IMMUNOEXPRESSION OF Ki-67 AND CYCLOOXYGENASE-2 IN ODONTOGENIC KERATOCYST AND DENTIGEROUS CYST: A COMPARATIVE STUDY

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ABSTRACT

Aims: To evaluate the expression of Cyclooxygenase-2 (COX-2) in odontogenic keratocyst (OKC) and dentigerous cyst and correlate it with Ki-67 expression in these lesions.

Material and Methods: This study was carried out on 32 paraffin embedded specimens of dentigerous cyst and OKC. 16 samples obtained from healthy mucosa served as controls. Slides prepared from paraffin-embedded sections were immunohistochemically stained for Ki-67 and COX-2 and immunoreactivity of the two markers was evaluated using index of positivity and staining intensity. Statistical analysis was performed using Mann-Whitney and Spearman’s correlation tests.

Results: Ki-67 and COX-2 expression in OKC and dentigerous cyst was found to be significantly higher than those observed in healthy mucosa groups (p < 0.05). The expression of the two markers was significantly higher in OKC than in dentigerous cyst. A positive correlation was found between the two markers in both lesions.

Conclusions: Increased expression of Ki-67 and COX-2 in OKC is an indicator of the high mitotic activity of these lesions compared with dentigerous cyst. COX-2 is implicated in odontogenic epithelial proliferation in OKC and dentigerous cyst.

Key words: Ki-67, COX-2, OKC, dentigerous cyst.

INTRODUCTION

Odontogenic cysts are those arising from epithelium involved in tooth development. They differ significantly in their incidence, clinical behavior, histological architecture and consequently treatment options (Regezi et al, 2008).

Because of its specific histological and clinical characteristics, odontogenic keratocyst (OKC) was classified as a cystic odontogenic neoplasm and the term keratocystic odontogenic tumor was used (Philipsen, 2005). However recently, it has been categorized as an odontogenic cyst according to World Health Orgaization (WHO) 2017 (Adel et al, 2017).
On the other hand, dentigerous cyst accounts for 20% of oral developmental cyst and is considered the most common developmental cyst in the oral cavity (Shibata et al, 2004). It has a low recurrence rate. However, longstanding lesions can produce local destruction, bony expansion, root resorption, or displacement of teeth (Kolokythas et al, 2012). The epithelium of dentigerous cyst may show neoplastic transformation into ameloblastoma, squamous cell carcinoma or intraosseous mucoepidermoid carcinoma (Neville et al, 2009).

Despite being both derived from odontogenic epithelium, there is great difference in developmental mechanism, biological behavior and treatment management of OKC and dentigerous cyst. Therefore, identifying the mechanisms underlying the lesion growth and behavior has been matter of concern (Maryam et al, 2015).

COX-2 is a cytokine inducible enzyme involved in the synthesis of prostaglandins (PGs) from arachidonic acid (Zha et al, 2004).

Under basal conditions, COX-2 expression is highly restricted; however, COX-2 is dramatically upregulated during inflammation (Crofford, 1997).

Several studies have detected significant COX-2 overexpression in a wide range of preneoplastic and malignant conditions including colorectal cancer, stomach, esophagus, breast, prostate and head and neck carcinomas (Castealo et al, 2003; Davies, 2003; Hussain et al, 2003; Manchana et al, 2006; Itoh et al, 2003; Kanekura et al, 2000; Kawai et al, 2002; Li et al, 2004; Masunaga et al, 2000; Miyata et al, 2003; Nozoe et al, 2005).

The exact mechanisms by which COX-2 encourages tumorigenesis include synthesis of PGs which stimulate PGs receptors with subsequent stimulation of cellular proliferation, induction of angiogenesis, inhibition of apoptosis, and stimulation of invasion/ motility by adjustment of cell adhesion molecules (Telliez et al, 2006).

Moreover, COX-2-dependent prostaglandin release can suppress antigen presentation and immune activation in cancer (Greenhough et al, 2009).

Ki-67 is a nuclear protein that can be detected in all proliferating cells, whereas resting, non-cycling cells (G0 phase) lack Ki-67 expression. It is usually used in research studies as an independent prognostic marker of tumor proliferation to investigate the growth characteristics of the lesion (Adelsperger et al, 2000).

The aim of the present study is to evaluate the expression of COX-2 in both OKC and dentigerous cyst and correlate it with the epithelial proliferation in these lesions.

**MATERIAL AND METHODS:**

**Case selection:**

A total of 32 paraffin blocks (16 dentigerous cysts and 16 OKC) were retrieved from the archives of Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Minia University. Clinical records for the patients were reviewed and information was gathered regarding age, gender and location of the lesions. A four micron section was cut from each block, stained with Hematoxylin and Eosin (H&E) staining and re-examined for confirmation of the diagnosis. The control group included 16 samples which were harvested from healthy mucosa taken by punch biopsy from operculum covering upper or lower wisdom. Considering the effect of inflammation on the expression of COX-2, in this study, non-inflammatory samples were used.

**Immunohistochemical staining**

It was performed using the standard method (avidin biotin peroxidase) (Bratthauer, 2010). Paraffin blocks were cut into 4 μm thick and mounted on positively charged glass slides (Optiplus, Biogenex, Milmont Drive, CA, USA) for
immunostaining with anti-Ki-67 and anti-COX-2 antibodies.

The paraffin embedded tissue sections on positively charged slides were deparaffinized in xylene, rehydrated through graded alcohols to water and treated with endogenous peroxidase in 0.3% H₂O₂ for 30 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM citrate buffer, pH 6.0 for 10-20 min followed by cooling at room temperature for 20 min. The sections were then incubated with the primary monoclonal mouse antihuman Ki-67 antibody (clone MIB-1, IS626, Ready to use, Dako, Denmark) and the primary antibody rabbit polyclonal anti-COX2 antibody (Thermo Scientific, Lab vision, Kalamazoo, MI, USA) with dilution of 1/500 for 30 min at room temperature in a humidified chamber. After washing with phosphate buffer solution (PBS), the slides were treated with the biotin labeled link antibody and then the streptavidin conjugated horse radish peroxidase was used.

The diaminobenzedine chromogen was applied to visualize the antigen antibody reaction. All these reagents belong to the universal Labeled Streptavidin-Biotin 2 System, Horseradish Peroxidase (code no.K0673 DakoCytomation, Glostrup, Denmark). All the slides were immersed in Mayer’s hematoxylin for counter staining. Finally, the sections were covered by cover slips using aqueous mounting medium. Negative controls were stained in the same technique but with omitting of the primary antibody and treating them with PBS instead.

Immunohistochemical evaluation

The ordinary light microscope (Leica, Germany) was used to detect and localize the immunostaining of the two antibodies. For Ki-67, cells with nuclear staining were considered positive while cells with cytoplasmic staining were considered positive in case of COX-2 antibody.

Five random fields in each specimen were captured using magnification (X400). Photomicrographs were captured using a digital camera (Leica, Germany) mounted on the light microscope.

Immunohistochemical analysis followed the semi-quantitative markers expression in the epithelial compartments of the lesions. It was calculated as an index of positivity (IP) for each used biomarker by reporting the number of marked cells by the total number of 500 cells identified at magnification of X400, followed by multiplying the result by 100. The percentage of positively stained cells in representative high power (X400) fields was determined and scored as follows: 0, no identified staining of the odontogenic epithelium or unnoticeable staining; 1, <10% staining; 2, 10-50% staining; and 3, >50% staining (Barboza et al, 2005).

Also the staining intensity was assessed using image analysis software (Image J, 1.41a, NIH, USA) and rated on a scale from 0 to 3 as follows: 0, no staining at all; 1, weak staining; 2, moderate staining; and 3, strong staining.

Degree of immunoreactivity was obtained by the sum of score of IP and staining intensity score with a maximum score of 6. A final score of 0 was regarded as negative, 2 as weak, 3 or 4 as mild, and 5 or 6 was considered as strong immunoreactivity (Mohammed et al, 2017).

Statistical analyses

The Statistical Package for the Social Sciences (SPSS) 19.0 software (IBM SPSS, Armonk, NY, USA) was used for analyzing the results. Quantitative data were presented by mean, standard deviation and range. Mann-Whitney U test was used to compare differences between two independent groups. The correlation between the Ki-67 and COX-2 expression in dentigerous cyst and OKC was assessed using Spearman rank correlation analysis.
The difference between groups was considered statistically significant at $P \leq 0.05$.

RESULTS

Clinical data

This study has been carried out on 48 specimens (16 dentigerous cysts, 16 OKC and 16 healthy mucosa) available for study at the archives of the Department of Oral and Maxillofacial Pathology, Minia University. Clinicopathological data of the lesions are summarized in table 1.

TABLE (1) Clinicopathological characteristics of the involved specimens

<table>
<thead>
<tr>
<th>Group (n=16)</th>
<th>Total</th>
<th>OKC</th>
<th>Dentigerous cyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>31.2</td>
<td>28.3</td>
<td>32.6</td>
</tr>
<tr>
<td>Range</td>
<td>21-57</td>
<td>21-45</td>
<td>24-57</td>
</tr>
<tr>
<td>Gender n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17(53.1%)</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Female</td>
<td>15(46.9%)</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Location, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxilla</td>
<td>10(31.3%)</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Mandible</td>
<td>22(68.7%)</td>
<td>13</td>
<td>9</td>
</tr>
</tbody>
</table>

Ki-67 protein expression

Ki-67 protein expression was detected as yellowish-brown nuclear staining in the epithelial cells of dentigerous cysts and OKC.

Ki-67 positive staining was detected in 28 cases including 13 cases of dentigerous cyst and 15 cases of OKC.

In dentigerous cyst, positive cells were mainly found in the basal and suprabasal cells layers (Figure 1). Ki-67 expression was absent in 18.75%, weak in 56.25%, moderate in 25%, and strong in 0% of odontogenic epithelial cells of the cyst lining (Table 2).

In OKC, positive cells were found mainly in the basal and suprabasal cells layers (Figure 2). Ki-67 expression was negative in 6.25%, weak in 31.25%, moderate in 25% and strong in 37.5% (Table 2).

The staining intensity and number of Ki-67 positively stained cells (IP) were higher in OKC than dentigerous cyst.

COX-2 protein expression

COX-2 protein expression was detected as yellowish-brown cytoplasmic staining of different intensities in the epithelial cells of dentigerous cysts and OKC.
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In dentigerous cysts, positive cells were observed throughout the cystic wall (Figure 3). COX-2 staining intensity was absent in 12.5%, weak in 31.25%, moderate in 56.25%, and strong in 0% of odontogenic epithelial cells in dentigerous cyst samples (Table 2).

OKC showed diffuse widely distributed COX-2 expression in epithelial cells (Figure 4). COX-2 expression was negative in 0%, weak in 18.75%, moderate in 50% and strong in 31.25% of OKC samples.

Higher number of COX-2 positively stained cells and stronger expression were found in OKC compared to dentigerous cyst.

TABLE (2): The immunohistochemical staining of Ki-67 and COX-2 in terms of staining intensity in the study groups

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dentigerous cyst (n=16)</th>
<th>OKC (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (Score=0)</td>
<td>3(18.75%)</td>
<td>1(6.25%)</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak (Score=1)</td>
<td>9(56.25%)</td>
<td>5(31.25%)</td>
</tr>
<tr>
<td>Moderate (Score=2)</td>
<td>4(25%)</td>
<td>4(25%)</td>
</tr>
<tr>
<td>Strong (Score=3)</td>
<td>0(0%)</td>
<td>6(37.5%)</td>
</tr>
<tr>
<td>COX-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (Score=0)</td>
<td>2(12.5%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak (Score=1)</td>
<td>5(31.25%)</td>
<td>3(8.75%)</td>
</tr>
<tr>
<td>Moderate (Score=2)</td>
<td>9(56.25%)</td>
<td>8(50%)</td>
</tr>
<tr>
<td>Strong (Score=3)</td>
<td>0(0%)</td>
<td>5(31.25%)</td>
</tr>
</tbody>
</table>

TABLE (3): The immunohistochemical staining of Ki-67 and COX-2 in terms of Median index of positivity (IP) in the study groups

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dentigerous cyst</th>
<th>OKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (Score=0)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>&lt;10% (Score=1)</td>
<td>7(5.2%)</td>
<td>3(6.4%)</td>
</tr>
<tr>
<td>10-50% (Score=2)</td>
<td>4(37.6%)</td>
<td>7(42.5%)</td>
</tr>
<tr>
<td>&gt;50% (Score=3)</td>
<td>2(54.3%)</td>
<td>5(77.5%)</td>
</tr>
<tr>
<td>COX-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (Score=0)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>&lt;10% (Score=1)</td>
<td>2(4.9%)</td>
<td>1(8.6%)</td>
</tr>
<tr>
<td>10-50% (Score=2)</td>
<td>6(33.5%)</td>
<td>7(39.8%)</td>
</tr>
<tr>
<td>&gt;50% (Score=3)</td>
<td>6(57.7%)</td>
<td>8(71.2%)</td>
</tr>
</tbody>
</table>
Statistical results

Ki-67 and COX-2 immunoreactivity (as represented by the sum of score of IP and score of staining intensity) was found to be significantly higher in OKC and dentigerous cyst in relation to normal oral mucosa as shown in table 4, 5.

TABLE (4): Comparison between normal mucosa group and dentigerous cyst group using Mann-Whitney U test regarding Ki-67 and COX-2 immunoreactivity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dentigerous cyst Mean±SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score</td>
<td>Ki-67 Marker 2.37±1.54</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>COX-2 Marker 3.44±1.63</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

P value is significant when it is ≤0.05

TABLE (5): Comparison between normal mucosa group and OKC group using Mann-Whitney U test regarding Ki-67 and COX-2 immunoreactivity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OKC Mean±SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score</td>
<td>Ki-67 Marker 3.93±1.80</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>COX-2 Marker 4.56±1.21</td>
<td>0.04</td>
</tr>
</tbody>
</table>

P value is significant when it is ≤0.05

OKC showed significantly higher expression of both Ki-67 and COX-2 compared to dentigerous cyst (p=0.01 and 0.04 respectively) (Table 6).

There is positive correlation between the two markers in both dentigerous cyst and OKC (P=0.02 and 0.0001 respectively) as shown in table 7.

### DISCUSSION

COX-2 is a gene induced by growth factors, oncogenes and carcinogens and it is overexpressed in many human tumors including those of the oral cavity and head and neck (Juhng et al, 2004; Itoh et al, 2003; Sakurai et al, 2007; Renkonen et al, 2002)

The exact role of COX-2 in neoplastic development and growth has been contributed to its ability to resist apoptosis, promote cell growth,
induce angiogenesis and enhance cell motility and adhesion (Wu et al., 2010).

The present study was carried out to elucidate the possible role of COX-2 in two odontogenic cysts (dentigerous cyst and OKC) and correlate its expression with a well-known proliferation marker, Ki-67.

Regarding clinical data, the incidence of cystic lesions was more prevalent among male (53.1%) and the lesions were more frequent in the mandible (68.7%). These results are in accordance with those of Mesgarzadeh et al., 2008 whose findings showed increased incidence of OKC and dentigerous cyst in male more than female with the mandible being the most common site.

COX-2 and Ki-67 immunoreactivity was evaluated using two parameters, staining intensity and index of positivity. Both lesions showed increased COX-2 and Ki-67 expression in relation to healthy oral mucosa.

These results are fully compatible with those of Gadbail et al., 2009 who detected higher proliferation index in OKC when compared to dentigerous cyst and healthy mucosa and it has been associated with higher intrinsic proliferation potential of OKC, which may explain the local aggressive behavior of that lesion.

Our study showed significant difference in COX-2 expression between dentigerous cyst and OKC. This may explain the invasive behavior of OKC when compared to less invasive clinical behavior of dentigerous cyst and this is supported by the notion that high expression of COX-2 is related to the aggressive behavior of oral tumors (Mendes et al., 2011).

The High expression of COX-2 was highly correlated with high expression of Ki-67 in the studied lesions.

The ability of COX-2 to stimulate cell proliferation can be explained by the fact that COX-2 leads to synthesis of PGE2 with subsequent stimulation of EP4 receptors which leads to induction of the functional expression of early growth response factor 1 (EGR-1) which in turn controls Cyclin D1, a key regulator of cell cycle progression (Guillemot et al., 2001).

Furthermore, prostaglandin E2 inhibits tumor necrosis factor and induces IL-10 which has inhibitory effects on the immune system. It also appears that increased expression of COX-2 changes cell adhesion and response to regulatory signals and also it inhibits apoptosis (Mohan and Epstein, 2003; Williams et al., 2000; Itoh et al., 2003).

Our results in addition to available data from previously mentioned studies suggest that COX-2 may induce tumorigenesis in pathways affecting the proliferation of cells in dentigerous cyst and OKC.

**CONCLUSIONS**

COX-2 may serve as predictive biomarker of the clinical behavior of dentigerous cyst and OKC and this could lead to the development of new therapeutic pathways such as molecular targeted therapies of patients affected by these lesions.

**REFERENCES**


