Evaluation of Cell Cycle-Related Indicators in Plexiform Ameloblastoma

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ABSTRACT

Background: Ameloblastoma (AB) is a locally invasive neoplasm with a potentially destructive behavior. The molecular mechanisms that regulate its cell growth are not yet fully understood. Intervention between G1 cell cycle regulatory proteins could participate in its pathogenesis. P53 gene status implied that p53 mutation might play a role in its tumorigensis; though few mutated genes were perceived. In the absence of p53 mutations, down-regulation of p21 could be suggested. Moreover, apoptotic pathways including Bcl2 were also reported to be involved in its progressive growth. Deregulation of these cell cycle regulatory proteins may be reflected on its proliferative potential. Immunohistochemical detection of PCNA represents a useful marker for the proliferating cellular fraction of AB.

Purpose: To determine whether cell cycle alteration plays a role in oncogenesis of plexiform ameloblastoma (PAB).

Material and Methods: A panel of cell cycle-related antibodies, including p53, p21, Bcl2 and proliferation marker PCNA, were examined immunohistochemically in 18 cases of PAB; three of them were recurred ones. Immunoexpression was scored using a semi-quantitative scale and statistically analyzed.

Results: The investigation revealed decreased immunoreactivity for p21 compared to intense immunoexpression for p53 and Bcl2. As regards to the proliferative potential, majority of cells exhibited immunolabeling for PCNA particularly at the periphery of the neoplastic odontogenic strands as well as in the endothelial cell lining of the stromal blood vessels.

Conclusions: Abnormalities in components of the cell-cycle regulatory machinery might play a role in the development of PAB. Furthermore, the peripheral cell layer is the port for these deregulations; hence it is thought to maintain the proliferating fraction that in turn expanding the neoplastic strands.

Key Words: Plexiform ameloblastoma – p53 – p21 – Bcl2 – PCNA.

INTRODUCTION

Odontogenic tumors are remarkable among oral lesions because of their clinical and histological heterogeneity. Ameloblastoma (AB) deserves special attention, not only because of its particular biologic behavior; exhibiting great infiltrative potential, high recurrence rate and capacity to metastasize; but also due to its relatively high frequency among odontogenic neoplasms; representing between 13 and 24%; depending on the population studied [1-3].

P53-cell cycle regulation system was proved to play a critical role in a variety of neoplasms including odontogenic tumors. The expression ratio of p53 in tooth germs was found to be significantly lower than those in AB, suggesting that p53 might be associated with its oncogenesis, tissue structuring and cytodifferentiation [4].

P21 protein mediates cell cycle arrest with relative increase of cell in G0/G1 phase and decrease of cells in G2/M phase to guard against DNA replication in cells that harbored damaged molecules [5,6].

Expression of p21 protein was detected in most epithelial cells including tooth germs and AB. It was established that p21 is well preserved in AB as compared with tooth germs, indicating that the odontogenic epithelium is strictly regulated by this factor [7,8].

Furthermore, apoptosis-related factors including Bcl2 were proposed to share in the pathogenesis of AB. The number of apoptotic...
cells in AB was significantly greater than in normal odontogenic epithelium, implying that apoptotic cell death serves to characterize the cyto-differentiation and cellular activity of AB [9,10].

Deregulation of aforementioned cell cycle regulatory proteins in AB may be reflected on its proliferative potential. Proliferating cell nuclear antigen (PCNA) is a nuclear protein synthesized in the late G1 and S phase of the cell cycle and its immunohistochemical detection represents a valuable indicator for the proliferating cells in AB [11,12].

Additionally, an intimate relation was illustrated between expression pattern of these biomarkers and prognostic outcome of AB [8,13,14].

From a histologic standpoint, AB exhibits distinct microscopic characteristics with variable histological patterns. It has been asserted that various levels of immunoreactivity displayed in each variant [15], in view of that context plexiform AB (PAB) was chosen in the present study resorting for consistency.

Although, several immunohistochemical studies have been described in the English literatures as an attempt to understand the mechanism of development of AB [4,9], there are no reports studying the above mentioned indicators together to clarify their possible intermingled role in its pathogenesis.

The current study was conducted to highlight the potential role of this panel of cell cycle-related indicators involving p53, p21, Bcl2 and proliferation marker PCNA in pathogenesis of PAB.

MATERIAL AND METHODS

Case selection:

This retrospective study included 18 cases diagnosed as PAB; during the period from 1990 to 2007; three out of them were documented to be recurred ones. Cases were selected by the availability of paraffin blocks in archives of Oral Pathology Departments, Faculty of Oral & Dental Medicine, Al-Azhar and Cairo Universities. The confirmation of the diagnosis was carried out by conventional histologic re-examination based on the criteria of World Health Organization Histological Typing of Odontogenic Tumors [16]. The clinicopathological data were recorded according to the surgical pathology reports of patients and summarized in Table (1).

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
<th>Recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>F</td>
<td>Mandible</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>F</td>
<td>Maxilla</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>M</td>
<td>Mandible</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>M</td>
<td>Mandible</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>32</td>
<td>F</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>33</td>
<td>F</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>F</td>
<td>Maxilla</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>38</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>40</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>14</td>
<td>40</td>
<td>M</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>42</td>
<td>F</td>
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<td>No</td>
</tr>
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<td>16</td>
<td>43</td>
<td>F</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>45</td>
<td>F</td>
<td>Mandible</td>
<td>No</td>
</tr>
<tr>
<td>18</td>
<td>50</td>
<td>M</td>
<td>Maxilla</td>
<td>No</td>
</tr>
</tbody>
</table>

M: Male. F: Female.

Immunohistochemical procedures:

Serial sections of 4μ thickness were cut from each formalin fixed, paraffin embedded tissue block, mounted on charged slides and dried. The slides were first deparaffinized, then dehydrated in graded ethanol concentrations.

In order to inhibit endogenous peroxidase activity, tissue samples were incubated with 0.3% hydrogen peroxide in methanol for 10min. After rinsing with water, the slides were placed in a glass dish filled with 10mmol/L sodium citrate buffer of pH 6.0. Between each stage of all procedures, careful rinses were performed with several changes of phosphate-buffered saline (PBS) of pH 7.2 for 5min.

The immunohistochemical staining was done according to the manufacturer’s instructions using: Anti P53 (clone 318-6-11, DAKO Corp., Carpinteria, CA) at a dilution 1:100 with heat-induced epitope retrieval on a NexES instrument (Ventana Medical System, Tucson, AZ), Anti P21 (clone SX118, Venta Medical system, Inc, Tucson, AZ) at a 1:50 dilution, Anti BCL2 (clone L 124, Venta Medical system, Inc, Tucson, AZ) at a 1:50 dilution and Anti PCNA (clone PC10, Venta Medical system, Inc, Tucson,
AZ) at a 1:50 dilution. Slides were then incubated for 30 min. with biotinylated goat secondary antiseraum; that is covalent to rabbit and mouse serum.

To enhance immunoreactivity, tissue sections were boiled in a microwave oven twice; 5 min. each; in addition to heat-induced epitope NexES on instrument (Ventana Medical System, Tucson, AZ). Detection of immunoreaction through antigen-antibody visualization was carried out using dianinobenzidine (DAB) in PBS containing 40% hydrogen peroxide (DAKO universal kit, Glostrup, Denmark). Sections were lightly washed under running tap water for 10 min. and counterstained with Mayer’s haematoxylin. Thereafter, the slides were allowed to cool in dry air and then mounted. Negative control were carried out on consecutive sections by omission of the primary anti-body resulting in no detectable staining.

**Evaluation of immunostaining:**

The immunostained sections were examined using light microscope to assess the prevalence of positive cases. Image analysis (software Leica Qwin, Germany) was employed to detect the percentage of positive cells that was measured in the form of an area and area percent inside a standard measuring frame of an area equal 11434.9 µm² per 10 fields using a magnification x400. The positive cells were masked by a blue binary color using the software computer system for measurement of the mean values.

Tumor cells with unequivocal staining of the cytoplasm or nucleus were considered positive. Grading of immunoperoxidase was evaluated using four-point semiquantitative scale based on the percentage of positive cells and scored as follows: Negative (0-19%), mild (20-39%), moderate (40-59%) and strong (60-100%) [17].

**Statistical analysis:**

The semiquantitative data were obtained and statistically analyzed. Chi-square-Fisher exact test was used for analysis. Mean and standard deviation were presented descriptive values for quantitative data. Non parametric t test (Mann Whitney test) was used for comparing mean of each two independent groups. Pearson’s correlation analysis and Spearman Rho correlation that measured the association between quantitative variables were performed. p value was considered significant at 0.05 levels.

**RESULTS**

Clinicopathological information related to PAB cases revealed nearly equal prevalence in males and females (5:4 respectively), with mean age of 34±6.5 ranging from 18 to 50 years this difference was statistically insignificant (p=0.406). Most of the cases were mandibular with three cases presented with evidence of recurrence.

**Immunohistochemical observations:**

Through microscopical investigation and according to semiquantitative grading scale immunopositivity, all the eighteen cases of PAB demonstrated p53 nuclear staining, where the positivity was mainly located in the peripheral cell layer; twelve cases out of eighteen were moderately positive and six cases showed mild positivity (Fig. 1).

Regarding p21 immunoreactivity, eleven cases out of eighteen demonstrated mild positivity; the immunostaining was scattered separately throughout the tumor cells (Fig. 2).

For Bcl2 immunodeposition, fifteen cases out of eighteen were moderately positive and two were mild. The positivity was mainly located in the peripheral layer with few cells in the central compartment having the immunoreactivity (Fig. 3).

Finally, all cases revealed PCNA nuclear staining; the positivity was scattered throughout the tumor cells in both the peripheral and central layers. Twelve cases out of eighteen were strongly positive and six cases were moderately positive (Fig. 4).

**Statistical findings:**

Statistical analysis revealed significant difference regarding the degree of positivity within Bcl2 immunoreactive cells (p<0.05). Moreover, highly significant difference was recorded between PCNA immunopositivity on one side and the other three utilized antibodies on the other side (p<0.0001). The summary of the results were tabulated and represented graphically (Table 2, Figs. 5,6).
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Table (2): Immunohistochemical findings of the studied antibodies (n=18).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Degree of staining</th>
<th>N.C.</th>
<th>R.C.</th>
<th>N.C.</th>
<th>R.C.</th>
<th>N.C.</th>
<th>R.C.</th>
<th>Total positive cases out of 18 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>Negative</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>18 (100%)</td>
</tr>
<tr>
<td>P21</td>
<td>Mild</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>12</td>
<td>3</td>
<td>11 (61%)</td>
</tr>
<tr>
<td>BCL\textsubscript{2}</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>17 (94%)*</td>
</tr>
<tr>
<td>PCNA</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>–</td>
<td>18 (100%)**</td>
</tr>
</tbody>
</table>

N.C: New cases. R.C.: Recurrent cases.
* Significant difference within Bcl\textsubscript{2} cells (p<0.05).
** Highly significant difference between PCNA immunostaining and other groups (p<0.000).

Fig. (1): A case of plexiform ameloblastoma disclosing considerable number of cells with nuclear immunoreexpression for p53 particularly at the periphery of the proliferating strands (X 400 DAB chromagen).

Fig. (2): A case of plexiform ameloblastoma showing few cells with nuclear immunoreactivity for p21 (arrows) (X 400 DAB chromogen).

Fig. (3): A case of plexiform ameloblastoma showing considerable number of cells with cytoplasmic immunostaining for Bcl\textsubscript{2} (X 400 DAB chromagen).

Fig. (4): A case of plexiform ameloblastoma showing large number of cells with nuclear immunolabeling for PCNA mainly at the periphery of the proliferating odontogenic strands. Note the immunoexpression in the wall of blood vessels (arrow) (X 400 DAB chromagen).


**DISCUSSION**

Briefly, the existing study demonstrated that the cell cycle regulatory proteins; p53, Be2 and proliferation marker PCNA; were positively expressed in tumor cells in the majority of the examined cases meanwhile, p21 was less expressed.

In parallel with the current findings, enhanced expression of p53 protein was evident in variant patterns of AB [1,18]; with p53 protein presented the strongest quantitative labeling index in the PAB [4].

Differences in p53 protein expression in different types of AB have implicated tumor suppressor gene alteration as a potential oncogenic mechanism; denoting the early event of neoplastic transformation [2].

P53 immunoreactivity signifies the prognostic status of the tumor where verification of more than 10% p53-positive cells may give an indication for a tendency to recurrence [1]. Besides, it was recognized that p53 positivity had a reduced overall survival compared to patient subgroups with marker negativity [13].

In support, the semiquantitative scale for the three studied recurrent cases indicated that more than 20% of their cells are p53-positive.

Through increased frequency of immunoreactive cells or increased staining density, overexpression of p53 protein was suggested to be a valid screening method for predicting underlying genetic changes in AB subtypes [19].

Despite overexpression of p53 at protein level was assumed to define a mutational status of p53 gene, DNA sequencing revealed that AB harbor scarce mutated p53 gene [2,18,20].

It was supposed that in the absence of p53 mutations, p53 inactivation is possible via complex formation with other proteins that may physically block the growth suppressor sites [21].

Accordingly, p21 immunoeexpression pattern may provide insight into the pathogenesis and biology of AB.

It was reported that increased cellular proliferation, in general is associated with decreasing p21 expression and this was accounted particularly in AB [7,22-24].

This observation is documented by the findings of the present investigation where concomitant low expression of p21 was detected in sparse cells in a minority of examined cases.

Antitumor targeting regimes pointing at p21 gene induce its upregulation with enhancement
of apoptosis and inhibiting angiogenesis [25,26]. Thus its impairment in the current studied cases might reflect decrease in the apoptotic potential with augmentation of the proliferative power and angiogenesis.

Moreover, decreased or lost p21 expression was found to be associated with recurred and malignantly transformed AB; hence its monitoring was anticipated to be a prognostic predictive marker of great efficacy [8,25]. This is mirrored in the existing study where all the recurrent cases were immunonegative for p21.

Regarding the cytoplasmic Bcl2 immunopositivity, the present results’ interpretation disclosed more concurrent expression with the peripheral layer of the odontogenic strands more than the central cells.

This finding is consistent with that cited in earlier studies where most of the outer layer cells were predominantly stained by Bcl2 antibody, indicating anti-apoptotic proliferating site in the outer layer [14,27,28].

The Bcl2 protein is therefore thought to play a role in maintaining the stem-cell population in the peripheral layers of the tumor nests from which proliferating cells can be recruited [29].

It was declared that among the Bcl2-family; Bcl2 was the most ubiquitously expressed protein in AB; indicating that this tumor has much more apoptosis-inhibiting proteins rather than apoptosis-modulating proteins [27]. This is obvious in the recorded statistical findings that presented significant difference within the Bcl2 immunoreacted cells advocating increase cellular activity through apoptosis suppression.

The expression of Bcl2 was recognized to be stronger in recurred or malignantly transformed AB, suggesting its participation as an indicator of prognosis in AB [14]. In parallel is the observed increase in immunostaining of the three examined recurrent cases compared to the newly diagnosed ones.

At the present work, as a consequence to the anti-apoptotic influence residing in the peripheral cell layer, these cells in all inspected cases reflect over-expression of PCNA as a sign of intensify proliferative activity.

Similar notes were reported by other investigators where co-expression of PCNA was frequently dominanted in the outer zone of the tumor strands that regards as the proliferating portion acting as reserve of central cells of AB [4,11,15,30,31].

It was indicated that PCNA expression correlates with the clinico-biological behavior of AB with recurrent cases presented higher PCNA positive cell counts more than other subtypes [16]. This is reflected in the current work where all the investigated recurrent cases were strongly immunoreacted for PCNA; where more than 60% of cells are positive.

An interesting observation was recorded in the existing study representing PCNA immunodetection in the endothelial cells lining the blood vessels denoting enhancement of angiogenesis. This is in accordance with the hypothesized angiogenesis augmentation with low p21 co-expression [23].

Overall finding of the statistical analysis revealed high significant difference between PCNA immunoreacted cells and the other studied antibodies. This might explained in view that deregulation of these antibodies deprive their main function as guard against proliferation that reflected in increase in PCNA immunopositive cells.

However, PCNA expression probably results not only form cellular proliferation, but also from other sources, including impairment of DNA repair as well as other factors influencing messenger-RNA of the proliferating cell nuclear antigen. Owing to these factors, PCNA can be found in cells that are not in the cellular cycle. Another important issue that should be considered is that PCNA has a considerably longer half-life; approximately 20 hours; compared to the rapid cell cycle time [4].

The deregulated DNA repair may be predisposed by defective p53 function and this hints for the co- and over-expression of PCNA and p53 biomarkers in the inspected odontogenic strands in the present study mainly in the peripheral fraction [4].

Based on the previous scenario, there are presumably other variables, such as epigenetic changes or potential mutations in oncogenes that may be responsible for virulent behavior of PAB.
Nevertheless, it might be hypothesized that overexpression of p53 in PAB not necessarily denote mutated p53 gene but rather impairment in p21 as it is well known that p53 is dependently regulated by this gene. In sequence, loss of the main function of p53 as guardian of the genome at the cell-cycle check point may allow cells anchorage defective genes to escape and continue their mitotic division [27].

Besides, this is reflected on the apoptotic machinery; that is partly monitored by Bcl2 through its anti-apoptotic effect [29]; where malfunctioning cells not ordered to suicide but enhanced its proliferative activity that represented by overexpressed PCNA.

Yet, the exact mechanism underlying these reciprocal effects needs additional work to be elucidated and hopefully to serve as surrogate markers to predict outcome of PAB.

From these data we can conclude that, abnormalities in several components related to the dynamics of the cell cycle could participate in the tumorigenesis of PAB and the peripheral portion of the odontogenic strands of PAB might be the harbor for the cell cycle deregulations; hence it is thought to have its impact on maintaining the proliferating cells that in turn expanding the neoplastic strands.

REFERENCES
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