The evaluation of p16 and Ki67 immunoexpression in ameloblastomas

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Abstract
In this study, we investigated the p16 and Ki67 immunoexpression in 19 ameloblastomas in order to highlight some correlations of these markers with the aggressive variants of tumors. The p16 immunoreaction was present in 90.9% of cases; the highest scores are present in the typical follicular and in the intraluminal unicystic variant, at the opposite pole being the granular cells variant. In these cases, the maximum reaction was observed at the level of the stellated reticulum cells while the lowest reaction was present at the level of cubico-cylindrical peripheral cells of the neoplastic islands. The Ki67 immunoreaction was present in all cases, the highest scores being present in the typical follicular variant, opposite being the ameloblastoma with granular cells cases and that with acanthomatous differentiation type. The immunostained cells were located predominantly at the periphery of the tumoral islands but also in the stellated reticulum cells in the central area. The p16 and Ki67 markers may be useful for distinguishing different types of ameloblastomas in terms of aggressiveness.

Keywords: ameloblastoma, p16, Ki67.

Introduction
The ameloblastoma is an epithelial odontogenic benign tumor, morphologically similar to the enamel organ but which does not produce calcified materials such as enamel or dentin. The tumor is classified by World Health Organization (WHO) in the group of odontogenic tumors derived from the odontogenic epithelium with fibrous, mature stroma [1]. According to the WHO definition (1992), the ameloblastoma is a polymorphous benign neoplasm, but locally invasive, consisting of the proliferation of odontogenic epithelium, usually with follicular or plexiform appearance, at which it is associated a fibrous stroma [1].

The ameloblastomas are tumors with ambiguous behavior due to their clinical and histological characteristics, which are contradictory and incongruous. Thus, if the histology aspect of the tumor is benign, the clinical behavior is invasive and destructive, being also reported rare cases of pulmonary metastases, which have a pulmonary centre in 88% of the cases [2].

There are numerous genetic and molecular changes, which seem to promote the growth and the multistage development of odontogenic tumors [3]. Thus, solid ameloblastoma variants are locally aggressive and recur in case of an inadequate excision, unlike the unicystic ameloblastoma with a distinct prognosis, less aggressive than other variants [4, 5].

Cellular proliferation has an orderly progression through the cellular cycle, which is regulated by the complex formed of cyclins and cyclin-dependent kinase. P16 functions as an inhibitor of cyclin-dependent kinases: CDK4 and CHK6, blocking the division cellular cycle in the control points of G1, G2 / M phases. The cyclin-dependent kinases blocking prevents the phosphorylation of the retinoblastoma’s protein RB1 and thus prevents the exit from G1 phase of the cellular cycle, which makes p16 to act as a negative regulator of the normal cells proliferation.

The study of proliferation markers (PCNA, Ki67) showed the existence of some expression differences between the different types of ameloblastoma [6, 7], but also between different areas of the same variant [8]. The Ki67 index may be useful for differentiating the benign from malignant tumors, but sometimes the ameloblastomas may have an increased proliferative activity similar to the ameloblastic carcinomas [9].

In this study, we aimed immunoexpression analysis of p16 and Ki67 in various forms of ameloblastomas, in order to highlight some correlations of these markers with the aggressive variants of tumors.

Materials and Methods
The study included 19 ameloblastomas, which came from the Clinic of Oral and Maxillofacial Surgery, Emergency County Hospital of Craiova, Romania. The surgical excision pieces were fixed in 10% buffered formalin, processed by the usual histological technique and diagnosed in the Laboratory of Pathological Anatomy, Emergency County Hospital of Craiova. Later, there were realized 4 \( \mu \)m sections, which were processed by immunohistochemical HRP/LSAB2 technique for p16 (MoAHu DCS-50, Santa Cruz Biotechnology, 1:100...
dilution, 0.1 M citrate, pH 6) and Ki67 (MIB-1 MoAHu, Dako, 1:50 dilution, 0.1 M citrate, pH 6). For the external control, it was used the colorectal carcinoma for Ki67 and the tonsil for p16. The semiquantitative analysis of the expression of these markers was performed in epithelial tumor compartments (basal, stellated reticulum, squamous, granular), calculating the index of positivity (IP) for each biomarker used, by reporting the number of labeled cells to the total number of identified cells at a microscope of ×40, then multiplying the result by 100, for each case counting 500 cells from each epithelial compartment. Also, the intensity of the reaction was assessed as low, moderate or intense according to the criteria established by Barboza et al. [10].

Results

The study included a total of 22 cases diagnosed with ameloblastoma, of which 18 follicular ameloblastomas, typical forms (15 cases) or variants of these (three cases), and four unicystic ameloblastomas, luminal form (two cases) and intraluminal form (two cases). The analyzed cases were diagnosed at patients aged between 31–62 years, predominantly in males (58.3%). Most of the tumors were located at the mandibular level (86.3%).

The immunoexpression of the p16 protein was present in 20 of the investigated cases (90.9%), the biomarker being cytoplasmatic and nuclear. The semiquantitative analysis of immunoreaction for p16, according to the different histological variants of ameloblastomas revealed the presence of high scores in the follicular typical variant (75.5±12.4) and intraluminal unicystic variant (66.8±12.2), at the opposite pole being the ameloblastomas with granular cells (12.3±7.3). An intermediate position was presented by the acanthomatous variant (39.2±9.7) (Table 1).

Table 1 – The distribution of cases and p16 and Ki67 medium IP in ameloblastomas

<table>
<thead>
<tr>
<th>Histopathological type</th>
<th>P16 immunostain</th>
<th>Ki67 immunostain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive cases</td>
<td>IP</td>
</tr>
<tr>
<td>Typical follicular ameloblastoma</td>
<td>15/15</td>
<td>75.5±12.4</td>
</tr>
<tr>
<td>Acanthomatous ameloblastoma</td>
<td>2/2</td>
<td>39.2±9.7</td>
</tr>
<tr>
<td>Ameloblastoma with granular cells</td>
<td>1/1</td>
<td>12.3±7.3</td>
</tr>
<tr>
<td>Unicystic luminal ameloblastoma</td>
<td>1/2</td>
<td>28.6±11.9</td>
</tr>
<tr>
<td>Unicystic intraluminal ameloblastoma</td>
<td>1/2</td>
<td>66.8±12.2</td>
</tr>
</tbody>
</table>

IP – Index of positivity.

For all the variants of investigated ameloblastomas, the maximum reactivity was observed in stellated reticulum cells and a lower reactivity in peripheral cubico-cylindrical cells of the neoplastic islands (Figure 1A). A moderate reactivity was present at the neoplastic proliferation from the acanthomatous variants in areas of squamous metaplasia (Figure 1B). The lowest reactivity was noticed in ameloblastomas with granular cells, the immunoreaction was present in the nucleus of neoplastic cells, but with a lower intensity in granular cells.

In the unicystic ameloblastomas, the highest reactivity was found in the intraluminal version. In both cases, luminal and intraluminal, the reactivity for p16 was present in the intermediate and superficial layers of the papery epithelial of the cystic walls and in the intraluminal neoplastic proliferations (Figure 1C).

The statistical analysis indicated the specific reactivity p16 for the stellated reticulum cells (p<0.05, ANOVA), the immunoreaction distribution being characteristic (Pearson index <0.3).

The investigation of Ki67 index indicated positivity in all the analyzed cases. The semiquantitative analysis of the immunoreaction for Ki67 revealed the presence of high scores for typical follicular variant (6.7±4.3), at the opposite pole being the ameloblastoma cases with granular cell (2.3±2.1) and that with differentiations of acanthomatous type (4.1±3.8). In the four cases of unicystic ameloblastoma, the values recorded for Ki67 are close to those obtained in the conventional version of ameloblastoma for luminal subtype (5.6±3.3) and slightly higher in the intraluminal subtype (6.2±4.1) (Table 1).

The cells showed nuclear stain and were located predominantly in the periphery of tumor islands of follicular ameloblastoma but also in the cells of the stellated reticulum from the center. For both cellular components, peripheral and central, were recorded values below 10%, the highest index being at the level of peripheral cells (7.4%), while for the central compartment environment Ki67 had values of 11% (Figure 1D). In the cases of granular and carcinoma cells, we did not notice a positive immunoreactivity for Ki67.

For the two cases of unicystic ameloblastoma, the reactivity pattern for Ki67 was almost identical to that of the follicular ameloblastomas, but in the areas of squamous epithelium the reactivity was higher for the luminal version (Figure 1E), while for IP medium Ki67 intraluminal variant the reactivity was higher at the level of proliferative intraluminal nodules (Figure 1F).

The statistical analysis revealed specific reactivity Ki67 for peripheral columnar cells (p<0.5, ANOVA), the distribution of immunostain being characteristic (Pearson index <0.3). The chi-square test did not reveal associations of marker expression with the histological type of solid ameloblastomas (p>0.05, chi-square).

Discussion

The biological major aspect of the ameloblastoma is its locally invasive behavior, which is also responsible for the high rate of postoperative recurrence, even in conditions of a radical surgical therapy. Some aspects, as regards both the pathogenesis and the invasive growth of tumors, remain unclear. In this sense, there are investigated molecular mechanisms of cell proliferation from ameloblastomas, their identification can be a basis for therapy and also helpful in assessing the prognosis of tumors.

P16 (cyclin-dependent kinase inhibitor) is a tumoral suppressor protein encoded by the CDKN2A gene [11, 12], which acts as a negative regulator of proliferation of normal cells and it is involved in the replicative senescence process. A number of studies have shown that the methylation of CDKN2A gene functions as a major mechanism of tumorigenesis in many human cancers [13, 14].
In our study, we observed the immunostaining for p16 in 90.9% of investigated cases, the highest scores being present in the follicular solid type and respectively in the unicystic intraluminal type. At the opposite pole was situated the ameloblastoma variant with granular cell. Qualitatively and statistically, we observed a higher reactivity in the central cellular compartment, the stellated reticulum cells showing maximum reactivity. The lowest reactivity was observed in the ameloblastoma cases with granular cell. A moderate reactivity was present at the neoplastic proliferation level from the squamous metaplasia version.

Kumamoto *et al.* showed the overexpression of p16 in the vast majority of neoplastic cells from ameloblastoma concluding that the odontogenic epithelium would be found under the control of this oncoprotein [15]. In addition, Artese *et al.* investigating the immunohistochemical expression of p16 in odontogenic tumors, including ameloblastomas, found a tendency to positivity mainly in the central compartment of tumoral cells for tumors with a low risk of recurrence and a similar reactivity of both compartments in tumors with high risk of recurrence [16]. This fact would suggest on the one hand that p16 might control the odontogenic epithelium and on the
other hand, that p16 and its location would influence the biological behavior of odontogenic tumors, explaining partly the infiltrative growth of some tumors [16].

Suzuki et al. found significant statistics differences as regards the p16 immunoexpression according to the histological variant of ameloblastoma namely the highest intensity was recorded in follicular ameloblastoma, followed by the ananthomatous as the minimum of reactivity being recorded in the plexiform type [17]. The authors suggested that such an immunophenotype p16 is suggestive for the varying degrees of differentiation, which can be developed by these tumors. Furthermore, the authors have established a direct correlation between the recurrence rate and the rate of expression of this marker in different varieties of ameloblastoma. Thus, they showed that if in the plexiform ameloblastoma the p16 expression rate was the lowest, and also the recurrence rate was the lowest at the opposite pole was situated the follicular variant where the maximum reactivity was recorded and the highest rate of recurrence [17]. Subsequently, Khojasteh et al. noticed methylations of p16 gene in all specimens of ameloblastic carcinoma investigated, suggesting the role played by p16 gene alterations in the progression of these lesions [18]. However, the same authors showed that in case of ameloblastoma there were recorded methylations of p16 gene but without evident characters of histological malignancy. Therefore, the authors concluded that p16 gene alterations in ameloblastomas may further predispose to the evolving of malignant transformation, but the hypermethylation of this oncogene are not a direct indicator of malignancy cynical behavior [18].

The estimation of the degree of proliferation in many human tumors may be predictive as regards their biological behavior and it is also useful in assessing therapeutic response in determining the risk of recurrence. One of the most used, but also the most reliable marker of cellular proliferation is considered to be Ki67 [19], a non-histone nuclear protein strictly associated with cellular proliferation [20], present in all active phases of the cellular cycle (G1, S, G2, and mitosis) and absent at the “resting” cells phase (G0).

In our study, the immunoreactivity for Ki67 was present in all analyzed cases. The semiquantitative analysis of immunoreaction for Ki67 revealed the presence of high scores of IP in typical follicular variant, at the opposite pole being the cases of ameloblastoma with granular cells type and the differentiations ananthomatous ameloblastoma type. The highest reactivity was recorded at the basal cells at the periphery of neoplastic islands, the stellated reticulum cells rarely becoming positive for this marker. In the cases of squamous cells and the granulated ones, we did not notice the immunoreaction for Ki67.

Kumamoto reported that the highest reactivity recorded in ameloblastomas is at the level of cells that are adjacent to the basement layer, but that it would be lower in granular ameloblastomas compared to that of plexiform and follicular variants [21]. Regarding various types of solid ameloblastomas, most studies have shown that the largest proliferative activity would be in the follicular ameloblastoma [6, 7, 22, 23]. Kim and Yook have not reported the existence of significant differences of proliferation index at Ki67 between different histological types of solid ameloblastoma [24]. Instead, Piattelli et al. stated that the plexiform ameloblastoma would have the highest index of proliferation, followed closely by the ananthomatous version [25]. On the other hand, Rizzardi et al. stated that the variant of granular cells of ameloblastoma have the highest proliferative activity, followed by the plexiform variant, while the follicular and ananthomatous variants are placed at the bottom as values of the proliferation index at Ki67 [26].

As regards the basic histological variants of the ameloblastoma, the majority of studies have shown that the lowest proliferative activity would be in the unicystic ameloblastoma and hence the low rate of recurrence in this version of ameloblastoma [6, 7, 22, 25, 27]. However, in two studies were recorded high values of proliferation index at Ki67 in unicystic ameloblastoma comparing with the solid and multicystic versions, suggesting that the low rate of recurrence in the unicystic form could be explained by its particular morphology, which would provide an easier and greater accessibility to the surgeon to remove the lesion [28, 29]. In the same way advocates the study undertaken by Rizzardi et al., which showed that there is a significantly lower recurrence rate in the peripheral and unicystic options of ameloblastoma, despite the proliferative index at Ki67 higher than the other ameloblastoma variants [26].

Recently, Abdel-Aziz and Amin indicated that there are significant differences as regards the proliferative activity between the recurrent and non-recurrent forms of ameloblastoma, finding the highest values of the proliferation index at Ki67 in recurrent ameloblastomas [30]. This fact was also found by other authors, but without a net statistical significance [26–31]. These results highlight the importance of determining the proliferation index at Ki67 in assessing the evolutionary biological behavior of various ameloblastomas developed in the human tumoral pathology.

Conclusions

The immunophenotype of ameloblastomas indicated that the p16 protein was highly expressed in the solid follicular variant, the minimum of reactivity being recorded in the ananthomatous version respectively in the granular cell version; suggestive aspect for a less aggressive behavior and a terminal differentiated phenotype of the last two variants of ameloblastomas as against the typical form. The typical variant of follicular solid ameloblastoma had the highest proliferative potential. The lower proliferative potential is present in the ananthomatous and granular cells version. The p16 and Ki67 immunoexpression can provide information as regards the progression of ameloblastomas and may be markers of tumoral aggressiveness.

Contribution Note

All authors contributed equally to the manuscript.

References

The evaluation of p16 and Ki67 immunoeexpression in ameloblastomas

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