Immunohistochemical expression of protein 53, murine double minute 2, B-cell lymphoma 2, and proliferating cell nuclear antigen in odontogenic cysts and keratocystic odontogenic tumor

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ABSTRACT

Introduction: Even though odontogenic cysts share a similar histogenesis, they show different growth and differentiation profile due to differences in the proliferative cellular activity.

Aims: We perform an immunohistochemical assessment of protein 53 (p53), proliferating cell nuclear antigen (PCNA), B-cell lymphoma 2 (bcl-2), and murine double minute 2 (MDM2) expression in odontogenic cysts and keratocystic odontogenic tumor analyzing their correlation with the biological behavior of these lesions.

Materials and Methods: By the streptavidin–biotin-peroxidase method with antibodies against p53, PCNA, bcl-2, and MDM2 proteins, 11 radicular cysts, 11 dentigerous cysts, and 11 keratocystic odontogenic tumor were analyzed. The non-parametric Mann–Whitney U-test and Kruskall–Wallis test (P ≤ 0.05) were used to analyze the data.

Results: Immunopositivity for PCNA was observed in all cases appraised, predominantly in the suprabasal layer of keratocystic odontogenic tumor epithelial lining (SD ± 19.44), but no significant differences were found among the groups of lesions. Bcl-2 immunoexpression was observed especially in the basal layer of keratocystic odontogenic tumor. PCNA LI was significantly higher than bcl-2 LI in keratocystic odontogenic tumor. MDM2 and p53 immunoexpression were not detected in the lesions studied. Among the evaluated lesions, the keratocystic odontogenic tumor showed different immunoexpression of the proliferation and apoptosis markers.

Conclusion: The results of this study suggest that the keratocystic odontogenic tumor presents distinct biological behavior of the odontogenic cysts, as for the processes of proliferation, apoptosis, and differentiation, reinforcing the information in favor of the neoplastic nature of this lesion.

Key words: B-cell lymphoma 2, dentigerous cyst, keratocystic odontogenic tumor, murine double minute 2, protein 53, proliferating cell nuclear antigen, radicular cyst
and OKC demonstrating more significant reactivity in the epithelial lining of OKCs. Moreover, half of the analyzed cases of OKCs (10 cases) showed epithelial dysplasia, of which 8 cases were p53 immunopositive.

A study performed by Carvalhais et al. revealed no p53 immunoeexpression in odontogenic cysts and tumors. On the other hand, murine double minute 2 (MDM2) immunoeexpression was found to be higher in ameloblastomas compared with RGs and there was increased MDM2 reactivity in OKCs.

Piatelli et al. found a B-cell lymphoma 2 (bcl-2) labeling index (LI) of 50% in OKCs and the absence of this protein in others odontogenic cysts, suggesting that disturbances in apoptosis may be related to the aggressive biological behavior of OKCs. The authors also observed a total proliferating cell nuclear antigen (PCNA) cell count of 23.5% in OKCs, especially in the suprabasal layer, suggesting that OKCs exhibit a suprabasal proliferative compartment, which could be related to its unfavorable clinical course and high recurrence rate. Comparing OKCs and DCs, Kichi et al. observed a highest p53-positive index in the intermediate layer of OKC epithelial lining, and a predominance of bcl-2-positive cells in the basal layer of OKCs.

In 16 cases of syndromic and non-syndromic OKC, observing that p53 and cyclin D1 immunoeexpression were not detected in either group. Furthermore, PCNA expression showed no significant differences between syndrome and non-syndrome OKC. Based on the controversial results in the literature, the aim of this study was to perform an immunohistochemical assessment of p53, PCNA, bcl-2, and MDM2 expression in the epithelial lining of RGs, DCs, and KCOTs to analyze their correlation with the biological behavior of these lesions.

**MATERIALS AND METHODS**

**Tissue specimens**

This study evaluated 11 cases of RGs, 11 cases of DCs, and 11 cases of keratocystic odontogenic tumor (KCOTs) randomly selected from the files of the Pathological Anatomy Service of the Oral Pathology Discipline at the Dentistry Department of the Rio Grande do Norte Federal University. This experiment was submitted to and approved by the Bioethics Committee of the referred institution.

**Immunohistochemical methods**

Paraffin tissue sections of 3 µm thick, were deparaffinized and immersed in methanol with hydrogen peroxide to eliminate endogenous peroxidase activity. For antigen retrieval, the sections for p53, MDM2, and PCNA were treated by heating in a Steamer (96°C) citrate buffer (pH 6.0) for 30 minutes. In the case of bcl-2, antigen retrieval was performed using an EDTA solution (pH 9.0) for 30 minutes (Steamer at 96°C). After treatment with normal serum, the specimens were incubated in a humidity chamber with the antibodies for p53, MDM2, PCNA, and bcl-2 [Table 1]. Following incubation with the primary antibodies, the sections were washed twice in phosphate buffered saline (PBS), incubated with an appropriate biotinylated secondary antibody, and treated with streptavidin–biotin complex (Dako) for 30 min at room temperature. The sections were visualized with dianminobenzidine (D5637; Sigma Chemical, USA), and finally counterstained with Mayer’s hematoxylin and coverslipped. Breast adenocarcinoma known to be positive for all the antibodies was used as the positive control. For a negative control, a slide was stained for each antibody, with the omission of the primary antibody.

**Evaluation of staining**

Microscopically each stained section was evaluated for the presence of immunoreactivity by counting positive cells in basal and suprabasal layers of the cysts under high-power magnification (×1000). The quantitative assessment of immunoreactivity to the antibodies used was realized by counting 1000 cells in 10 fields randomly selected. Following cell counting, the LI was calculated in each section by dividing the number of positive cells by the total number of cells.

**Statistical methods**

Positive counting cells (LI for p53, PCNA, bcl-2, and MDM2) were presented as mean ± standard deviation. The Kruskall–Wallis test was used to analyze differences in LIs among the different lesions and the Mann–Whitney U-test to analyze differences in LIs between the cellular layers of KCOT. A $P \leq 0.05$ was considered to be significant.

**RESULTS**

All studied cases were immunopositive for PCNA. Overall, RGs and DCs demonstrated fewer PCNA-positive cells, which were more common in areas of inflammation [Figures 1 and 2]. The highest number of PCNA-positive cells was observed in KCOT ranging from 12.2% to 78.2% and a mean of

**Table 1: Antibodies used for immunohistochemistry**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clonality</th>
<th>Dilution</th>
<th>Treatment</th>
<th>Incubation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bcl-2 (124)*</td>
<td>Polyclonal (IgG1/Kappa)</td>
<td>1:50</td>
<td>Steamer, 96°C, citrate pH 6.0</td>
<td>30</td>
</tr>
<tr>
<td>MDM2 (SMP14)*</td>
<td>Monoclonal (IgG1/Kappa)</td>
<td>1:25</td>
<td>Steamer, 96°C, citrate pH 6.0</td>
<td>30</td>
</tr>
<tr>
<td>p53 (DO-7)*</td>
<td>Monoclonal (IgG2b/Kappa)</td>
<td>1:80</td>
<td>Steamer, 96°C, citrate pH 6.0</td>
<td>30</td>
</tr>
<tr>
<td>PCNA (PC10)*</td>
<td>Monoclonal (IgG2a/Kappa)</td>
<td>1:50</td>
<td>Steamer, 96°C, citrate pH 6.0</td>
<td>30</td>
</tr>
</tbody>
</table>

*Dako, Glostrup, Denmark, PCNA=Proliferating cell nuclear antigen, MDM2=Murine double minute 2, bcl-2=B-cell lymphoma 2, p53=Protein 53, IgG=Immunoglobulin G
Odontogenic cysts: p53, MDM2, bcl-2, PCNA

42.6% (SD ± 19.44). Nevertheless, no significant differences were found among the groups due to the high variation coefficient of positivity [Table 2].

The predominant suprabasal distribution of PCNA positivity was a consistent finding in the epithelium of KCOT and the total number of positive cells was significantly higher in this layer [Table 3] [Figure 3].

Bcl-2 immunoexpression was seen only in KCOT, especially in the basal layer [Figure 4]. The Kruskall–Wallis test demonstrated that PCNA LI was significantly higher than bcl-2 LI in KCOT. Finally, p53 and MDM2 immunoexpressions were not detected in the lesions studied.

DISCUSSION

Comparative studies in odontogenic cysts that have investigated the growth and differentiation of epithelial cells demonstrated differences in the proliferative activity of these entities. Even though odontogenic cysts share a similar histogenesis, these studies were able to indicate the more aggressive biological behavior of OKCs now designated by the World Health Organization as a keratocystic odontogenic tumor (KCOT). [9-11]

Our results showed a higher number of PCNA-positive cells in KCOTs; however, no differences could be found between this lesion and the studied cysts. It is important

<table>
<thead>
<tr>
<th>Specimen</th>
<th>N</th>
<th>Mean±SD</th>
<th>IC (95%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCOT</td>
<td>11</td>
<td>42.60±19.44</td>
<td>29.537-55.663</td>
<td>0.095*</td>
</tr>
<tr>
<td>DC</td>
<td>11</td>
<td>31.84±19.41</td>
<td>18.806-44.884</td>
<td></td>
</tr>
<tr>
<td>RC</td>
<td>11</td>
<td>26.45±24.40</td>
<td>10.061-42.848</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskall-Wallis test, KCOT=Keratocystic odontogenic tumor, DC=Dentigerous cyst, RC=Radicular cyst, SD=Standard deviation, PCNA=Proliferating cell nuclear antigen, IC=Confidence interval

<table>
<thead>
<tr>
<th>Epithelium layers</th>
<th>Mean±SD</th>
<th>IC (95%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>18.54±4.76</td>
<td>15.347-21.744</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Suprabasal</td>
<td>38.90±5.55</td>
<td>35.175-42.643</td>
<td></td>
</tr>
</tbody>
</table>

*Mann-Whitney U test, KCOT=Keratocystic odontogenic tumor, SD=Standard deviation, PCNA=Proliferating cell nuclear antigen, IC=Confidence interval

Figure 1: Moderate immunostaining of PCNA predominantly in basal cells of the inflammations areas of RC (original magnification ×400)

Figure 2: Weak immunostaining of PCNA predominantly in basal cells of DC (original magnification ×400)

Figure 3: Intense immunostaining of PCNA predominantly in suprabasal cells of KCOT (original magnification ×400)

Figure 4: Intense immunostaining of bcl-2 in the basal layer of KCOT (original magnification ×400)
to emphasize that some RCs showed an increased number of PCNA-positive cells in relation to the number observed in KCOTs. A high variation coefficient of PCNA positivity was also verified. These data are supported by others studies that demonstrated means of PCNA positivity ranging from 15.8% to 94.4%, confirming the high proliferative rate of KCOTs.\(^\text{[1,3,5,6]}\)

The differences in the number of PCNA-positive cells in these studies may be related to sample features, such as: Association with the nevoid basal cell carcinoma syndrome (NBCCS), presence of inflammation,\(^\text{[14]}\) dysplastic alterations in the epithelium, non-standardization of technical procedures, used antibody, and others. We understand that these data are not always available and sometimes the reports do not mention important criteria.

On the other hand, it is known that the NBCCS association does not strictly correlate with all clinical manifestations since the syndrome is transmitted as a hereditary autosomal dominant pattern having high penetrance but with variable expressivity. This is in agreement with the study performed by Barreto et al.,\(^\text{[15]}\) who attributed a possible molecular origin for KCOTs related to the loss of one copy of the gene-PTCH, a tumor suppressor gene involved in both NBCCS and sporadic KCOTs that occurs on chromosome 9q22.3-q31.

Regarding the evolution of odontogenic cysts, it is important to emphasize that when inflammation is present in the wall of the cyst, it must be accompanied by an alteration in expression of PCNA, due to the growth factors and cytokines released during the inflammatory process.\(^\text{[13]}\) Thus, the value of PCNA immunoexpression in diverse experiments must be strictly analyzed before taking it into consideration. Furthermore, PCNA immunoexpression does not only reflect cell proliferation and would be related to other phenomenon, such as DNA repair,\(^\text{[16]}\) stimulation of RNAm by autocrine/paracrine growth factors, and others.\(^\text{[17]}\) Thus, it is reasonable to suggest that high levels of PCNA expression may be found in non-proliferating cells.\(^\text{[18]}\)

It must also be emphasized that besides the higher levels of PCNA immunoexpression in KCOT, which are similar to the levels found in dysplastic and neoplastic lesions, the most diffuse topographical localization of labeled cells and their significant increase throughout suprabasal layer strengthen the distinct behavior of the lesion formerly known as OKC suggesting a neoplastic profile,\(^\text{[3,5,6]}\) justifying its current reclassifications as KCOTs.

In this study, one finding that distinguishes KCOTs from the cystic lesions availed is the basal localization of bcl-2-immunopositive cells. A comparative study performed by Piatelli et al.,\(^\text{[9]}\) demonstrated a strong bcl-2 immunoexpression in the basal layer of the epithelial lining of OKC and negative cells in DC and RC, suggesting that abnormal control of the cell cycle inhibits apoptosis in OKC, increasing cell life and stimulating its proliferation. The highest bcl-2-positive ratio in the basal layer of OKC epithelial lining observed by Kichi et al.\(^\text{[2]}\) supports this idea because bcl-2 inhibits apoptosis to facilitate cellular proliferation in the basal layer, whereas apoptosis occurs in the surface layer of OKCs in order to maintain the homeostasis of the thickness of the epithelial lining and allows the synthesis of large amounts of keratin in this layer of these lesions.

Our results demonstrated non-reactivity for p53 and MDM2, but PCNA and bcl-2 immunoexpression were marked findings which are in agreement with the findings of Carvalhais et al.,\(^\text{[13]}\) and Piatelli et al.,\(^\text{[5]}\) respectively. A significantly higher expression of PCNA-positive cells in KCOT was observed, especially in the suprabasal epithelial layer. This finding was also noted in preliminary studies of Li, Browne, and Matheus,\(^\text{[3]}\) El Mutardi et al.,\(^\text{[1]}\) Takahashi et al.,\(^\text{[6]}\) and Piatelli et al.\(^\text{[5]}\) The predominant suprabasal distribution of PCNA-positive cells in KCOT epithelial lining could be related to its aggressive clinical course and high risk of recurrence, due to the transference of the proliferative compartment to suprabasal cell layer.

In the reviewed literature, only a few studies indicate the use of p53 for differential diagnosis among odontogenic cysts. This can be explained by the low degree of mutation observed in odontogenic cysts, contrary to reports for KCOT except to the lesions related to NBCCS. In spite of the scarce literature in this regard, it can be observed that the results are discrepant and contradictory.

These conflicting results may be attributed to the complex biology of p53 or the distinct sources and availability of antibodies. For example, Lombardi, Odell, and Morgan,\(^\text{[8]}\) Slootweg and Li, Browne used CM1 monoclonal antibody and BP5312-1 polyclonal antibody for wild and mutant proteins, respectively. As performed by Carvalhais et al.,\(^\text{[13]}\) and Gadbach et al.,\(^\text{[7]}\) we used DO-7 monoclonal antibody, diluted 1:50, which is not able to recognize all p53 residues, only the N-terminal epitope located between amino acid 19 and 26, a placement that identifies mutated p53.

Since the time when the actual known KCOT was considered as an odontogenic cyst was discussed its neoplastic nature, the results of Lo Muzzio et al.,\(^\text{[4]}\) indicate that the p53 immunoexpression present in syndromic and non-syndromic OKC is related to its neoplastic potential which shows variation according to the inherent features of the patient and is not strictly correlated to NBCCS. Molecular evidence support the idea today accepted about the neoplastic nature of OKC that contributed to it’s reclassification as OKCT, as an allelic loss at two or more
Odontogenic cysts: p53, MDM2, bcl-2, PCNA

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loci of 9q22 leading to overexpression of bcl-1 and TP53 in the NBCCS. There is also evidence that the PTCH gene might be a significant factor in the development of sporadic KCOT (19). Evidence has shown that the pathogenesis of NBCCS and sporadic KCOTs can involves the allelic loss at 9q22 known as “loss of heterozygosity” (LOH). This event leads to the deregulation of the oncoproteins cyclin D1 and p53 in KCOTs.

The literature shows that LOH in the 9q22.3-q31 region has been reported for many epithelial tumors, including basal cell carcinomas, squamous cell carcinomas and transitional cell carcinomas, suggesting that LOH is a feature of tumorigenic tissue.

The recurrence rate could not be well analyzed in the present study since this information was lacking in almost all patient files. Nevertheless, some points must be taken into consideration. First, KCOT clearly demonstrates a highly proliferative activity which may be explained by its histogenesis, thought to arise from cell remains of dental lamina. Besides this, some morphological aspects could be attributed to its recurrence, such as the normally flat epithelium–connective tissue interface, the absence of rete ridges leading to the incomplete removal of the lesion and islands of odontogenic epithelium in the cystic wall.

It has been reported that MDM2/p53 form an autoregulatory feedback system that may be important in maintaining a fine balance between the ratio of MDM2 and p53 proteins within the cell. The coordinated interactions between these genes have been implicated to play a crucial role in transcriptional regulation of a set of growth genes and in mediating cell cycle progression following exposure to DNA damaging agents and could be closely related to tumorigenesis. MDM2 can inhibit p53 expression by mimicking the transactivation in the N-terminal domain of p53. On the other hand, MDM2 deregulation has been observed in various human malignancies.

Carvalhais et al. found MDM2 immunoexpression throughout the epithelium, except in the superficial layer, not only in syndrome but also in non-syndromic OKC and in 2 of 11 RC. The authors did not observe significant differences between MDM2 immunoexpression in sporadic OKC and syndromic OKC. Regarding the p53 protein, no reactivity was detected in the lesions studied. Based on these findings, it was concluded that the absence of p53 staining might be associated with DNA integrity or a short p53 protein half life. Possible mutation without p53 stabilization and accumulation, and the deletion of the p53 gene must also be taken into consideration.

Lombardi, Odell and Morgan and Li, Browne detected p53 immunoexpression in OKC using monoclonal and polyclonal antibodies against wild and mutated p53 and suggested that p53 overexpression represents the physiological expression of normal p53 protein instead of a mutant type.

According to Slootweg, who studied p53 and Ki-67 expression in OKC, ameloblastomas, and odontogenic carcinomas, p53 overexpression may indicate an increase synthesis or a low rate of p53 degradation, resulting in growth regulation disturbances. The author also supports a relationship between p53 overexpression and aggressive behavior. Nonetheless, our findings were not able to corroborate this hypothesis since all cases were p53 immunonegative.

The findings above are in agreement with the study of Gadbiel et al. that analyzing the actual proliferative activity (APA) of epithelium of KCOT, RC, and DC using Ki-67 LI and AgNOR count per nucleus and the expression of p53, observing that Ki-67 positive cells were highest in suprabasal cell layers of KCOT with uniform distribution and this cellular layer showed higher actual proliferative activity. The p53 immunolabeling was dense and scattered in basal and suprabasal cell layers in KCOT. The authors concluded that the quantitative and qualitative differences of the proliferative activity and the p53 protein expression in sporadic KCOT may be associated with intrinsic growth potential that could play a role in its development and explain locally aggressive biological behavior and that the p53 protein expression in odontogenic lesions can be useful to predict the prognosis.

In contrast to the result of Carvalhais et al., that suggest a neoplastic origin for OKC due to the high MDM2 immunoexpression observed in their study, we did not observe immunostaining for this protein, but based on our result, we concluded suggesting that the OKC presents distinct biological behavior than the odontogenic cysts, as for proliferation, apoptosis and differentiation processes, supporting the novel designation of the OKC as neoplasm and alert to the clinicians for the adequate treatment considering the aggressive clinical behavior and recurrence rate of this lesion.

REFERENCES

Odontogenic cysts: p53, MDM2, bcl-2, PCNA


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