC. P63 expression in the odontogenic epithelium is negatively correlated with the degree of the inflammatory infiltrate but not with epithelial cell proliferation or the size of the cyst.

1. Introduction

The radicular cyst (RC) is an odontogenic inflammatory cyst containing an epithelial lining enclosed within a fibrous capsule. RC is thought to be caused by inflammation of granulation tissue, resulting in stimulation of epithelial cells.[1]

P63 is a member of the P53 family. Currently, there are at least ten different isoforms of P63 that are thought to be encoded by the P63 gene. Among them, five possess the transactivating domain (TAp63), whereas the remaining five do not (ΔNp63). Biologically, these related isoforms have reversed actions. In fact, TAp63 forms are capable of transactivating p53 target genes, while ΔNp63 forms act in a dominant and negative mode towards p53.[2] Isoforms of the P63 carrying out diverse functions by affecting different pathways. Further, a range of pathways is involved in controlling P63 expression.[3] P63 was found to be a key regulator of epidermal development through studies in human diseases that are associated with P63 mutation and in different animal models.[4] Epidermal cell development and proliferation are important in several biological and pathological events like odontogenic cysts and tumors. The epithelial cells were found to predominantly express ΔNp63, which is crucial to the process of growth and regeneration like those that happen in normal proliferating cells.[5] The impact of P63 on tumors and other diseases is still being discovered.[3] Specifically, P63 could have a role in the pathogenesis, persistence, and progression of the odontogenic cysts. Understanding the role of P63 in the pathogenesis and biological behavior of odontogenic cysts could help in finding targets for non-surgical management of these lesions.

Cellular proliferation is an important factor in determining the

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biological and pathological profile of odontogenic cysts. Ki-67 is widely used as a proliferation marker because proliferating cells express it actively. Except for the resting cycle (G0), it is present during all active phases of the cell cycle.[6]

Several studies have investigated P63 expression in RC.[7–11] Intriguingly, our group have confirmed such correlation between P63 and Ki-67 expressions in dentigerous cyst and odontogenic keratocyst.[12] The aim of this study was to identify the expression of P63 and its relation to odontogenic epithelial cell proliferation, severity of the inflammatory infiltrate and size of RCs. In fact, studying P63 and Ki-67 expressions in RCs as well as their clinical and pathological correlates helps in the histopathological differentiation of this lesion from other odontogenic lesions. Additionally, it facilitates the development of a non-surgical treatment for this most common odontogenic condition.

2. Methods

2.1. Sample selection

In this retrospective cross-sectional study, 30 cases of paraffin-embedded RCs were randomly selected from the archive of the pathology laboratory at Tongji Hospital of Tongji Medical College (Wuhan, China). There was a definite diagnosis of RC in all samples, as well as an adequate tissue component. All H&E slides and pathology reports of the cases were reviewed and approved for the study. Data from patients' medical records were retrieved, including their age, gender, and location of lesions. The Institutional Review Board at Tongji Medical College approved this study in accordance with the Declaration of Helsinki (Ref. TJ-C20100108). Consent was obtained from all subjects. The diagrammatic representation of the methodology is shown in figure (1).

2.2. Immunohistochemistry protocol

A standard streptavidin-biotin-peroxidase complex (SABC) immunostaining method was used. A 5 μm thick tissue section was cut from a formalin-fixed paraffin-embedded samples. The sections were deparaffinized in xylene, dehydrated in alcohol, and washed in distilled water. The antigen was unmasked by immersing the samples in 0.01 M citrate buffer that was heated to boiling in a microwave oven after 3% H2O2 was added to inhibit internal peroxidase activity. Afterwards, the samples were treated with goat serum for a period of 50 min at room temperature. A diluted 1:100 solution of primary antibodies was then incubated with the sections at 4 °C overnight. Both primary antibodies were diluted at 1:100 and consisted of polyclonal rabbit anti-human Ki-67 antigen (Catalog No. PB9026, Wuhan Boster Biological Technology, Ltd., Wuhan, China) and polyclonal rabbit anti-human P63 antigen which mainly recognizes ΔNp63 isoforms (Catalog No. BA1326, Wuhan Boster Biological Technology, Ltd., Wuhan, China). Then after, the samples were treated with a biotinylated secondary antibody (10 μg/ml) at room temperature for 2 h. After that, the tissue sections were incubated with SABC (Wuhan Boster Biological Technology, Ltd.). Lastly, the tissue sections were developed with 3,3′-Diaminobenzidine substrate and counterstained with Mayer's hematoxylin. For both antibodies, the positive control was normal skin. Meanwhile, a negative control sample was incubated in PBS instead of the primary antibodies.

2.3. Evaluation of immunohistochemical expression and the inflammatory infiltrate

The histological slides were analyzed using a light microscope (Olympus BX43, Center Valley, PA). Immunohistochemical staining of P63 was detected in the epithelium and the connective tissue wall of the studied samples. The positivity was mainly nuclear and, to a lesser extent, cytoplasmic. Nevertheless, Ki-67 staining was exclusively nuclear in the odontogenic epithelial cells of RC. The scoring of Ki-67 in the cystic epithelium and P63 staining in both epithelium and connective tissue wall was achieved by counting the percentage of positive cells out of the total cells in 10 continuous and representative high-power fields

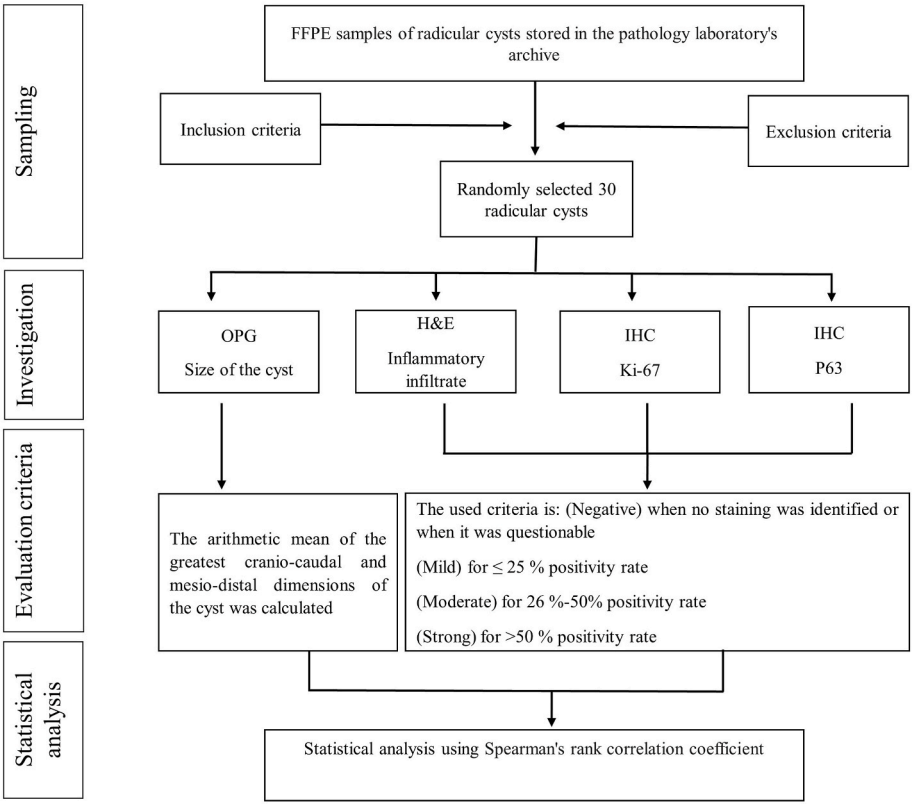

Fig. 1. A diagrammatic representation of the chronology of the methodology.

(400× magnification). The scores were 0, when no staining was identified or when it was questionable; mild for ≤25% positivity rate; moderate for 26 %-50% positivity rate, and strong for >50% positivity rate. Chronic inflammation in the cystic capsule is classified as mild if the proportion of chronic inflammatory cells is less than 25%; moderate for 26–50% positivity rate, and strong for >50% positivity rate.

2.4. Radiographic evaluation

Using standard panoramic images, the arithmetic mean of the greatest cranio-caudal and mesio-distal dimensions of the cyst was calculated.

2.5. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 28.0 software (IBM SPSS, Armonk, NY, USA) was used to analyze the data. We calculated the frequency as well as the percentage of variables and assessed the correlation between the parameters using Spearman's rank correlation coefficient. A statistically significant result was defined as ($P < .05$).

3. Results

The study included 30 RC samples. Eight (26.7%) of them were females and 22 (73.3%) of them were males. There were six cases located in the mandible (20%), while the remaining 24 cases were located in the maxilla (80%). The mean age was 38.97 years.

The studied RC samples contained fibrous connective tissue walls that were lined by a stratified squamous epithelium of variable thickness, or by epithelial hyperplasia. The inflammatory cells like neutrophils, plasma cells and lymphocytes were a common finding in the studied samples. Occasionally, cholesterol clefts and Russell bodies were found in the wall. There were three cases in which the inflammatory infiltrate was weak, nine cases in which it was moderate, and 18 cases in which it was strong (Table 1; Fig. 2).

For Ki-67 positivity, only nuclear staining was regarded as a positive result while almost nuclear but some cytoplasmic staining was recommended for P63 staining. Positively stained P63 and Ki-67 cells were present invariably in all layers of the odontogenic epithelium (Fig. 3). Additionally, P63 was expressed in several inflammatory infiltrate cells as well as other cells of the cystic wall, including endothelial cells and fibroblasts (Fig. 3).

Epithelial P63 expression was absent in four, weak in 10, and moderate in 16 cases (Table 1). In the connective tissue wall of RC, P63 expression was absent in two cases, weak in 24 cases, and moderate in four cases (Table 1). Ki-67 was found to be weakly expressed in 12 cases, moderately expressed in 13, and strongly expressed in five cases (Table 1).

Statistical analysis using Spearman's correlation test showed non-significant correlation between Ki-67 expression in odontogenic epithelium and expression of P63 in odontogenic epithelium (rho = 0.110, p = .563) and fibrous capsule (rho = 0.160, p = .399). Nevertheless, we found a positive correlation between Ki-67 expression in the odontogenic epithelium and the size of the RC (rho = 0.450, p = .013). The inflammatory infiltrate was negatively correlated with the expression of P63 in the odontogenic epithelium (rho = −0.428, p = .018), and the size of cysts (rho = −0.728, p < .001) but not with P63 expression in the fibrous capsule (rho = −0.051, p = .790), nor with Ki-67 expression in the epithelium (rho = −0.094, p = .621) (Table 2).

Table 1
Expression of P63 and Ki-67, and degree of inflammatory infiltrate in the studied radicular cysts.

	Absent	Weak	Moderate	Strong
P63 expression in odontogenic epithelium	4 (13.3%)	10 (33.3%)	16 (53.3%)	0 (0%)
P63 in the connective tissue capsule	2 (6.7%)	24 (80.0%)	4 (13.3%)	0 (0%)
Ki-67 expression in odontogenic epithelium	0 (0%)	12 (40.0%)	13 (43.3)	5 (16.7%)
Inflammatory infiltrate	0 (0%)	3 (10.0%)	9 (30.0%)	18 (60.0%)

4. Discussion

The primary antibody against P63 used in our study detected the ΔNp63 isoforms. We found a high positivity rate for P63 expression in variable cells of RC. Specifically, stronger expression was found in the odontogenic epithelium than in the connective tissue wall. In the current study, the epithelial positivity rate of P63 was 86.66%. Several previous studies investigated P63 expression in RC.[7–11] Positivity rates were 85.7%,[7] 91.43,[9] and 100%[8,10,11] in the odontogenic epithelium of the studied cysts. It is clear that P63 expression in the present study is in comparison with the available findings.

The comparison of P63 expression in RC with other odontogenic cysts was contradictory in the previous studies. Several studies found that P63 expression is higher in odontogenic keratocyst than dentigerous cyst[7–9,12] and RC.[7–9] Other studies found a higher positivity rate of P63 in the odontogenic epithelium of RC than that of dentigerous cyst[8] and apical granuloma.[11] Contrary, no significant difference was observed in P63 expression among RC, odontogenic keratocyst, and dentigerous cyst.[10] The high expression of ΔNp63 in RC samples could help in the existence and persistence of these intra bony lesions. Indistinguishably, ΔNp63 expression is increased in the cells of squamous cell carcinoma of the head and neck which helps in their continued survival.[13]

In the present study, P63 positively stained cells were distributed impartially through all layers of the epithelial lining of RC. This finding is in accordance with another study that found a high P63 positivity rate in all layers of RC.[9] However, several studies found that the staining of P63 was mainly in the basal and parabasal layers with a weaker expression in other layers.[7,10] Another study found that the positivity was also in the intermediate layer in addition to the basal and parabasal layers.[8] This controversy in the location of P63 expression in the previous studies could be attributed to the diversity in the studied P63 isotypes. Most of the previous studies addressed P63 expression in general and were not designed to find the specific main isoforms of P63, let alone, evaluating the different subtypes of the two main isoforms. Therefore, each of the subtypes could have a predominant location within the epithelium. In fact, the balance between the two main isoforms, ΔNp63 and TAp63, is necessary for regulating cellular fates, such as apoptosis versus survival, maintenance of uncommitted stem cells versus differentiation, and tumor suppression versus tumorigenesis.[14] P63 positivity was also found in the connective tissues of our samples. Correspondingly, a previous study also noticed such positivity in the connective tissue of RC in addition to the epithelium.[11] Contrary, another study demonstrated that ΔNp63 isoform is restricted to the epithelium. Meanwhile, TAp63 protein was present in both epithelium and connective tissue walls like endothelium and lymphoid cells.[13]

In our study, all RCs were found to express Ki-67 varying in intensity. However, P63 staining was negative in 13.3% of the epithelium and 7.6% of capsules in the studied RCs. Similarly, a previous study found that over one third of RCs studied showed negative staining for P63.[7] This negativity of P63 might indicate a better state of disease because ΔNp63 helps maintain the integrity of the epithelium of a lesion, as here in RCs. Noteworthy, in the present study methodology, we used a score of 0 when the staining was questionable, along with a condition of non-identification of the staining. This could also explain why some samples were negative for delta P63 staining. It has been proposed that inhomogeneous tissue fixation, inefficient immunostaining or other preanalytical factors can result in questionable and negative

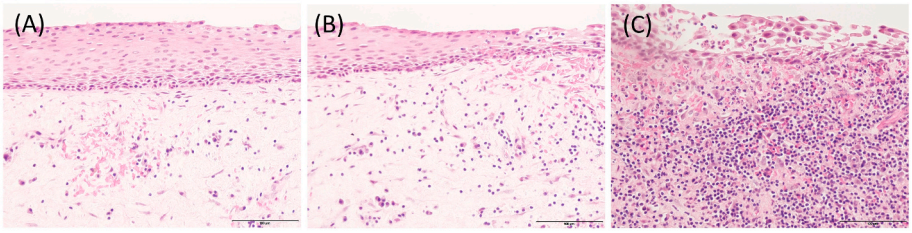

Fig. 2. Inflammatory infiltrate in the connective tissue capsule of the studied radicular cyst (HE X 40). (A) the inflammatory infiltrate is mild; (B) the inflammatory infiltrate is moderate; and (C) the inflammatory infiltrate is strong.

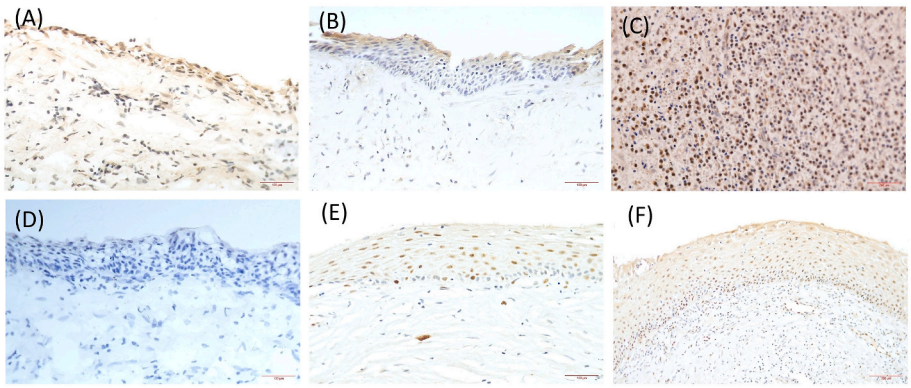

Fig. 3. Immunohistochemical staining of P63 and Ki-67 in radicular cysts (magnification, ×400). (A) Strong P63 positivity through the epithelium and connective tissue, (B) moderate P63 positivity mainly through the epithelium, (C) strong P63 positivity in the inflammatory cells throughout the connective tissue, (D) absence of P63 staining in the epithelium and connective tissue wall, (E) moderate Ki-67 staining in the nucleus of the positive cells throughout the whole epithelium, and (F) strong Ki-67 positivity throughout epithelium and in many cells in the connective tissue capsule.

Table 2
Correlations between P63, Ki-67, inflammatory infiltrate, and size of the studied radicular cysts.

	P63 expression in odontogenic epithelium	P63 expression in the connective tissue capsule	Ki-67 expression in odontogenic epithelium	Inflammatory infiltrate	Size of the cyst
P63 expression in odontogenic epithelium	rho = 1.000 p = .	rho = −.284 p = .186	rho = .110 p = .563	rho = −.428* p = .018*	rho = .228 p = .226
P63 expression in the connective tissue capsule	rho = −.284 p = .186	rho = 1.000 p = .	rho = .160 p = .399	rho = .051 p = .790	rho = −.020 p = .918
Ki-67 expression in odontogenic epithelium	rho = .110 p = .563	rho = .160 p = .399	rho = 1.000 p = .	rho = −.094 p = .621	rho = .450* p = .013*
Inflammatory infiltrate	rho = −.428 p = .018*	rho = .051 p = .790	rho = −.094 p = .621	rho = 1.000 p = .	rho = −.728** p < .001**
Size of the cyst	rho = .228 p = .226	rho = −.020 p = .918	rho = .450* p = .013*	rho = −.728** p < .001**	rho = 1.000 p = .

* = Significant.
** = Highly significant.

immunohistochemical staining.[15]

In the current results, there was a negative correlation between the degree of the inflammatory infiltrate in the connective tissue and P63 expression in the odontogenic epithelium of RC. Comparably, a previous study demonstrated a declined expression of P63 in epithelium next to areas of severe inflammatory infiltrate, attributing it to the inhibitory effect of the inflammatory infiltrate to P63 protein production.[9] Another study also found a negative correlation but not reaching the significance between P63 expression and the immune reaction in samples of dentigerous cyst.[16] Besides, it has been shown that decreased ΔNp63 and adjuvant increased p53 expression in oral lichen planus and graft versus host disease when compared with normal oral mucosa, indicating a synchronized action of these two proteins in protecting the tissue from DNA damage of the underlying chronic inflammation.[13]

Cellular proliferation is regarded as an important factor in determining the biological and pathological profile of odontogenic cysts. Indeed, Ki-67 gives an impression of the behavior of related lesions.[6] Ki-67 was already used as a proliferative marker for evaluating the proliferative activity in odontogenic cysts.[17–21] The current study evaluated the expression of Ki-67 in the odontogenic epithelium of RC in order to determine the proliferative capacity of the epithelial cells in this cyst and correlate it with P63 expression. It has been concluded that epithelial cells are more important than connective tissue cells when considering the biological behavior of odontogenic lesions.[20] The previous studies demonstrated a variable positivity rate of Ki-67 in RCs. It was 53.8%,[17] 60%,[18] and 93.33%.[21] Additionally, there is controversy when comparing the expression of Ki-67 in RC with other odontogenic cysts. It has been found that Ki-67 expression in odontogenic keratocyst was more than that of RC[17,19,20] and dentigerous cyst.[21] This significance was confined to the suprabasal layer than the basal layer.[18] Moreover, Ki-67 expression was more in RC than dentigerous cyst[19] and vice versa.[21]

In contrast to several previous studies that found expression of Ki-67 is mainly in the basal epithelial layer,[17,18,20] we found that Ki-67 positive cells were distributed impartially throughout the epithelium of RC. In fact, the thickness and layers of epithelium of RC is not constant but vary in different samples depending on the duration of the lesion and the severity of the inflammatory infiltrate. The present findings are

consistent with the results of a previous study that found that all epithelium showed positivity with a little more presence in the basal layers of RC.[21]

In the present study, we did not find a significant correlation between the degree of inflammatory infiltrate and Ki-67 expression in the odontogenic epithelium. A contradiction is present when correlating the severity of inflammation with epithelial cell proliferation of the RC. Ayoub et al. demonstrated a higher Ki-67 expression with the increase in the inflammatory infiltrate in the connective tissue wall of RC, assuming that chronic inflammation triggers epithelial cell proliferation.[17] Similarly, the mean of Ki-67 expression in the lining epithelium of RC with intense subepithelial inflammation was significantly higher than those with less subepithelial inflammation.[22] In addition, it has been shown that higher expression of Ki-67 in the dental follicle with marked inflammatory changes than with less inflammation.[19] Although there is evidence that inflammatory change initiates RC formation by reactivation of the epithelial rests of Malassez,[23] this action is not necessary to be regarded as the direct stimulator of the odontogenic epithelial cells proliferation and other pathways should be considered in this concern. In accordance with our results, a previous study found that there was no correlation between adjacent inflammation and both proliferative markers, Ki-67 and PCNA, in the epithelium of odontogenic keratocyst.[24]

It has been proposed that inflammatory infiltrate may vary in type and density in RC walls based on the duration of the lesion.[25] The current results revealed a negative correlation between the degree of the inflammatory infiltrate and the size of the RCs. Jurisic et al. found that higher TNF-a levels were associated with more inflammatory cells and smaller radicular cysts, which is consistent with our results.[26] A noteworthy finding in the present study was the positive correlation between Ki-67 expression and RC size. In other words, the larger RCs could have more aggressive behavior than the smaller ones. Comparably, a previous study concluded that high Ki-67 expressions in odontogenic keratocysts correlate with aggressive clinical behavior and a high recurrence rate.[20] Furthermore, the mean expression of Ki-67 in RCs was higher when the peripheral cortical plate was perforated than when the cortical plate was intact.[27] However, the former study did not find a positive correlation between Ki-67 expression and the size of RCs, proposing that the intrinsic proliferative ability of the epithelial lining cells may have contributed to these differences in RC proliferative ability.

We found a non-significant correlation between Ki-67 expression in the odontogenic epithelium and P63 expression in both the odontogenic epithelium and connective tissue wall of the studied cysts. This is in concurrence with a previous study that did not find such correlation in samples of dentigerous cyst, unicystic ameloblastoma and ameloblastoma.[16] Intriguingly, our group have previously found such correlation in samples of dentigerous cyst and odontogenic keratocyst.[12] Indeed, RC differs from odontogenic keratocyst and dentigerous cyst in that it is an inflammatory cyst while odontogenic keratocyst and dentigerous cyst are developmental in origin. Differences in the correlations of Ki-67 and P63 in various lesions could be helpful in the histopathological differentiation of these lesions. In this regard, it could be helpful in differentiating RC from dentigerous cyst and odontogenic keratocyst. Moreover, these results could indicate a different role for P63 in the pathogenesis of RC from other developmental jaw cysts. We further recommend the use of different cell markers to specifically identify cell populations inside RC. Previous studies had no consensus about utilizing P63 as a marker of the aggressiveness of the odontogenic lesions. Some of them found that P63 expression might be helpful in identifying cysts with a more invasive and aggressive phenotype,[7,8] another study proposed that P63 could not be used as a marker for detecting the aggressiveness of odontogenic lesions.[10]

P63 was concluded to be involved in differentiation and proliferation of odontogenic epithelial cells.[10] Basically, ΔNp63 is proposed to maintain the proliferative capacity of the related cells by blocking the action of p53.[28] It is conceivable that the control of odontogenic cellular proliferation in any lesion is the product of the activity and interactions of multiple pathways and not dependent on a single factor. For example, Soluk et al. demonstrated that higher proliferative activity in odontogenic keratocyst and ameloblastoma is due to increase in the pro-apoptotic protein, bax and reduction of the anti-apoptotic protein, bcl-2 in epithelial cells of RC in comparison to odontogenic keratocyst and ameloblastoma.[20]

A strength of this retrospective study is that the histopathological section was approved and then revised by the researchers. Additionally, it is a correlational study that correlates immunohistochemical markers with clinical findings like cyst size. Furthermore, immunohistochemical markers were evaluated numerically, then graded, allowing for unbiased analysis. The results of this study must be viewed in the context of certain limitations. In the current study, samples of control groups were not included. RC encompasses reactive tissues that replace healthy bone, thus there is no true tissue equivalent to serve as a control. Furthermore, this study uses conventional histopathology to identify the various inflammatory cells and the connective tissue capsule of RC based on their appearance and distribution patterns. Indeed, cell-specific markers can provide more precise results. However, another staining may interfere with the immunohistochemistry of the current methodology. Additionally, a more comprehensive approach to targeting the proliferative capacity of the odontogenic epithelium and its relation to P63 protein through different pathways by genomic, transcriptomic, and proteomic analyses is recommended.

5. Conclusions

There is high expression of P63 throughout the odontogenic epithelium and connective tissue capsule of the RC. The expression of P63 in the odontogenic epithelium is negatively correlated with the degree of the inflammatory infiltrate but not with epithelial cell proliferation or the size of the cyst. Additionally, the larger RCs showed a higher proliferation rate of odontogenic epithelial cells. The identification of this pattern of P63 and Ki-67 expression in radicular cysts allows histopathological differentiation from other odontogenic lesions and may assist in the development of non-surgical treatment of radicular cysts. For a better understanding of the role of P63 protein in odontogenic epithelium proliferation, further studies with genomic, transcriptomic, and proteomic analyses are recommended.

Declaration of competing interest

The authors declare no conflict of interest.

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