Original Article

Protein 53, B-Cell Lymphoma-2, Cyclooxygenase-2, and CD105 Reactivity in Keratocystic Odontogenic Tumors: An Immunohistochemical Analysis

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Abstract

Objective: The objective is to evaluate and compare immunohistochemically, the biological behavior of keratocystic odontogenic tumor (KCOT) with normal oral mucosa by analyzing cell proliferation, angiogenesis, and antiapoptosis using cyclooxygenase-2 (COX-2), protein 53 (p53), B-cell lymphoma-2 (Bcl-2), and CD105 (endoglin). **Materials and Methods:** The present study comprised 30 cases of KCOT in the study group, in which the expression of COX-2, p53, Bcl-2, and CD105 were analyzed and compared with that of control group consisting of 30 normal oral mucosae. **Results:** The results were evaluated based on a number of positively stained cells; among 30 samples of KCOT, 22 samples were p53 positive, 23 samples were Bcl-2 positive, 18 samples were COX-2 positive, whereas all the above markers were negative in all the 30 samples of the normal oral mucosa and CD105 mean vascular density in KCOT 13.8 in normal oral mucosa. **Conclusion:** The present study suggests that angiogenesis, cell proliferation, and antiapoptosis may be the possible factors contributing for the unique biological behavior of KCOT.

Keywords: *Angiogenesis, antiapoptosis and immunohistochemistry, cell proliferation, keratocystic odontogenic tumor*

Introduction

Odontogenic keratocyst is known to be a unique developmental cyst compared to other odontogenic cysts due to its high recurrence rate with inherent proliferative potential and aggressive nature, leading to a marked ability for destroying bone[1,2] and because of such peculiar behavior, the World Health Organization (WHO) has reclassified odontogenic keratocyst as a benign neoplasm and designated it as "keratocystic odontogenic tumor" (KCOT), but according to the WHO classification of Head-and-Neck pathology in 2017, this neoplasm KCOT was reclassified as odontogenic keratocyst. Mostly, KCOTs occur sporadically, but about 4%–5% of KCOTs were found to arise in association with nevoid basal-cell carcinoma syndrome, which has a high tendency to transform into basal cell carcinoma.[3,4]

Several immunohistochemical studies were conducted using various markers of proliferation apoptosis and angiogenesis to examine biological behavior of KCOTs. The proliferative activity of the epithelial lining of KCOTs showed strong expression for protein 53 (p53), proliferating cell nuclear antigen, and Ki-67 than in any other types of odontogenic cysts.[5-8]

Materials and Methods

The present study comprised study group with 30 cases of KCOT, which were randomly selected from the archives of the Department of Oral Pathology and Microbiology, Kamineni Institute of Dental Sciences, Narketpally, Nalgonda (District), Telangana, and control group consisting 30 normal oral mucosae. All the samples in the present study, that is, study and control groups were selected with the clearance of Institutional Ethical Committee.

Immunohistochemistry

Paraffin wax-embedded tissue blocks of 30 KCOTs were randomly selected from the archives and sectioned into four sections of 3-μm thickness each with rotary microtome. The normal healthy oral mucosae of 30 individuals were obtained, fixed, processed, embedded with wax, and sectioned into four sections with the same thickness of 3 μm. All the sections (study and control groups) were taken onto SuperFrost Plus

How to cite this article: Nimmanagoti R, Nandan SR, Kulkarni PG, Reddy SP, Keerthi M, Pupala G. Protein 53, B-Cell Lymphoma-2, Cyclooxygenase-2, and CD105 reactivity in keratocystic odontogenic tumors: An immunohistochemical analysis. Int J App Basic Med Res 2019;9:27-31.

Received: 19 April, 2018.
Accepted: 02 January, 2019.

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Access this article online

Website: www.ijabmr.org

DOI: 10.4103/ijabmr.IJABMR_138_18

Quick Response Code:

Glass Slides, deparaffinized by placing the slides in the hot air oven at 70º C for 20 min, and further rehydrated by taking the tissue sections through two changes of xylene, absolute, and 80% and 70% alcohol for 5 min each. The slides were immersed under running tap water for 2–3 min.

Antigen retrieval

Microwave-based antigen retrieval was done in tris ethylenediaminetetraacetic acid-buffered saline solution which was then heated in a microwave oven for three to five times depending on the antibody used at 100°C temperature for a period of 5 min each. All the slides were allowed to cool to room temperature, and all the reagents stored in the refrigerator were brought to room temperature (24°C–28°C) before immunohistochemical staining. Tissue sections were allowed to dry during the staining procedure and then washed gently with phosphate-buffered solution (PBS) three times for 3 min each and excess of buffer solution was tapped off. The sections were then covered with peroxide block for 10 min followed by PBS wash three times for 3 min each. After tapping off the excess buffer from the slide, the sections were covered with Prediluted (Biogenex) (p53, B-cell lymphoma-2 [Bcl-2], cyclooxygenase-2 [COX-2], and CD105) Monoclonal Rabbit Antibody and incubated for 45 min at room temperature followed by PBS wash three times for 3 min each. Target binder was added to the tissue sections and left for 10–12 min, followed by PBS wash three times for 3 min each. After tapping off the excess buffer, the tissue sections were then incubated with secondary antibody for 10–12 min and washed gently with PBS three times for 3 min each. Excess buffer was tapped off and tissue sections were covered with freshly prepared substrate chromogen (diaminobenzidine) solution for 10 min followed by gentle washing with distilled water for 2 min. The sections were then immersed in Mayer's hematoxylin for 2 min and washed gently under running tap water for bluing. Finally, the tissue sections were dehydrated through series of alcohols for 5 min each, respectively. Then, the sections were immersed in xylene for clearing and later mounted by using DPX.

p53 expression was evaluated based on a number of positively stained nuclei of the cells, whereas Bcl-2 and COX-2 expressions were evaluated based on a number of positively stained cells. Three high-power fields (40X) were selected randomly in the tissue section to determine the stained cells per 100 counted cells in full thickness of the epithelium in each field (LI). The mean labeling index was calculated for these three fields. If the mean of labeling index is more than 5, then the case is considered as positive, if it is ≤5, then the case is considered as negative.[6,7] CD105 expression was evaluated by microvessel density by assessing the endothelial cells lining the blood vessels by their brown cytoplasmic staining; the highest density of staining determined by low-power view (×10) was selected and then under three high-power views (×40), a number of CD105-positive endothelial-lined blood vessels were counted and the mean of these three fields was considered as mean vascular density (MVD).

Statistical analysis

SPSS version 19 (IBM corp., Hyderabad, Telangana, India) was used for the statistical analysis. The significance of the results obtained from the control and study groups was statistically analyzed by independent sample *t*-test. $P < 0.05$ was considered to be statistically significant. Mean and standard deviation of cases and controls were also determined.

Results

In the present study, among the 30 (100%) samples of KCOT, 22 (73%) samples were p53 positive, 8 (27%) samples were negative, whereas 23 (77%) samples were Bcl-2 positive, 7 (23%) samples were negative, 18 (60%) samples were COX-2 positive, 12 (40%) samples were negative, and all the normal oral mucosae were negative to the p53, Bcl-2, and COX-2. The mean value of mean vascular density stained with CD105 in normal oral mucosa was 4.1; in KCOT, it was 13.8.

Discussion

Odontogenic keratocyst is a unique developmental cyst that has a greater tendency to reoccur more frequently and histologically; the epithelial lining shows greater propensity to dysplasia and malignant transformation compared to other jaw cysts.[2,9] Multiple odontogenic keratocysts which constitute as a part of basal cell nevus syndrome show a higher epithelial mitotic rate, frequent basal-cell budding, more odontogenic rests, and satellite cysts in patients with the basal cell nevus syndrome compared to those without syndrome.[3]

Cell proliferation is considered to be a vitally important biological process to all living organisms as it helps in maintenance of growth and tissue homeostasis, but in some situations, when there is increase in cell proliferation, it is considered as one of the first indicators for development of any pathology or it even suggests recurrence of any previous lesion. Based on the concepts of the cell cycle, different methods are available to assess the rate of proliferation. Immunohistochemistry is the recent advancement that has become popular in understanding the mechanisms underlying growth regulation and identification of proteins that are preferentially synthesized in proliferating cells.[5]

Normally tumor suppressor genes encode proteins that maintain required number of cells by suppressing proliferation, but in tumorigenesis, these genes get mutated that result in loss of function. p53 is a tumor suppressor gene that has a key role in deciding the fate of cells, by eliminating the cells that have sustained genetic damage and if there is any damage to the p53 gene itself, it results in abnormal cell proliferation.

Mitochondrial pathway is the major mechanism of apoptosis in all mammalian cells, with its role in both physiologic and pathologic processes. Balance between pro- and antiapoptotic members of the Bcl family (B-cell lymphoma) controls the mitochondrial permeability and prevents leakage of mitochondrial proteins that have the ability to trigger cell death.[10] Bcl-2 gene is located on chromosome 18q21.3 which encodes a protein capable of inhibits apoptosis, thus facilitating cell survival independently of cell division.[11] As Bcl-2 is an antiapoptotic protein, in the present study, Bcl-2-positive cells were detected exclusively in the basal layer. Bcl-2 inhibits apoptosis to facilitate cellular proliferation in the basal layer, whereas apoptosis maintains the homeostasis of the thickness of the lining epithelium and allows the synthesis of large amounts of keratin in the surface layer of KCOTs. Which helps in maintaining the balance between cellular proliferation and cell death.

In the present study, p53 and Bcl-2 labeling index of KCOT is very high when compared to the normal oral mucosa. This suggests that lining epithelium of KCOT shows continuous proliferation and antiapoptotic activity; these findings were similar to other studies. Hence, our study strengthens the classification of KCOT as an odontogenic tumor and should contribute to its aggressive clinical behavior. According to the present study, mean labeling index of p53 is approximately eight times more when compared to the normal oral mucosa. In all the positive cases of KCOT, p53 expression was more in the parabasal layers as compared to the basal layers [Figure 1]. Present p53 results are consistent with the recent and old studies.[12-14] This suggests that greater proliferative activity in KCOT, thus explaining their more aggressive clinical behavior.

In regard to Bcl-2 staining, among the 30 samples of KCOT, 69% showed Bcl-2 positive and 31% were Bcl-2 negative, whereas all the normal oral mucosa samples were negative to the Bcl-2 staining. The antiapoptotic activity of Bcl-2 is approximately nine times when compared to the normal oral mucosa and in all KCOTs, the expression of Bcl-2 was exclusively found in the basal layers of the lining epithelium as compared to the other layers [Figure 2]. Present results were consistent with other studies.[13,15,16]

COX-2 levels have been found to be elevated in various tumors. In fact, regulation of COX-2 expression is physiologically vital for PGE2 synthesis. Enhanced synthesis of prostaglandins, a consequence of upregulation of COX-2, can increase cell proliferation, promoting angiogenesis, and inhibiting immune surveillance, thus leading to the progression of disease. In the present study, COX-2 staining was positive in 60% of KCOT cases in the epithelial layers [Figure 3] and 40% of cases were negative, whereas all the normal oral mucosae are negative to the COX-2 staining. The mean labeling index of COX-2 is approximately 8 times more when compared to normal oral mucosa. The results of this study suggest that COX-2 can be considered as an indirect marker for proliferation activity of the KCOT which induces the proliferation of the epithelial cells. Results of the present study were consistent with other studies.[17,18] There is a limited number

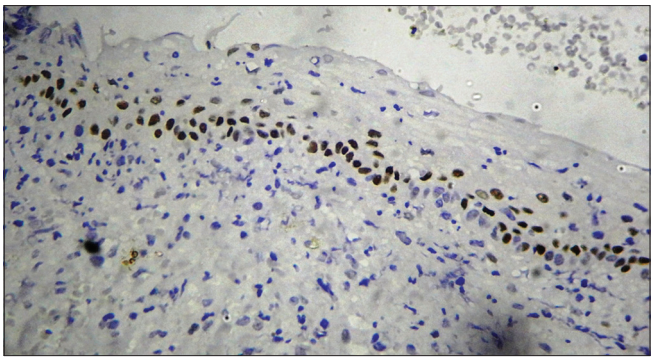

Figure 1: Protein 53 antibody staining in keratocystic odontogenic tumor (immunohistochemical, ×400)

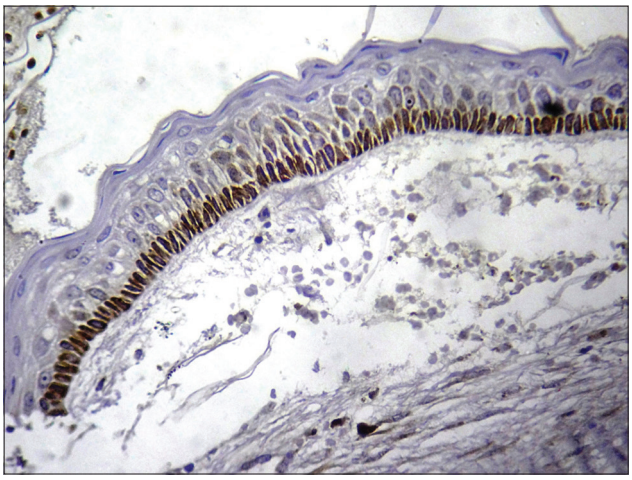

Figure 2: Bcl-2 antibody staining in keratocystic odontogenic tumor (immunohistochemical, ×400)

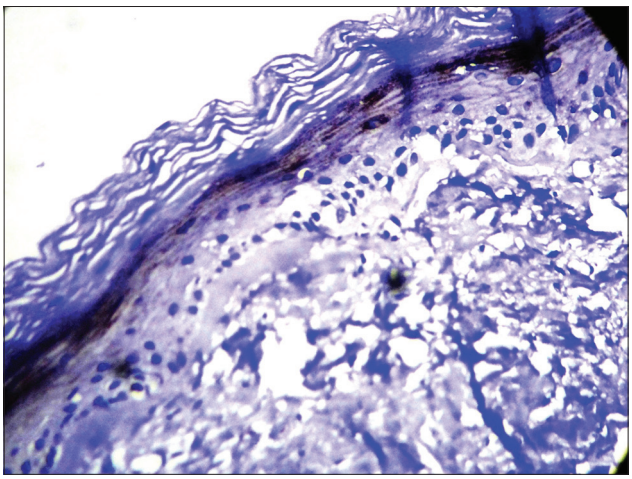

Figure 3: Cyclooxygenase-2 antibody staining in keratocystic odontogenic tumor (immunohistochemical, ×400)

of studies regarding COX-2 staining in KCOT; the findings of the present study suggest that COX-2 can be used as a potential marker to analyze the biological behavior of the KCOT.

Angiogenesis, the neoformation of the blood vessels from preexisting microvessels, is essential to numerous physiological and pathological processes, such as cell nourishment and cancer and ischemic disease progression. This complex process involves remodeling of the extracellular matrix and proliferation and migration of the endothelial cells. Mean vascular density (MVD) has been reported to be an independent prognostic indicator of outcome in a variety of human malignancies, with increased MVD correlating with overall survival rates.

The mean vascular density of KCOT is approximately three times when compared to normal oral mucosa. CD105 expression was observed in the endothelial cells [Figure 4]. The results of present the study were consistent with Kumar *et al*. 2015[8] where the mean vascular density in KCOT was 11 and Jamshidi *et al*. 2014,[19] where the mean vascular density was 13. Since neoplastic tissues require oxygen and nutrients to continue their growth and development, they induce neovascularization. MVD can predict the growth of the tumor and patient's survival and this value is related to the aggressiveness of the tumor. The growth of the tumor does not only necessitate an increase in the number of blood vessels but it also depends on factors such as protein molecules expressed in the endothelial cells. Angiogenesis may be associated with the different biological behaviors of the KCOT which suggests that the angiogenesis have an important role in tumor progression and invasiveness of KCOT. Increased angiogenesis in KCOT may contribute to the locally aggressive biological behavior. Assessment of tumor angiogenesis may prove very valuable in predicting the recurrent cases of KCOT.

Several authors have documented the existence of cell proliferation, antiapoptosis, and angiogenesis in KCOT using single marker or combination of two markers or three by comparing with other odontogenic cysts or tumors, with the best of our knowledge, none of them have considered the combination of p53, Bcl-2, COX-2, and CD105 antibodies reactivity in KCOT.

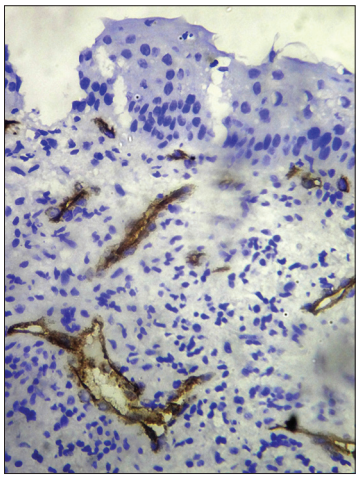

Figure 4: CD105 antibody staining in keratocystic odontogenic tumor (immunohistochemical, ×400)

The possible limitation of the present study is that it does not compare the reactivity of these markers in the other odontogenic cysts or tumors, and samples of KCOT were selected randomly without considering the age, gender, site of occurrence, radiological findings, primary or recurrent case, and any association of syndromes.

Further studies with inclusion of above-mentioned findings can be considered while selecting the samples and correlating with the molecular events such as cell proliferation, antiapoptosis, and angiogenesis of KCOT, which may reveal more specific characters of the KCOT.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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