Immunohistochemical differential expression of p16 proteins in follicular type and plexiform type ameloblastoma

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ABSTRACT

Background: Differences in histopathological features that describe the growth mechanism and biological behaviour of follicular and plexiform ameloblastomas are associated with benign, aggressive and destructive tumour markers. p16 has inhibitory interactions between cyclin D and CDK 4/6 to block the cell cycle and alterations related to severity. Purpose: This study intends to evaluate and determine differential expressions of p16 protein in follicular and plexiform ameloblastomas. Methods: This is a descriptive analytics study. A total of 21 specimens consisting of follicular and plexiform ameloblastomas and healthy gingiva tissues as the negative control were examined using the immunohistochemistry assay. The analysis of p16 protein expression was interpreted by immunoreactive scoring. Statistical analysis was conducted using SPSS software with the Mann–Whitney test. A p-value <0.05 shows the significance of the change in expression. Results: An increased expression of p16 protein was found in the follicular ameloblastoma type (2.13 ± 1.808) and the plexiform type (4.44 ± 2.506) in comparison to the negative control group (0 ± 0). The increase of p16 expression in the follicular and plexiform ameloblastomas was significant compared to the negative control group (p-value <0.05); however, there was no significant difference between either type of ameloblastoma (p-value >0.05). Conclusion: The highest intensity of p16 protein expression was found in the plexiform type, even though it was not significantly different from the follicular type ameloblastoma.

Keywords: ameloblastomas; follicular; immunohistochemistry; plexiform; p16 protein expression

INTRODUCTION

The most common types of ameloblastomas are follicular and plexiform, whose clinical findings and specific clinical behaviours are associated with histopathological appearance. Given that appearance, this tumour shows signs of being a benign tumour, although clinically, it is aggressive and destructive.1 Ameloblastoma is an odontogenic tumour in tooth-forming tissue that grows slowly and locally invasive. Usually, the patient is unaware until the inflammation enlarges. Its recurrence is high, and its spread is expansive and infiltrative, giving the impression of malignancy.2

Ameloblastoma is commonly found in the mandibular and maxillary areas, with swelling resulting in facial deformity. On clinical examination, ameloblastoma does not have a specific feature because the stain of the tumour tissue is the same as the surrounding tissue.3 In addition, the consistency can be soft or hard, with no pain and paraesthesia and no ulceration of the mucosa around the tumour tissue.4 The growth of ameloblastoma is influenced by oral infection, tooth extraction, trauma to the teeth or jaws and genetic factors, such as tumour suppressor genes (TSG) and oncogenes (c-myc gene and ras gene). The loss of function of TSG, which plays a role in controlling cell proliferation and preventing cells from becoming malignant, causes tumour formation.5-7

p16 is a group of TSG called MTS1 (multiple tumour suppressor 1), CDKN2 (cyclin-dependent kinase inhibitor 2) and p16INK4a, which functions as an inhibitor of
the interaction between cyclin D and cyclin-dependent kinase (CDK) 4 and 6; it blocks the cell division cycle in G1 phase-control points.\textsuperscript{8,9} High throughput technology analyses using microarray gene expression profiling offer a technology to classify the tumour subtypes, such as breast cancer,\textsuperscript{10} bladder cancer\textsuperscript{11} and pancreatic adenocarcinoma.\textsuperscript{12} However, because of the high cost and complexity of laboratory procedures, alternate immunohistochemistry (IHC) assay is used to identify subtypes of tumour classification. The identification of the subtype and the clinical impact of the tumour can be used to determine the success of treatment due to tumour biological properties and behaviour.\textsuperscript{13}

Based on its role, p16 can be used as a marker of the cell-cycle phase to study pathophysiological conditions, such as abnormal cell differentiation and tumour prognosis.\textsuperscript{14,15} This study aims to determine the differential expression of p16 mutant proteins in the formation of follicular and plexiform ameloblastomas. This may inform future investigations into the molecular mechanism and increase potential therapeutics for Indonesian ameloblastoma.

**MATERIALS AND METHODS**

This descriptive analytics study uses 21 paraffin block-stained specimens from the Department of Anatomical Pathology Laboratory of Prof. Dr. Margono Soekarjo Hospital, Purwokerto and the Asri Medical Centre (AMC), Yogyakarta. The paraffin blocks were cut with a microtome, deparaffinised, rehydrated and then subjected to IHC staining to observe the p16 expression using the anti-CDKN2A/p16INK4a antibody (Abcam ab108349, USA) with a 30-minute incubation using 1:100 dilution. Counterstaining using Mayer’s hemalum was performed to determine the differences in three groups: follicular and plexiform types of ameloblastomas as well as healthy gingiva tissue as a negative control.\textsuperscript{16,17} All procedures followed the manufacturer’s recommendations.

Observation of the p16 protein expression was performed by 400x magnification in five viewpoints using a light microscope camera with Optilab® (Motic® B2-series, USA) and software Raster Image (US National Institutes of Health, USA). Observation with a grading picture of cells recorded as positive and the reaction intensity were as follows:\textsuperscript{13,18} grade 0 (no cells recorded) and negative reaction intensity; grade 1 (>10%–50% of recorded cells) and weak staining intensity; grade 2 (>10%–50% of recorded cells) and medium staining intensity (2); grade 3 (>50%–80% of recorded cells) and strong staining intensity; and grade 4 (>80% of recorded cells) and powerful staining intensity.

Based on a previous study, immunohistochemistry p16 expression analysis was carried out based on grading status and intensity scores, with expression scores ranging from 0 to 12.\textsuperscript{18} Immunoreactive scores are categorised as 1–4 positive scores (+), weak definition; 5–8 positive scores (++), moderate definition; and 8–12 positive scores (+++), strong definition. Data analysis was carried out using SPSS software version 22 (IBM Corp Version 23, Chicago, IL). Cohen’s kappa coefficient was used to test the validity of the examination by two observers. The analysis of differences in p16 expression was carried out using the Kruskal–Wallis non-parametric test. The Mann–Whitney test was performed to determine significant differences between groups; a p-value <0.05 indicates a significant difference.

**RESULTS**

In this study, the specimen sample consisted of follicular and plexiform ameloblastomas (Table 1). Immunohistochemical staining was conducted to evaluate the expression of p16 by discoulouration. The brown and dark brown discoulouration in follicular and plexiform ameloblastoma specimens showed mutant p16 expressions (Figure 1). The differential in colour intensity indicated weak positive,
moderately positive and strong positive differences in immunosuppression scores (Figure 1). The mean value of the difference in the expression of mutant p16 from the results of immunohistochemical examinations in the sample group are shown in Table 1. Two observers analysed mutant p16 expression by multiplying the positive cell grading and the reaction intensity from five fields of view observations.

The kappa coefficient test analysis showed a significance value of p = 0.000 with an ideal value of 0.598. In our study, the mean p16 expression for the follicular ameloblastoma group was 2.13 ± 1.808, for the plexiform ameloblastoma group 4.44 ± 2.506 and for the healthy gingival epithelium control group 0 ± 0. The analysis of differences in the expression of p16 showed a significant difference (p-value <0.05) in the follicular ameloblastoma, the plexiform ameloblastoma and the control groups (can be seen in Table 2). The expression of p16 mutant proteins showed differences between the control group compared with the follicular type ameloblastoma (p-value <0.05) and the control group compared with the plexiform type ameloblastoma (p-value <0.05), whereas the follicular type ameloblastoma compared with the plexiform type ameloblastoma group showed no significant difference (p-value = 0.071; p-value >0.05) (Figure 2).

Table 2. Different expressions of p16 mutant proteins

<table>
<thead>
<tr>
<th>Variable I</th>
<th>Variable II</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Control</td>
<td>Follicular</td>
<td>0.028</td>
</tr>
<tr>
<td>Control</td>
<td>Ameloblastoma</td>
<td>0.005</td>
</tr>
<tr>
<td>Control</td>
<td>Plexiform</td>
<td>0.005</td>
</tr>
<tr>
<td>Follicular</td>
<td>Ameloblastoma</td>
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</tr>
<tr>
<td>Follicular</td>
<td>Plexiform</td>
<td>0.071</td>
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<tr>
<td>Ameloblastoma</td>
<td>Ameloblastoma</td>
<td>0.071</td>
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![Figure 2](image)

Figure 2. Immunohistochemical expressions of p16 mutant proteins on follicular and plexiform ameloblastomas and healthy gingiva control group.

DISCUSSION

Ameloblastoma is a tumour with a high incidence, unrelated to age and gender and with no specific clinical symptoms. Histopathological and radiographic examinations are the gold standard for diagnosis in the incidence of ameloblastoma, with several types often found, namely follicular, plexiform and adenomatous. Different types of ameloblastomas can represent characteristics such as aggressiveness, recurrence and severity. Increased p16 expression in the incidence of ameloblastoma can provide an overview of the severity of its role as a tumour-suppressor gene in inhibiting the uncontrolled proliferation process. Increased expression of p16 in both types of ameloblastomas compared to the healthy gingival tissue, with the highest expression of p16 shown in the plexiform type ameloblastoma.

Increased expression of the p16 mutant ameloblastoma indicated the incidence of a malignancy. On the other hand, the wild-type p16 protein is difficult to detect in normal conditions because it has a short half-life. Increased expression of mutant p16 causes failure of cell proliferation in the G1 phase so that it is often found in follicular and plexiform types of ameloblastomas. The imbalance of cell cycle regulatory pathways involving p16-RB can impair cell proliferation, ultimately leading to unrestricted proliferation and tumourigenesis.

The p16 protein expressed in the G1 phase is a product of the CDKN2A gene, a tumour suppressor gene (anti-oncogene) that can prevent the overgrowth of cells in the G1 phase. The p16 protein acts as a negative regulator of cell proliferation. In normal cells, wild-type p16 is expressed and binds to CDK4 and CDK6 so that cyclin D and protein kinase complexes are inactive. Decrease or inactivation of p16 causes CDK4/6 to bind to cyclin D, causing an active protein kinase complex. The protein kinase complex triggers the phosphorylation of pRb so that pRb is inactive. Inactivation of pRb causes the release of the transcription factor E2F so that the cell enters the S phase. Continuous E2F transcription will cause normal cells to become ameloblastoma.

The results showed an increase in mutant p16 expression in follicular and plexiform ameloblastoma types. These results are supported by the research of Kumamoto et al. (2001), who demonstrated over-expression of p16 in most neoplastic cells from ameloblastoma so that odontogenic epithelium would be found to be under the control of this oncoprotein. Another study also showed the immunohistochemical expression of p16 in odontogenic tumours, including ameloblastomas, finding a particularly positive trend in tumour cell nuclei for tumours with low recurrence risk and a similar reaction for the nucleus and cytoplasm of tumours with high recurrence rates.
The difference in p16 expression in follicular type ameloblastoma was not significantly different from the plexiform type ameloblastoma group. This shows that the two groups have similar characteristics of mutant p16 expression. The results of this study are supported by a previous study that showed that there was no statistically significant difference in the expression of positive p16 in the central cells of low-risk and high-risk odontogenic tumours; in both groups, the results were equally high.\(^{32}\) Another study showed that the expression of the tumour suppressor p16 was not significantly different in odontogenic keratoses and unicystic ameloblastomas.\(^{13}\) This suggests that the invasive growth of odontogenic keratosis and the cystic behaviour of unicystic ameloblastoma are closely related to the state of p16 expression in the lesional epithelium. A candidate tumour marker can be used to analyse mutant p16 protein expression changes in follicular and plexiform ameloblastomas. However, it cannot be used as a progression marker between follicular and plexiform ameloblastoma groups.

This study has several limitations: the limited number of specimens involved and the clinical data that may have risk factors associated with p16 protein expression. In addition, we believe that our findings impact the understanding of factors associated with p16 protein expression. In addition, the highest increase in the expression of the p16 protein is shown in the plexiform type’s ameloblastoma.

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REFERENCES


