The evaluation of Ki67, p53, MCM3 and PCNA immunoeexpressions at the level of the dental follicle of impacted teeth, dentigerous cysts and keratocystic odontogenic tumors

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Abstract
The aim of this study is to analyze the immunoeexpression of Ki67, p53, MCM3 and PCNA markers in epithelial remnants of dental follicles of impacted teeth and to identify a possible correlation between the immunoeexpression of these markers in dentigerous cysts and keratocystic odontogenic tumors in order to evaluate their evolutionary behavior. Materials and Methods: A total of 102 cases were included in the study and divided into three subgroups: the first subgroup consisted of 62 cases with dental follicles of impacted teeth, the second included 20 cases of dentigerous cysts and the third subgroup comprised a number of 20 cases with keratocystic odontogenic tumors. Immunomarking with the four antibodies was performed. Results: A positive marking was obtained in over 60% of the dental follicles for all markers. Statistically significant differences were also obtained in dentigerous cysts and keratocystic odontogenic tumors for Ki67, p53 and MCM3. Assessment of the four antibodies in the two layers of keratocystic odontogenic tumors shows a positive correlation between Ki67 and MCM3 both for the basal and parabasal layer, with slightly increased values in the latter. Conclusions: In order to determine the proliferative capacity of epithelial remnants in the dental follicles, Ki67 and PCNA, Ki67 and MCM3 are the most useful markers in practice; they have similar behavior and are more likely to help in distinguishing between dentigerous cysts and keratocystic odontogenic tumors.

Keywords: dental follicle, impacted teeth, dentigerous cysts, keratocystic odontogenic tumors, immunohistochemistry.

Introduction
The dental follicle (DF) is an ectomesenchymal tissue involved in the formation of periodontal components during tooth development that is present around impacted teeth as well. Its structure may contain islands of epithelial residues persisted from tooth blade fragmentation, which can maintain their proliferative and differentiation capacity and can generate a pathology associated with dental inclusion. Most often, this pathology has cystic features (dentigerous cysts, keratocystic odontogenic tumors) but in certain circumstances, it can turn into tumoral processes (ameloblastoma) [1–4].

Odontogenic cysts are common lesions that can occur in the jawbone and can be developmental (dentigerous cyst, eruption cyst, odontogenic keratocyst, orthokeratinized odontogenic cyst, gingival cyst of the newborn, gingival cyst of the adult, lateral periodontal cyst, glandular odontogenic cyst) or inflammatory (apical cyst, residual cyst) [5]. Although they have a similar histopathological feature, they have a different clinical behavior.

Dentigerous cysts (DCs) are the most common developmental odontogenic cysts, associated with the crowns of an unerupted or impacted tooth. Their origin is from the reduced enamel epithelium between the follicle and the tooth crown. Usually asymptomatic lesions may be able to transform into more serious lesions, such as unicystic ameloblastoma, odontogenic adenomatoid tumor, squamous cell carcinoma, mucoepidermoid carcinoma. Histologically, the cyst lining resembles reduced enamel epithelium, cyst wall composed of thin fibroconnective tissue with or without nests of odontogenic epithelium, dystrophic calcifications and inflammatory cells [5].

The term ‘odontogenic keratocyst’ created confusion in the past as it was considered a developmental cyst. Odontogenic keratocyst is currently seen as a neoplastic lesion with an aggressive and progressive potential, a higher rate of recurrence and a specific histopathological appearance, therefore, according to the latest World Health Organization (WHO) Classification 2005 it is referred to as keratocystic odontogenic tumor (KCOT) [6].

In the literature, most often described immunohistochemical markers for assessing the histopathological features of these lesions are Ki67 and p53 [7–9]. Other less studied markers are MCM2, MCM3, PCNA and BCL2, which are mainly used to understand their biological behavior [10–12]. The results of the existing
studies show the need for clinical, histopathological and immunohistochemical evaluation of the dental follicle, in order to understand their cystic transformation ability and of the cystic lesions as well, so as to assess their recurrence, progression or malignant transformation.

The aim of our study was to analyze the immunexpression of the Ki67, p53, MCM3 and PCNA in epithelial odontogenic rests from dental follicles of impacted teeth in order to assess their proliferative capacity, as well as their cystic transformation potential. Our objective was also to identify a possible correlation between the immunexpression of markers studied in dentigerous cysts and keratocystic odontogenic tumors in order to evaluate their evolutionary behavior. This study was actually “born” because of the scarce information on this subject in the national and international literature.

Materials and Methods

The study material was selected from the archives of the Department of Pathology, Emergency County Hospital of Tîrgu Mureș, Romania, after the surgical procedures performed in the Clinic of Oral and Maxillofacial Surgery of Tîrgu Mureș.

After previous assessment, the study included 102 cases divided into three groups: the first group consisted of 62 cases with dental follicles from impacted teeth, the second group consisted of 20 cases of dentigerous cysts and the third group comprised 20 cases of keratocystic odontogenic tumors. The dental follicles were removed in local anesthesia without dental components. For developmental cysts and keratocystic odontogenic tumors, we used enucleation or cystectomy separately from the bone component.

The collected materials were formalin-fixed, dehydrated and paraffin-embedded. We did not used decalcification procedures. Sections of 3–5 μm were obtained from specimens and stained with Hematoxylin–Eosin (HE).

Cases that met the inclusion criteria were studied immunohistochemically in the Laboratory of Immunohistochemistry, Department of Histology, University of Medicine and Pharmacy of Tîrgu Mureș.

A 3–4 μm sections from paraffin embedded blocks were made. The slides with tissue sections were treated with xylene, followed by descending grades of alcohol and rehydration with water. The slides were then transferred to specific buffer for antigen retrieval, after were allowed to cool and washed in phosphate-buffered saline (PBS). Peroxidase and protein blocking were done using 3% hydrogen peroxide. The slides were incubated with primary antibodies. We used: monoclonal rabbit anti-human p53 clone 318-6-11 (1:50, 30 minutes, DAKO-10069125), monoclonal mouse anti-human Ki67 antigen clone MIB-1 (1:100, 30 minutes, DAKO-00090217), monoclonal mouse anti-human MCM3 protein clone 101 (1:50, 30 minutes, DAKO-0081318) and mouse monoclonal anti-polymerase cell nuclear antigen PCNA clone (1:400, 30 minutes, DAKO-0003603). The detection system used was Ultra Vision LP Detection, System Large Volume HRP Polymer, DAB, Ready-To-Use (LHL 110224A, LabVision). PBS washes were performed after every step during the immunostaining procedure. The working protocol applied for each marker took into account the manufacturer’s guidelines and included the use of positive and negative control reactions of the technique. The cross-sections were counterstained with Hematoxylin.

Results of immunohistochemical reactions were interpreted by two pathologists. The following parameters were used to evaluate primary antibodies staining: nuclei with brown color regardless of staining intensity were regarded as positive. To assess the proliferation index from the dental follicle, 100 epithelial cells from the remaining islands were counted in its structure, which identified the percentage of labeled cells for each marker. In case of the cysts, 100 cells from the basal (b) layer and 100 cells from the parabasal (pb) layer were counted and the percentage of immunohistochemically labeled cells was determined. Reading was performed with the help of an OLYMPUS BX46 microscope at a magnification of ×40 HPF (high-power field).

Results were introduced in a database. Statistical analysis was performed using Med Calc Software, Version 12.5.0.0. Data were considered nominal or quantitative variables. Nominal variables were characterized using frequencies. Quantitative variables were tested for normality of minimax–maxima or by mean and standard deviation (SD), when appropriate. A chi-square test was used in order to compare the frequencies of nominal variables. Quantitative variables were compared using t-test or Mann–Whitney test. The correlation between quantitative variables was assessed using Spearman’s rho (rank correlation coefficient). The level of statistical significance was set at $p<0.05$.

The study was approved by the Ethics Committee of University of Medicine and Pharmacy of Tîrgu Mureș (No. 30/16.03.2015).

Results

The results of immunohistochemical reactions from epithelial remnants in the structure of dental follicles were variable for the 62 cases. Positive results were obtained for PCNA and Ki67 in 60 (96.77%) and in 46 (90.32%) cases, respectively, while for MCM3 and p53 we obtained positive reactions in 42 (74.19%) and 40 (64.51%) cases, respectively (Figure 1).

The Mann–Whitney test was applied in order to compare the differences between median values associated with basal versus parabasal layers for each parameter; initially for DC and then for KCOT. Statistically significant differences were obtained in DC and KCOT for Ki67, p53 and MCM3, the values identified in the basal versus parabasal layer for PCNA for both cyst types being similar (Table 1, Figure 2).

The Mann–Whitney test was used to compare medians of corresponding values obtained for each marker in the basal and parabasal layer, initially for dentigerous cysts (DCs) and keratocystic odontogenic tumors (KCOTs). We obtained statistically significant differences in the basal layer for Ki67 and MCM3, and statistically significant
values for PCNA in the parabasal layer (Table 2, Figure 3).

The immunoexpression of the four antibodies in dentigerous cysts (DCs) and keratocystic odontogenic tumors (KCOTs) was evaluated in the basal (b) and parabasal (pb) layer.

In order to evaluate the correlation between the four markers, we compared the results by applying Spearman’s rho test for both dental follicles and cystic lesions. Analyzing the immunoexpression of the four antibodies at the level of the dental follicle, we obtained a statistically significant positive correlation between Ki67 and p53 ($p=0.0001$), Ki67 and MCM3 ($p=0.002$), and MCM3 and p53 ($p=0.002$).

Table 1 – The immunoexpression of the four antibodies in the basal (b) and parabasal (pb) layer evaluated on the dentigerous cysts (DCs) and keratocystic odontogenic tumors (KCOTs)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>DCs</th>
<th>Median</th>
<th>Min–Max</th>
<th>p</th>
<th>KCOTs</th>
<th>Median</th>
<th>Min–Max</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Ki67</td>
<td>b</td>
<td>16</td>
<td>3–46</td>
<td>0.001</td>
<td>pb</td>
<td>5</td>
<td>3–16</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>pb</td>
<td>35</td>
<td>7–56</td>
<td></td>
<td></td>
<td>38</td>
<td>13–53</td>
<td></td>
</tr>
<tr>
<td>MCM3</td>
<td>b</td>
<td>7.5</td>
<td>4–39</td>
<td>0.008</td>
<td>pb</td>
<td>20.5</td>
<td>10–45</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>pb</td>
<td>10</td>
<td>2–30</td>
<td>0.005</td>
<td></td>
<td>28</td>
<td>11–43</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>b</td>
<td>23.5</td>
<td>2–33</td>
<td>0.005</td>
<td>p</td>
<td>17.5</td>
<td>10–53</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>pb</td>
<td>96</td>
<td>14–98</td>
<td>0.14</td>
<td>PCNA</td>
<td>92</td>
<td>32–98</td>
<td>0.59</td>
</tr>
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<td></td>
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</tbody>
</table>

For dentigerous cysts, we evaluated the four antibodies initially in the basal and then in the parabasal layer. We obtained a positive correlation for the basal layer between Ki67 and the MCM3 ($p=0.002$) and a negative correlation between p53 and PCNA ($p=0.0001$). For the parabasal layer, we obtained a positive correlation between Ki67 and MCM3 ($p=0.001$) and a negative correlation between MCM3 and PCNA ($p=0.005$).

In keratocystic odontogenic tumors, assessment of the four antibodies in the two layers shows a positive correlation between Ki67 and MCM3 both for the basal and parabasal layer with slightly increased values in the parabasal layer (Figure 4).
Figure 2 – The expression of Ki67 and MCM3 in keratocystic odontogenic tumors (KCOTs) and dentigerous cysts (DCs). KCOTs: (A) Ki67 immunostaining, ×200; (B) MCM3 immunostaining, ×200. DCs: (C) Ki67 immunostaining, ×100; (D) MCM3 immunostaining, ×200.

Figure 3 – The expression of p53 and PCNA in keratocystic odontogenic tumors (KCOTs) and dentigerous cysts (DCs). KCOTs: (A) p53 immunostaining, ×200; (B) PCNA immunostaining, ×200. DCs: (C) p53 immunostaining, ×200; (D) PCNA immunostaining, ×100.
that MCM3 can be considered a potential marker for that the two markers behave similarly and we can conclude between MCM3 and Ki67 in the dental follicle, which markers [20–22].

Our aim was to identify those markers that might indicate the possibility of cystic or tumor transformation of these epithelial remnants during a lifetime and which could be useful in the differential and positive diagnosis as well as in the prognostic evaluation of the studied cystic lesions.

According to the literature, among the markers we used in order to highlight the proliferative activity in the dental follicle, the most commonly used and described is Ki67 [13]. Some studies have shown that using this marker the possibility to anticipate morphological changes in the dental follicle is relatively low [14, 15]. MCM immunoexpression is highlighted both in normal proliferating cells as well as in the tumoral ones [16]. The major protein involved in the regulation of the apoptotic process is p53. Some authors report a weak or undetectable expression of this marker in the molar dental follicle [17]. PCNA positive cells are regarded as cells undergoing a division process [17, 18], therefore the immunoexpression of this protein at the level of dental follicle epithelial remnants could be another useful marker in the assessment of their cystic transformation ability.

Analyzing the immunoexpression of markers in dental follicle epithelial remnants of impacted teeth in our study, we can state that PCNA showed a positive marking in almost all cases (60 cases). Even if we obtained a positive immunomarking in fewer cases for Ki67, p53 and MCM3, we obtained a statistically significant correlation among these three markers.

Some authors have taken into consideration the dental follicle size when performing the immunohistochemical study [19]. However, they found no correlation between the size of the dental follicle and Ki67 and PCNA expression, which may support the similarity of the dental follicle behavior to that obtained in our study, as our results showed no significant correlation between the two markers [20–22].

The present study demonstrated a positive correlation between MCM3 and Ki67 in the dental follicle, which that the two markers behave similarly and we can conclude that MCM3 can be considered a potential marker for determining the proliferative activity of epithelial cell remnants in the dental follicle.

We also obtained a significant statistical correlation between p53, Ki67, and MCM3, respectively, which means that there is a close association between cell proliferation and apoptosis.

Based on the assessment of the immunoexpression results of the four antibodies in the basal versus the parabasal layer for the two types of cystic lesions, we can state that there is a significant difference between the two median values, in the two layers, for both groups in Ki67, MCM3 and p53.

We have also obtained highly significant values for these antibodies in the third group versus second group, as the difference of the median values in the two layers is significantly higher for the keratocystic odontogenic tumors than for the dentigerous cysts. After evaluation of the maximum values of positive cells in the two layers, these are much higher in DCs than in KCOTs, while expression of these markers in the parabasal layer was very tight as value for both types of cysts.

Comparing the immunoexpression results of the four antibodies in the two types of lesions and assessing the initially obtained values for the basal and then for the parabasal layer, we can state that there are significant differences between the two cystic lesions for the parabasal layer in MCM3 and Ki67. Even if for the parabasal layer we obtained significant differences only for PCNA, analyzing the median values and the minimum and maximum number of positive cells in this layer respectively, we recorded higher positive values in KCOT than in DC. This indicates the fact that although they are considered benign lesions, their biological evolution is more aggressive, development of this type of cyst being based on maintenance of the proliferative capacity of cells in the parabasal layer [19, 20]. The result is consistent with data from the literature, which considers this type of cyst a benign odontogenic tumor, similar to ameloblastomas [14, 23].

p53 had a stronger expression in DCs in both layers, which is consistent with other results in the literature [23]. The behavior of this marker in the DC can be explained through the fact that cellular stress represented by local inflammation is responsible for higher values in this marker.

Conclusions

The positive marking we obtained in all four antibodies in a large number of cases in first group indicates the proliferative activity of cells in the epithelial remnants.

Figure 4 – The correlation of Ki67 and MCM3 in the basal layer (A) and the parabasal one (B) in keratocystic odontogenic tumors.

Discussion

Although the importance of the proliferation capacity of cells from the epithelial remnants of dental follicles is not fully documented, development of cystic and tumoral lesions is closely related to these epithelial remnants. The proliferative potential may be assessed through immunohistochemical methods using specific markers for different stages of the cell cycle.

Figure 4 – The correlation of Ki67 and MCM3 in the basal layer (A) and the parabasal one (B) in keratocystic odontogenic tumors.

Even if we obtained a positive marking we obtained in all four antibodies in a large number of cases in first group indicates the proliferative activity of cells in the epithelial remnants.
By demonstrating the proliferative capacity of dental follicle, we can state that the removal of the impacted teeth can be considered in preventing a possible cystic or tumoral transformation of epithelial residues. Statistically significant differences in the basal layer, with higher values for DCs for Ki67 and MCM3, demonstrate that these two markers behave similarly and are more likely to predict the distinction between dentigerous cysts and KCOTs. The results of our study confirm the more aggressive character of KCOTs as documented in the literature and that certain inflammation factors have positive influences on the proliferative ability of odontogenic epithelium in DCs. In everyday practice, in order to differentiate and evaluate the biological behavior of dentigerous cystic lesions, determination of Ki67 or MCM3 immunexpression for the basal layer and the PCNA for the parabasal layer is extremely useful.

Conflict of interests
The authors declare that they have no conflict of interests.

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