Evaluation of Ki-67 Antigen and Protein P53 Expression in Orthokratinized and Parakratinized Odontogenic Keratocyst

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Statement of Problem: Odontogenic Keratocysts (OKC) make up 10-12% of all developmental cysts with dental origin. OKCs are classified into parakeratotic and orthokeratotic types, with completely different clinical features. In order to investigate biological behavior of OKCs, an immunohistochemical study was designed, using Ki-67 antigen as proliferation marker and P53 protein as tumor suppressor gene.
Purposes: The aim of the present study was to investigate the expression of P53 and Ki-67 markers in two types of OKCs and to determine their relationship with the biological behaviour of OKC.
Materials and Methods: A total of 20 OKCs (parakeratotic n=10, orthokeratotic n=10) were stained immunohistochemically for Ki-67 and P53 protein by Biotin – Streptavidine method. Then, slides were studied quantitatively through optical lense (magnification=X10) and the number of positively stained cells was counted/mm BM.
Results: The average number of Positively stained cells for Ki-67 were 62.30±11.96 cells/mm BM in parakeratotic, and 29.90±4.90 cells/mmBM in orthokeratotic OKCs ($P<0.05$). Positive cells for Ki-67 were dominantly located in parabasal layer. Mean stained cells for P53 were 4.30± 2.21cells/mmBM in parakeratinized and 4.80±1.75 cells/mmBM in orthokeratotic types that was not statistically significant. ($P<0.58$)
Parakeratotic OKCs mostly occur in the lower jaw (90%), whereas just 50% of orthokeratotic OKCs occur in mandible ($P=0.05$)
Conclusion: Regarding other clinical features and the existence of daughter cysts, no significant statistical difference was found between two types of OKCs.

Key words: Immunohistochemistry; P53 Protein; Ki-67 antigen Parakeratinized Odontogenic Keratocyst; Orthokeratinized Odontogenic Keratocyst

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The term "Odontogenic Keratocyst" (OKC) refers to a lesion, first described microscopically by Shear, Pinborg et al in 1956.(1)

A special microscopic feature, an aggressive clinical course and a high risk of recurrence differs OKC from other odontogenic cysts and present it as a benign cystic neoplasm.(2) Therefore, it seems that the keratin zed lining epithelium is highly active and instead of degenerative processes, reveals a complete differentiation.(3)

Studies show that OKCs make up 10 to 12% of all developmental cysts with dental origin.(4)

It is generally accepted that this cyst has been originated from dental lamina and fibroblast growth factors (FGF2) might be the probable factor responsible for this process.[4]

Most authors believe that dentigerous and radicular cysts continue to enlarge as a result of increased osmotic pressure within the lumen of the cysts.

This mechanism does not appear to be true for OKCs, and their growth may be attributed to unknown factors, inherent in the epithelium itself or enzymatic activities in the fibrous wall.

OKCs tend to grow in an anterior- posterior direction within the medullary cavity of the bone, without causing obvious bone expansion.

Multiple OKCs are occasionally associated with basal cell nevus (Gorlin) syndrome (BCNS).[2]

Studies have shown that over expression of cyclin D1 and P53, at different stages, in BCNS- associated OKCs, can be considered as an important sign for mutant cellular phenotype, proving that more aggressive clinical course in BCNS- associated OKCs is resulted from an imbalance between the expression of P53 and cyclin D1, Which leads to the disruption of cellular proliferation control system.[5]

Differences in the expression of positive cells for P27, P21, Fas antigen and SSDNA-Positive cells in BCNS-associated OKCs suggest that they should be considered different from isolated OKCs.[6]

The histophatologic feature of OKCs is characterized by:

- A uniform thickness of stratified squamous epithelium, usually ranging from 6-8 cells thick and a prominent palisade, polarized basal layer of cells often described as having a "picket fence" appearance.
- The epithelium and connective tissue interface is usually flat, and rete ridge formation is inconspicuous.
- The luminal surface shows flattened parakeratotic epithelial cells, which exhibit a corrugated appearance.
- Satellite cysts, or odontogenic epithelial islands, are found within the cyst fibrous wall.[2]

A Study on the OKCs connective tissue has revealed that stroma not only plays a supporting structure role, but also has an important role in its neoplastic behavior. [7]

Some investigators have recognized a microscopic orthokeratotic variant. These cysts do not demonstrate the same microscopic and biologic features as parakeratotic OKCs. The risk of recurrence for parakeratotic type is 62-65%, whereas that of orthokeratotic type is reported just 2%.

Orthokeratotic type is not associated with basal cell nevus syndrome but it has been reported that neoplastic transformations in the epithelial layer of orthokeratotic OKCs are a little more.[8]

SEM studies on two types of OKCs have revealed that in parakeratotic type, projections and depressions on the cell surface, coupled with cytoplasmic and desmosome junctions, make a complex cell surface morphology, whereas in the orthokeratotic type a uniform structure covered by a thick layer of orthokeratin, is observed.[8]

Today, some investigators believe that the growth speed of a lesion indicates its future behavior pattern. High risk of recurrence in OKCs, is a serious complication for patients and make them candidates for multiple surgeries.

In this study, Ki-67 antigen was used as a proliferation marker, to investigate its immunoreactivity in the epithelial layers of OKCs. Studies show that ki-67 monoclonal antibody staining is the best method for measuring cell proliferation.[9]

It is believed that cell proliferation plays an important role in growth and development of odontogenic cysts and tumors. In some neoplasms, cells proliferative activity determines their aggression level and provides prognostic information.

Recently, by immunohistochemical techniques, the discovery of Ki-67 antigen has shown the importance of cell proliferation in breast, head and neck carcinomas.

Moreover, any change in the P53 gene expression, which is a nuclear phosphoprotein and cell growth and proliferation regulator, or the inactivity of its wild type, plays an important role in human cancers. In normal cells, P53 gene is a tumor suppressor and cell proliferation regulator and its inactivity leads to neoplastic changes.[10]

The possibility of neoplastic changes in different types of OKCs encouraged us to investigate the expression of these markers in the epithelial lining of parakeratotic and orthokeratotic OKCs by immunohistochemical staining.

Materials and Methods

A total of 20 formalin fixed paraffinized blocks, with maximum tissue volume and proper fixation, were selected from banked tissue blocks (1981-2000). All blocks were prepared by Department of Oral Pathology, Faculty of Dentistry, Tehran University of Medical Sciences.

All samples had the OKC's criterias, described by Pindborg. slides were also confirmed by two expert pathologists through light microscope (×40 magnification). Meanwhile, the clinical information about patients including, age, sex, the presence of an impacted tooth, the clinical signs of lesion, and the size of lesion were collected from their files.

Then, the samples were prepared for hematoxilin- Eosin staining and for Ki-67 antigen and P53 protein by immunohisto-chemical techniques.

A 5- micron section was prepared from each block and stained by H&E. The stained samples were reevaluated to ensure the best regions of the lesion with minimum inflammation and bleeding.

After measuring each sample, BM length equal or more than 8mm were selected for immunohistochemical staining.[11]

Immunohistochemical study of the samples was performed on 3µm sections by P53 P clone Do-(Dako Denmark) and Ki-67 antigen monoclonal antibodies.

First, all samples were deparaffinized and dried and placed in microwave in a fresh solution of Citrate/Hcl Buffer 10 mmol with pH=6.0 for ten minutes.

After cooling in room temperature, they were rinsed in phosphate buffered salin (PBS) and incubated with Anti Ki-67 antigen and Anti P53 protein antibodies, with 0.01 dilution, for one hour. Then sections were rinsed with PBS and incubated with biotinylated antibodies for 30 minutes, followed by washing with PBS and incubation for another 30 minutes with peroxidase labeled streptavidin.

Then, the samples were rinsed again with PBS and incubated with 3,3 Diamino Benzidine Hydrocholorid (DAB) chromogene, resulted in a brown product. Finally, all sections were stained by Ethyl- green, redehydrated and covered by a lamellae.

In both experiments, a positive and a negative control subject were used.

In order to evaluate the stained sections descriptively with Ki-67 and P53 antibodies, the number of positive cells in basal and suprabasal regions in lining epithelium of cysts were counted mm/BM by X10 magnification, with Eye Piece Gratitude (MIC 11406 EYE Piece, HWF, 10×, Euro max Microscopes, Holland)

The optical lense, has a measured surface with an area equal to 400 squares, and with the dimensions of 20î 20. With X10 magnification, used in this study, the stained cells were counted in 20 squares, equal to 7mm of BM. Cell nuclei with bright brown color, regardless of the color intensity, were considered as positive cells for Ki-67 and P53.

The following items were investigated quantitatively:

- BM length, based on mm (samples with a length ≥8mm were selected and evaluated.
- Total number of cells (mm BM)
- Total number of stained cells mm BM, for Ki-67 and P53.

4- Percentage of cells, which stained to the total number of counted cells.
- The total number of stained cells for Ki-67 and P53 in basal and parabasal layers.
In this study, t-test and fisher exact test were used for statistical analyses and $P<0.05$ considerd significant.

Results

Staining with Ki-67 antibody lead to a definite brown staining in nucleus, surrounded by a cream cytoplasm. The stained cells for Ki-67 marker were mainly detected in parabasal region.
The average staining for Ki-67 were 62.30±11.96 cells/mm BM and 29.7±4.90 cells/mm BM for parakeratotic and orthokeratotic OKCs, respectively.
The differences were statistically significant ($P<0.05$) (Table I and Fig. 1)
The average staining, in basal layer, for Ki-67, were 20.70±4.96 cells/mm BM and 8.70±1.94 cells/mm BM in parakeratotic and orthokeratotic OKCs, respectively. The differences were statistically significant ($P<0.05$).
Regarding to the cell staining, in parabasal layer, 80% of the parakeratotic OKCs showed more than 30 cells/mm BM, whereas in orthokeratotic types, 80% of the samples demonstrated less than 30 cells/mm BM.
The differences were statistically significant ($P<0.007$).
The reaction between monoclonal antibody with P53 protein resulted in a bright brown staining in the nucleus of the epithelial cells. Positive cells for P53 were mainly detected in the parabasal layer and totally a small number of cells, in both groups, were positive for P53.
The average number of positive cells for P53 was 4.30±2.21 cells /mm BM for parakeratotic types and 4.80±1.75 cells/mm BM for orthokeratotic cysts. The difference was not statistically significant.
In parakeratotic OKCs, in 70% of the samples, more than 2 positive cells for P53 were detected in basal layer, however, this parameter was 80% for orthokeratotic type. The difference was not statistically significant.
The average number of positive cells for P53, in parabasal layer were 45.33±10.08 cells /mm BM and 42±15.87 cells/mm BM for parakeratotic and orthokeratotic OKCs, respectively. The difference was not statistically significant.
The mean total number of counted cells was 336.20±83.22 cells/mm BM in parakeratotic type and 232.70±84.03 cells/mm BM in orthokeratotic type.
The total percentage of positively stained cells for Ki-67 to the total counted cells was 18.53% for parakeratotic type, whereas this number was 12.76% for orthokeratotic OKCs.
The ratio of total positively stained cells for P53, to the total counted cells were 1.27% and 2.06% for parakeratotic and orthokeratotic OKCs, respectively.

Discussion

A comparative study through Ki-67 and P53 markers and immunohistochemistry staining, has not been performed between two types of parakeratotic and orthokeratotic OKCs. The previous conducted studies, have mainly compared OKC with other cysts and odontogenic tumors.
A related study showed that there was a statistically significant difference between Ki-67 antigen and P53 protein expression in lesions with odontogenic epithelial origin.[12]
Odontogenic keratocyst, due to its aggressive behavior, high risk of recurrence and special biological characteristics, has always been the focus of a lot of studies.
Distinct differences in the biological behaviour of two defined types of this lesion, has been the main stone of the present research.
Findings of this study showed that the mean positively stained cells/mm BM for Ki-67 antigen is considerably more in parakeratotic

OKC than that of orthokeratotic type.

Today, several methods, such as immunohistochemistry with Bromodixy Uridine, Ki-67, PCNA markers and flowcytometry, are applied to evaluate cell proliferation rate. Some of these methods require frozen tissue sections which is a limiting factor, However most of the retrospective studies showed that PCNA and Ki-67 can be applied on paraffinized sections.[13]

Li et al. showed that although PCNA expression is related to the synthesis phase of cell cycle, but it does not necessarily have a close relationship with cell cycle. For example PCNA expression is also observed in DNA repairing process.[14]

The specific marker for cell proliferation, which is abundantly expressed in S stage of cell cycle and disappears immediately after mitosis, is Ki-67 antigen. This antigen is a preferable and trustful criterion in immunohistochemistry studies, showing cell proliferation rate.[14]

In this study, the proliferation rates of parakeratotic and orthokeratotic odontogenic keratocysts, by Ki-67 marker were compared.[2]

Parakeratotic type shows 40% recurrences after treatment, its aggressive behavior and tendency for recurrence can be attributed to Ki-67 expression as 63.3±11.96 mm BM, whereas this parameter is 29.70±4.90mm BM for orthokeratotic.[4]

Because of similar microscopic view and different clinical behaviour, it can be concluded that immunohistochemistry staining for Ki-67 marker, in addition to justifying biological characteristics, can be an effective method to differentiate between these two types of OKCs and finally help surgeon to make a strategic decision for treatment.

In other words, with 90% confidence it can be suggested that samples with positively stained cells for Ki-67 which have 19.90±39.50 cells/mm BM require more conservative treatments and those with 39.38+86.22 cells/mm BM need more radical treatments.[11]

On the other hand, comparing the results of this study with previous researches about Ki-67 expression, orthokeratotic OKC, demonstrates an obvious difference with other cysts of jaw such as dentigerous and radicular. It seems that more proliferative activity of orthokeratotic type, as compared with the mentioned cysts, results in a distinct clinical behavior, requiring more investigation.[11]

Findings show that positive staining for Ki-67 is mainly detected in parabasal cell layer, which is true for both types.

This can be justified as that such cells exist in a stage of cell cycle, followed by higher rate of Ki-67 expression.

This could be because basal cells are mostly a stem cell type, having a slow cell cycle and a long G1 phase.[15]

Although the mean value of P53 expression in orthokeratotic OKCs was more than parakeratoic type, this difference was not statistically significant.

Since more occurrences of neoplastic changes in the epithelial wall of orthokeratotic OKCs can be partly attributed to this phenomenon, complementary researches is suggested.

Considering the significant difference of Ki-67 antigen expression in the epithelial cells of parakeratotic and orthokeratotic odontogenic keratocysts in the present study and previous SEM studies, it can be concluded that these two types are two distinct entities with different histopathogenesis.[16,17]

More occurrence of orthokeratotic OKC with impacted tooth supports the theory that cystic changes of the tooth covering epithelium can be considered as a pathogenic factor.

Since the two types are significantly different in Ki-67 antigen expression, it can be related to the difference in their clinical behavior and can be considered an effective way in their definite microscopic recognition.

Therefore, investigating the pathologic and biologic relationship of different types of OKC, help the surgeon to make a proper decision and predict the risk of recurrence correctly and

finally develop immunohistochemical methods from research stage to ward applied- clinical stage.

A study in 1998 showed that odontogenic keratocysts make 38% of all growth-developmental cysts with odontogenic origin. This study was also conducted in the Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Tehran University of Medical Sciences and due to the fact that this center has a long time history for the admission of odontogenic lesions, the mentioned prevalence rate of OKCS is significant and applying sensitive diagnostic methods seems necessary.[18]

Because a lot of odontogenic cysts have keratinized epithelium and their histological differentiation based on conventional methods can result in errors, and also odontogenic keratocysts show a different behavior from other odontogenic cysts, immunohistochemical staining is helpful for exact diagnosis. Moreover, prospective studies to investigate the relationship between this marker and clinical behavior of odontogenic cysts are suggested.

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