Assessment of changes to the dental follicle in deep and partially impacted mandibular third molar
Abeer Kamal\textsuperscript{a}, Eman Allam\textsuperscript{c}, Mohammed Dehis\textsuperscript{b} Susan Zunt\textsuperscript{d} and L. Jack Windsor\textsuperscript{c}

\textbf{Introduction} Residual dental follicles surrounding the crowns of impacted teeth may represent a significant risk for their potential pathological changes. In this study, the expression levels of cell cycle proteins, proliferating cell nuclear antigen (PCNA), cyclin D1, and p21 were evaluated using immunohistochemistry.

\textbf{Patients and methods} Twenty-two impacted mandibular third molars in 17 healthy individuals were evaluated in this study. They were divided into two groups on the basis of the depth of impaction: the partially impacted group and the fully impacted group. The impacted teeth were surgically removed and the surrounding follicle was curetted and examined by immunohistochemistry to evaluate whether there was a difference in cell cycle regulation between the two groups.

\textbf{Results} PCNA was expressed in most of the epithelial cells with no significant difference between the groups. The p21 expression was significantly higher in the fully impacted group. Cyclin D1 expression was expressed at low levels with no significant difference between the groups. Weak cyclin D1 expression was consistent with high p21, a negative regulator of the cell cycle. The high p21 expression could be a compensatory mechanism to the high PCNA levels. These results suggest the possibility that dental follicles associated with full bony impactions possess different cellular activities compared with partial bony/soft-tissue impactions. \textit{Egypt J Oral Maxillofac Surg} 3:30–35 © 2012 The Egyptian Association of Oral & Maxillofacial Surgeons.

\textbf{Keywords}: cell cycle proteins, dental follicle, immunohistochemistry, impacted third molar

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Received 16 December 2011 accepted 5 January 2012

\textbf{Introduction and review of literature} One of the problems that usually oral and maxillofacial surgeons face during odontectomy of the third molar is the concept of meticulous exision of residual dental follicles. This tissue has the potential to change into cystic or tumor tissue such as dentigerous cyst, keratocecytic odontogenic tumor, or ameloblastoma. The present study was designed to evaluate the effect of the depth of impacted mandibular third molars on the potential behavior of the tissues of the residual dental follicle. Dental follicles are derived from odontogenic ectomesenchyme, which constitutes one of the components of tooth germs and develops into cementum, periodontal ligaments, and alveolar bone. Dental follicles remain adjacent to the crown of unerupted or impacted teeth. Radiographically, dental follicles appear as thin, semicircular radiolucent areas of less than 3 mm in width. Histologically, they show fibrous connective tissue with remnants of reduced enamel epithelium. The epithelium becomes thicker and more squamous in the presence of inflammatory changes. Dentigerous cysts, the second most common odontogenic cysts, are caused by expansion of these dental follicles and accumulation of fluid between the tooth crown and the epithelial component [1–4].

Various types of proteins are involved in the process of cell cycle regulation. It is a complex process and involves proliferating cell nuclear antigen (PCNA), cyclin D1, and p21. PCNA is a cell cycle phase/cell proliferation marker and is considered as one of the critical regulators of the cell cycle. PCNA is located in the nucleus and is associated with sites of DNA synthesis and the initiation of cell proliferation. Thus, its level is considered a good marker for cellular proliferation and growth rate estimations. This protein has been detected immunohistochemically in odontogenic lesions [5–7].

Cyclins are positive regulators of the cell cycle. Their concentrations rise and fall at specific stages throughout the cycle. They are classified into three groups: G1 cyclins, S-phase cyclins, and mitotic cyclins. They regulate the G1/S transition, the initiation of DNA replication, and the G2/M transition, respectively. Cyclin D1 is a G1 cyclin that accumulates and reaches a maximum level in the early G1 phase of the cell cycle. Binding of cyclin D1 to cyclin-dependent kinases (Cdks) 4 and 6 results in the phosphorylation and inactivation of the retinoblastoma protein; this leads to the release of the E2F transcription factor that permits cells to enter the S phase and replicate. In contrast, cyclin-dependent kinase inhibitors (e.g., p21) inhibit cell cycle progression by suppressing cyclin D1. Cyclin D1 and p21 are responsible for switching on and off the transition from the G1 to the S phase, respectively [8–11].

In the present study, expression levels of PCNA, cyclin D1, and p21 were examined in the dental follicular tissues surrounding partial bony/soft-tissue impactions and full bony impacted teeth to clarify the possible effect...
of the depth of impaction on cell proliferation and the potential behavior of dental follicles. The obtained data will elucidate the possible role of the depth of impacted mandibular third molars on the probable deleterious effect of dental follicles.

**Patients and methods**

Twenty-two dental follicles from 17 patients with at least one impacted mandibular third molar were included in this study. The patients were recruited from those attending the outpatient clinic of the Department of Oral and Maxillofacial Surgery, Faculty of Oral and Dental Medicine, Cairo University, Egypt. There were six female and 11 male patients with ages ranging from 18 to 49 years with a mean age of 26.9 ± 9.0 (Table 1). Before surgery, complete dental and medical histories and periapical and panoramic radiographs were taken for each patient. Informed consent was obtained from each patient. Exclusion criteria included the history or the existence of infectious complications and the appearance of enlarged tissues surrounding the impacted teeth. Periapical radiographs using a parallel technique were taken for all impacted teeth, and the radiographs were transferred to digital form using a digital scanner. The sizes of the dental follicles were measured using Scion Image program (Scion, Frederick, Maryland, USA). Dental follicles with a follicular width of 2.5 mm or less in any dimension were included in the study. The impacted teeth were distributed into two groups according to depth following Pederson’s classification [12,13].

All operations were carried out under local anesthesia by conventional third molar surgery. Dental follicular tissues were obtained by careful curettage during surgical removal of the teeth. The selected dental follicles were distributed into two groups according to the conditions of their related teeth. Group 1 consisted of 11 dental follicles surrounding impacted teeth with total or partial absence of bony coverage, and group 2 consisted of 11 dental follicles surrounding full bony impacted teeth (Figs 1 and 2).

**Tissue preparations**

After the dental follicles were surgically removed, the specimens were fixed in 10% buffered formalin for 24 h and embedded in paraffin wax. They were sliced into 4-μm-thick sections and processed for routine histological and subsequent immunohistochemical examinations. Histologically, the specimens were classified according to the type of epithelium and the degree of subepithelial inflammation. Epithelial components were divided into two groups: reduced enamel epithelium and stratified squamous epithelium. The degree of subepithelial inflammatory changes was further classified into two groups: none or slight, and moderate to severe.

**Immunohistochemistry**

Immunohistochemistry was carried out by means of a standard avidin–biotin–peroxidase procedure. Sections were cut from each of the specimens and mounted on positively charged slides. The sections were deparaffinized with xylene and rehydrated through a series of decreasing percentages of ethanol. Antigen retrieval was performed by treating the sections with 10 mmol/l citrate buffer (pH 6.0) at 95°C for 20 min and then returning to room temperature for an additional 20 min. Endogenous

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**Table 1 Clinical details of patients involved in study group 1 and 2**

<table>
<thead>
<tr>
<th>Case number</th>
<th>Number of impacted teeth</th>
<th>Age</th>
<th>Impacted tooth</th>
<th>Bony coverage</th>
<th>Mucosal coverage</th>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>26</td>
<td>MdL3rdM</td>
<td>Partial</td>
<td>Total</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
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<tr>
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<tr>
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</tr>
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<td>1</td>
<td>29</td>
<td>MdR3rdM</td>
<td>No</td>
<td>Partial</td>
</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>Total</td>
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</tr>
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<td>1</td>
<td>36</td>
<td>MdL3rdM</td>
<td>Total</td>
<td>Total</td>
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</tbody>
</table>

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**Fig. 1**

Partially impacted mandibular third molar in group 1.

**Fig. 2**

Totally impacted mandibular third molar in group 2.
peroxidase activity was then blocked by 10 min of incubation in a hydrogen peroxide block (Thermo Fisher Scientific/Labvision, Fremont, California, USA). The background signal was reduced by incubating the sections for 30 min with normal serum. Sections were incubated with primary antibody (anti-cyclin D1, anti-p21, or anti-PCNA) (Table 2) in a humidifying chamber. Sections were then incubated with a biotinylated universal secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature and then incubated with the ABC reagent (Vectastain Elite ABC kit; Vector Laboratories). Diaminobenzidine staining was performed to visualize the antigens in the tissue sections. Finally, sections were counterstained with Mayer’s hematoxylin and then dehydrated with increasing percentages of ethanol and xylene. The slides were mounted with Permount (Fisher Scientific, Hanover Park, Illinois, USA). An oral squamous cell carcinoma was used as a positive control, and sections for which the primary antibodies were omitted served as the negative controls.

**Evaluation of the staining**

Cells were considered positive when clear staining in the nucleus could be identified. PCNA, cyclin D1, and p21-positive cells were quantified using image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, Tennessee, USA). Images were captured using an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan) and a Sony DXC-390 camera (Sony Electronics Inc., New Jersey, USA). For each antibody, three random images from each sample were captured within a standard region of interest, and staining density was measured in pixels within this area (pixels/mm²). Subsequently, the average of the three measurements was used to represent the immunoreactivity of each sample. Statistical differences between the two groups were investigated using a two-tailed *t*-test. Differences were considered statistically significant when *P* value was less than 0.05.

**Results**

Histologically, all the dental follicles consisted of an epithelial component and fibrous connective tissue. The epithelial component was reduced enamel epithelium in eight specimens and stratified squamous epithelium in 14 specimens. The degree of inflammation in the underlying connective tissue was classified as none or slight in 12 specimens and as moderate to severe in 10 specimens. Immunohistochemical findings showed that all the specimens included in the study stained positively for PCNA and p21. However, some of the specimens (13.6%) showed no cyclin D1 staining. Positive PCNA staining was observed in most of the epithelial cell layers of the follicular tissues.

**Table 2** Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody (clone)</th>
<th>Species/type</th>
<th>Source</th>
<th>Dilution/incubation time</th>
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<tr>
<td>PCNA (PC 10)</td>
<td>Mouse monoclonal</td>
<td>Sigma Chemical Co. (St Louis, Missouri, USA)</td>
<td>1 : 3000/overnight at 4 °C</td>
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<tr>
<td>Cyclin D1 (SP 4)</td>
<td>Rabbit monoclonal</td>
<td>NeoMarkers (Fremont, California, USA)</td>
<td>1 : 50/overnight at 4 °C</td>
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<tr>
<td>p21 (CP 74)</td>
<td>Mouse monoclonal</td>
<td>NeoMarkers (Fremont)</td>
<td>1 : 50/1 h at 23 °C</td>
</tr>
</tbody>
</table>

PCNA, proliferating cell nuclear antigen.

**Fig. 3**

(a) Positive proliferating cell nuclear antigen (PCNA) expression along the epithelial layer of the follicular tissue and (b) islands of odontogenic epithelium within the follicular mesenchymal tissue showing positive PCNA expression (magnification, 200 ×).
Islands of odontogenic epithelium were identified in 10 cases (four from group 1 and six from group 2) and most of them also stained positively for PCNA (Fig. 3a and b).

For cyclin D1, discrete weak staining was observed in most of the specimens, and a predominantly focal distribution was observed in epithelial cells extending into the underlying mesenchyme. In contrast, staining for p21 was intense in the epithelial cell layer and in the odontogenic islands in all of the specimens and had a similar distribution as the PCNA (Figs 4 and 5).

Comparison of the overall expression of PCNA, cyclin D1, and p21 between the two groups revealed a significant difference for p21 ($P = 0.006$), whereas no significant difference was observed for cyclin D1 ($P = 0.29$) or PCNA expression ($P = 0.82$) (Table 3 and Fig. 6).

**Discussion**

There is a debate about the high incidence of histological change in dental follicles associated with an impacted third molar. This argument extends to include not only a long-standing symptomatic third molar but also the left or overlooked follicle after mandibular third molar odontectomy. The present study investigated the expression of cell cycle regulatory proteins in epithelial components of dental follicles associated with partial bony/soft-tissue impacted and full bony impacted third molars in order to find an answer for the hypothesis that the position of the impacted tooth may affect the cellular activities of the surrounding dental follicular tissues. The present research has selected three trustworthy immunohistochemical markers to achieve a reliable answer to this suggestion [14–17].

The choice of the markers used in this study, PCNA, cyclin D1, and p21, was based on their roles in regulating cellular proliferation, cell cycle progression, and cell kinetics. Cell proliferation plays an important role in several biological and pathological events. Cellular detection of PCNA is considered a reliable marker of cell proliferation. Cyclin D1 stimulates the cell cycle. It controls cell cycle transit from G1 to S phase by binding Cdks 4 and 6 and is thus considered a biological marker to predict cell cycle progression. p21 is the product of a suppressor gene that acts as an inhibitor of complexes formed by cyclins D, E, and A and their respective Cdks and thus functions as a regulator of cell cycle progression at G1 [18–22].
In the present study, PCNA-positive cells were detected in the reduced epithelium of the dental follicles and in the basal/suprabasal cell layers of the stratified squamous epithelium. Although a statistically significant difference was not detected between the two groups, the high expression levels of PCNA in the epithelial components in both groups suggested a high proliferation rate in the dental follicular tissues. These results of high PCNA expression are consistent with those of Cabbar et al. [4], who investigated the proliferative potential of dental follicles of asymptomatic impacted third molar teeth using Ki-67 and mini-chromosome maintenance protein 2 proliferation markers. They indicated that the odontogenic epithelium in follicular tissues of asymptomatic impacted third molars proliferates actively and may be an indicator of the differentiation potential of dental follicles. On the basis of these observations, they strongly supported the prophylactic removal of impacted third molars.

Expression of p21 was greater in group 2 than in group 1. It appears that the increased p21 expression may be a compensatory mechanism to the high PCNA expression and it represents a cellular attempt to balance the high cellular proliferation rate. Previous studies examining the expression of the p21 protein in tooth germs and follicular tissues showed different expression levels during distinct developmental stages [23,24]. Kumamoto et al. [25] reported that most odontogenic epithelial cells of human tooth germs at the initial stage of crown mineralization were reactive for the p21 protein. They suggested that the cellular differentiation of the odontogenic epithelium correlates with p21 expression. On the basis of their findings, it may also be concluded in this study that the increased p21 expression was related to the high degree of differentiation of the epithelial components in the dental follicular tissues.

The current study revealed that most of the dental follicular tissues exhibited decreased immunohistochemical staining for cyclin D1 as defined by the absence or decreased intensity of staining in the epithelial cells. In fact, these results correlate well with the p21 results, wherein it is expected that the level of cyclin D1 would decrease as the level of p21 expression increases. In addition, no significant difference in cyclin D1 expression between the two groups was identified. Although one might expect a higher cyclin D1 expression similar to the high PCNA expression, this was not the case. The absence of cyclin D1 in mitotic cells is probably due to the inactivation of this protein at the end of S phase and due to its short half-life [20,26].

**Conclusion**

This study showed that there was high PCNA expression in the epithelial components of the dental follicular tissues of both groups, confirming the highly proliferative nature of these tissues. There have been no previous studies comparing the expression of cell cycle proteins in relation to the depth of the impacted tooth. The significant difference observed in the level of p21 expression between dental follicles surrounding full bony and partial bony/soft-tissue impacted mandibular third molars suggests the possibility of differences in cell cycle regulation and cellular activities. Further studies using additional markers and techniques are needed to confirm the findings of this preliminary investigation. The obtained data will clarify the role of the impacted tooth and its relation to the degree of cellular proliferation in the overall evaluation of risk factor associated with deeply seated impacted teeth and their associated follicular tissue.

The findings of the present study have elucidated the possible deleterious behavior of cells of the dental follicle, particularly those associated with a deeply impacted mandibular third molar. Oral and Maxillofacial Surgeon should pay attention to this effect whether to monitor asymptomatic impacted mandibular third molar or during surgery to excise completely the residual follicle.

**Acknowledgements**

There are no conflicts of interest.

**References**


