

ORIGINAL ARTICLE

Evaluation and comparison of expression of p63 in odontogenic keratocyst, solid ameloblastoma and unicystic ameloblastoma

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ABSTRACT

Background and Objectives: The behavior of odontogenic lesions varies with some tumors behaving like a cyst and some cysts behaving like tumors. p63, a member of the p53 family of tumor suppressor genes has recently come into light in view of its role as an oncogene. The aim of the present study was to investigate the expression of p63 protein in OKC, Solid ameloblastoma, Unicystic Ameloblastoma and Follicular tissue. **Materials and Methods:** p63 expression was compared in 12 cases of OKC, 12 Solid Ameloblastoma, 14 cases of Unicystic ameloblastoma and 10 cases of Follicular tissue using immunohistochemical technique. All 48 cases were subjected to heat-induced antigen retrieval method using citrate buffer in a pressure cooker. Then the sections were stained with anti-p63 polyclonal antibody and visualized using super sensitive polymer HRP detection system. In each case, number of cells showing p63 positivity were assessed in two compartments - basal and suprabasal and compared.

Results: Statistical analysis showed that p63 expression in the suprabasal compartment in Odontogenic keratocysts was equivalent to that of central neoplastic cells of Solid Ameloblastoma and Unicystic Ameloblastoma type 3. Statistically significant difference in the expression of p63 was observed between OKC and Unicystic Ameloblastoma Type 1 and Solid Ameloblastoma and Unicystic Ameloblastoma Type 1. **Conclusion:** We conclude that the higher expression of p63 in these odontogenic lesions correlates well with their aggressive behavior and thereby suggesting alterations in treatment modalities.

Key words: Immunohistochemistry, odontogenic keratocyst, solid ameloblastoma, unicystic ameloblastoma

INTRODUCTION

Odontogenic lesions comprise a diverse group of lesions of varied behavior, ranging from innocuous hamartomatous proliferations to cysts with considerable growth and frankly malignant neoplasms with metastatic capabilities.

Odontogenic Keratocyst (OKC) is a distinctive developmental odontogenic cyst of epithelial origin with a potential for aggressive behavior, marked tendency for local recurrence,

and an association with Nevroid Basal Cell Carcinoma Syndrome (NBCCS).^[1]

The finding of clonal deletion mutations of genomic DNA in OKC supports the hypothesis that they are neoplastic in nature. Since, it shows characteristics of both cyst and benign tumor, very rightfully, it has now been renamed as 'Keratocystic odontogenic tumor' (KCOT), but with a far more aggressive nature when compared to other odontogenic cysts.^[1]

However this newly introduced concept of KCOT has not been given its due importance by several authors and surgeons who still continue to use the term OKC and use the older treatment modalities. Hence there is a need to accumulate further evidence to either support or disagree with the concept of KCOT.

A comparative study using a novel marker, which would compare the lesion with a well-established neoplasm could

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help in resolving the problem. Our study is an effort in the same direction.

Therefore we have compared OKC with Unicystic Ameloblastoma Type I which is at one end of the spectrum and Solid Ameloblastoma at the other end of the spectrum.

Recently, p63 has been proposed to be a specific marker of precursor/stem cells in many epithelial tissues and represents a candidate marker for assessing the proliferative activity of cells. p63 gene is a member of the p53 family and shares similar structure homology and functions with other p53 family members including p73. $\Delta Np63$ is the major isoform expressed which behaves in a dominant negative way opposite to that of p53.^[2]

Due to the almost restricted expression of p63 in epithelial cells, its infrequent mutation, as well as its overexpression in various solid tumors, it is suggested that it may play an oncogenic role in the regulation of proliferation and differentiation in premalignant and malignant lesions of epithelial origin. Overexpression of p63 has been noted in squamous and transitional cell carcinomas, as well as in certain lymphomas and thymomas. p63 expression has also been studied in OKC and other odontogenic cysts and tumors such as Dentigerous cyst, Radicular cyst and Ameloblastoma.^[3,4]

Hence, p63 expression may prove useful in assessing the proliferative activity and thereby comparing the biological behavior of these odontogenic lesions.

This study aims at evaluating the expression of p63 in OKC with Solid Ameloblastoma and Unicystic Ameloblastoma

Type I and Type III and comparing the same with each other thereby providing novel information about diagnostic as well as treatment modalities and prognosis.^[3]

MATERIALS AND METHODS

A total of 48 cases were evaluated. 12 cases of OKC, 12 cases of Solid Ameloblastoma, seven cases of Unicystic Ameloblastoma Type III, seven cases of Unicystic Ameloblastoma Type I and 10 cases of Follicular tissue as control were retrieved from the archives of our college. Normal skin was taken as positive control [Figure 1a] Syndrome associated OKCs and hybrid tumors were excluded.

Immunohistochemistry

Four micrometer thick sections were taken using a semi-automatic microtome (Microm HM 340E) and subjected to immunohistochemical study using p63 antibody as follows.

Procedure

Tissue sections of 4 μm thickness mounted on poly-lysine coated slides were incubated at 37°C overnight and then for an hour at 60°C before staining. The slides were deparaffinized in xylene and rehydrated through graded alcohols into water and subjected to antigen retrieval in a pressure cooker at 150°C for 30-35 min. The tissues were cooled to room temperature and incubated with peroxide block for 12 min to block endogenous peroxidase activity and subsequently for 10 min with protein block to eliminate background staining. The sections so treated were then incubated with primary antibody for 45 min followed by post primary for 30 min.

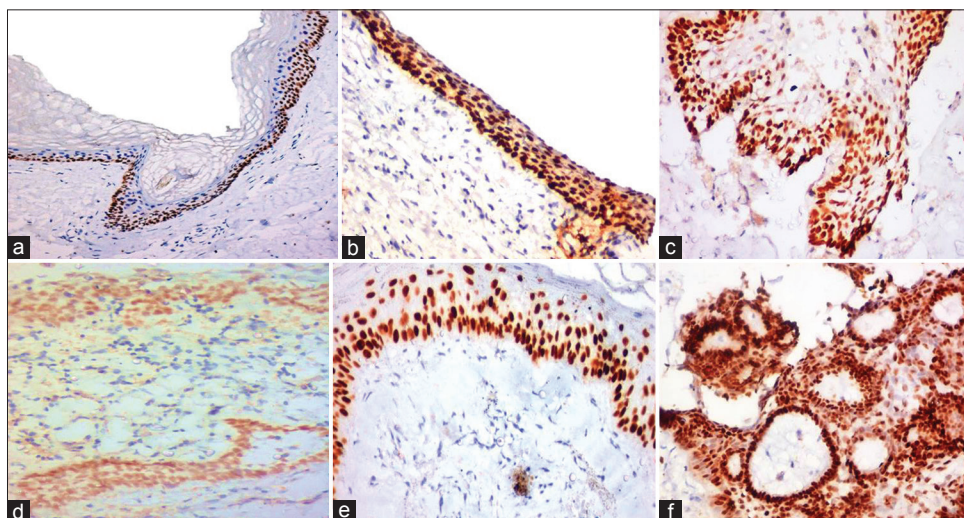


Figure 1: Photomicrograph showing Positive expression of p63 in the: (a) Basal and suprabasal layers of skin/control group (IHC stain, $\times 100$); (b) Basal and suprabasal layers of Follicular tissue (IHC stain, $\times 200$); (c) Basal and stellate reticulum-like cells in the cystic lining of Unicystic Ameloblastoma Type I (IHC stain, $\times 200$); (d) Basal and stellate reticulum-like cells in the cystic lining and mural islands of Unicystic Ameloblastoma Type III (IHC stain, $\times 200$); (e) Basal and suprabasal layers of OKC (IHC stain, $\times 200$); (f) Peripheral columnar and central stellate reticulum-like cells of Ameloblastoma (IHC stain, $\times 200$)

Subsequently, they were incubated with Novolink polymer for 30 min and finally with fresh 3, 3'-diaminobenzidine (DAB) chromogen for 1-2 minutes (prepared in a ratio of 1:20). The slides were then washed in water to remove the excess DAB and counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted with DPX and assessed for staining characteristics. Tris buffer was used as wash buffer as and when required.

The sections so stained were then viewed under the microscope and assessed for the staining characteristics.

Interpretation of staining

Normal skin was taken as positive control, which showed positive expression in the basal and parabasal layers. Presence of brown colored end product was indicative of positive immunoreactivity. The distribution of the stain in each case was observed in the cell nuclei.

RESULTS

The present study included a total of 48 cases which comprised of 12 cases of OKC, 12 cases of Solid Ameloblastoma, seven cases of Unicystic Ameloblastoma Type III, seven cases of Unicystic Ameloblastoma Type I and 10 cases of Follicular tissue. All 48 cases were subjected to immunohistochemistry using p63 antibody. Three cases showed positive immunoreactivity to p63 whereas 12 cases were negative.

Nuclear positivity for p63 was assessed and the cells were counted in five high power fields under 40x magnification using research microscope (Olympus BX 41).

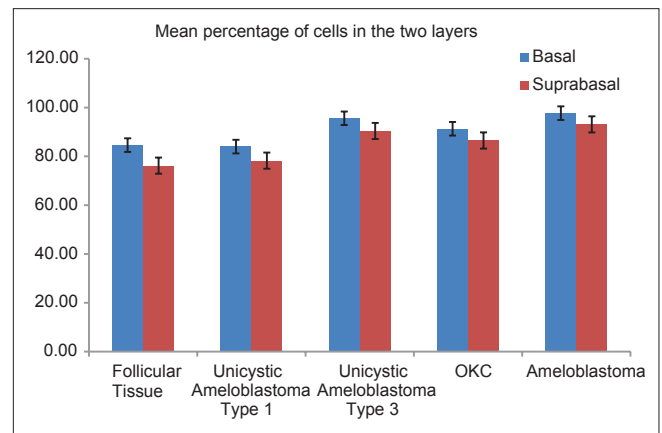
In each case nuclear staining of epithelial cells were evaluated in basal and suprabasal layers [Table 1]. Labeling index was

done in each case and subjected to appropriate statistical analysis.

The mean number of positive cells were compared between all the groups and tested for significance using t- test [Table 2 and Figure 2].

DISCUSSION

OKC has been one of the most controversial pathologic entities, ever since its initial description by Philipsen in 1956. It has long been of particular interest because of its potential for increased growth rate, local destructive behavior, high recurrence rate and its tendency for multiplicity, especially



	Basal	Suprabasal
Follicular Tissue	84.60	76.20
Unicystic Ameloblastoma Type 1	84	78.25
Unicystic Ameloblastoma Type 3	95.60	90.40
OKC	91.30	86.50
Ameloblastoma	97.70	93.10

Figure 2: Box-plot showing differences in mean percentage of positive cells between all the five groups

Table 1: Percentage of cells showing p63 positivity in all the five groups

% of positive cells in Follicular Tissue		% of positive cells in Unicystic Ameloblastoma Type I		% of positive cells in Unicystic Ameloblastoma Type III		% of positive cells in Odontogenic Keratocyst		% of positive cells in Solid Ameloblastoma	
Basal layer	Suprabasal layer	Basal layer	Suprabasal layer	Basal layer	Suprabasal layer	Basal layer	Suprabasal layer	Basal layer	Suprabasal layer
92	73	88	84	99	93	88	86	96	94
96	89	83	75	99	96	75	92	95	92
50	48	83	75	95	80	100	99	97	96
95	94	82	79	90	90	100	97	100	83
90	77	-ve	-ve	95	93	97	98	96	94
-ve	-ve	-ve	-ve	-ve	-ve	100	98	100	99
-ve	-ve	-ve	-ve	-ve	-ve	95	97	99	98
-ve	-ve					100	95	98	93
-ve	-ve					100	99	97	85
-ve	-ve					95	95	99	97
						-ve	-ve	-ve	-ve
						-ve	-ve	-ve	-ve

Table 2: Comparison of p63 indices with their respective Pp-values

Comparison of p63 indices	Basal P value	Parabasal P value	Results
OKC vs Solid Ameloblastoma	0.312	0.250	Not significant
OKC vs Unicystic Ameloblastoma Type III	0.88	0.08172	Not significant
OKC vs Unicystic Ameloblastoma Type I	0.021*	<0.0001*	Significant
Solid Ameloblastoma vs Unicystic Ameloblastoma Type III	0.16	0.4	Not significant
Solid Ameloblastoma vs Unicystic Ameloblastoma Type I	0.21	0.0009*	Significant
OKC vs Follicular tissue	0.159	0.072	Not significant
Solid Ameloblastoma vs Follicular tissue	0.207	0.102	Not significant
Unicystic Ameloblastoma Type III vs Follicular tissue	0.25	0.1334	Not significant
Unicystic Ameloblastoma vs Follicular tissue	0.95	0.832	Not significant

OKC: Odontogenic Keratocyst, *Denotes significance

when associated with Nevroid Basal Cell Carcinoma Syndrome. Thus the WHO in 2005 has rightfully reclassified OKC as a benign intraosseous neoplasm, recommending the term 'Keratocystic Odontogenic Tumor'. However, the precise nature of OKC and the reasons for its high recurrence rate still remain substantially unknown.^[5,6]

Authors have reported that the behavior of some OKCs is as aggressive as a benign neoplasm such as Solid Ameloblastoma. Studies have indicated that the mitotic index of OKC epithelium is similar to that of Solid ameloblastoma.^[7]

Unfortunately there is no consensus on a uniform treatment plan for OKC and the recommended surgical management varies from marsupialization to en bloc resection. The type of treatment chosen depends on several factors including patient age, size and location of the lesion and whether OKC is primary or recurrent. Because of the complications of radical surgery, marsupialization followed by enucleation has been suggested as a conservative approach by some authors.^[8]

Therefore there is a need to compare OKC with Unicystic Ameloblastoma Type I which is at one end of the spectrum and Solid Ameloblastoma at the other end of the spectrum.

p63, a member of the p53 superfamily, is a marker of stratified squamous epithelia and plays an important role in its development. Basal cells of normal human epithelium strongly express p63 proteins, predominantly the Δ Np63 isotype, but lose them as soon as these cells withdraw from the stem cell compartment. Thus p63 plays an essential role in maintaining the proliferative capacity of epithelial stem cells.^[9,10]

Overexpression of Δ Np63 is thought to block the growth-inhibitory and apoptosis-inducing activities of p53 or of signals that act through p53 thus maintaining the proliferative capacity of cells. Thus Δ Np63 might constitute an alternative mechanism to overcome p53-mediated cell cycle arrest and apoptosis. Increased expression of p63 has been observed in squamous cell carcinomas, nasopharyngeal carcinomas, salivary gland tumors, lymphomas and other lesions.^[3]

In accordance with these studies, our study was carried out with the aim of comparing the expression of p63 in the epithelial linings of OKC with that of solid Ameloblastoma, the well known locally aggressive odontogenic tumor and its clinically less aggressive variant Unicystic Ameloblastoma Type I in order to contribute more to the biological profile of these tumors. These tumors were compared to follicular tissue which acted as a negative control.

p63 expression was seen in the basal and suprabasal layers in OKC [Figure 1e] in accordance with previous studies by Lo Muzio *et al.*,^[3] Foschini *et al.*,^[11] and Gurgel *et al.*,^[12] where they suggest a greater proliferative potential in the suprabasal layers of OKC.

Positive expression of p63 was observed in the peripheral columnar and central stellate reticulum-like polyhedral cells of ameloblastic islands of Solid Ameloblastoma. Positivity was also seen in the cystic lining of Unicystic Ameloblastoma Type I and Type III and intramural nodules in Unicystic Ameloblastoma Type III [Figure 1c, d and f]. These results are in accordance with previous studies by Kumamoto *et al.*,^[13] respectively. p63 expression was analyzed and compared in the basal and suprabasal layers of OKC and Solid Ameloblastoma to note the difference in staining between the two groups. The Labeling Index for p63 was seen to be more in the basal layer (peripheral columnar cells) in case of Solid Ameloblastoma, while it was higher in the suprabasal layers of OKC, though the difference was statistically not significant [Table 2 and Figure 2]. Similar studies done using other proliferative markers PCNA, IPO-38, Ki-67 by Takahashi H *et al.*,^[14] Thosaporn *et al.*,^[15] Amaral *et al.*,^[16] and SolukTekkeşin M *et al.*^[17] concluded that proliferation indices are useful in predicting the different biological behavior of odontogenic lesions and also that KCOT showed a higher proliferative rate than Ameloblastoma. But in our study the proliferation in OKC is as high as in Solid Ameloblastoma.

The Labeling index in case of OKC in the basal and suprabasal layers was more when compared to Unicystic Ameloblastoma Type I and the change seen was statistically significant [Table 2].

The Labeling index in case of OKC in the basal and suprabasal layers was more when compared to Unicystic Ameloblastoma Type III though the change seen was not statistically significant [Table 2]. Our study shows that the behavior of OKC is equivalent to Unicystic Ameloblastoma Type III and that Unicystic Ameloblastoma Type I behaves innocuously.

Though no study has been done on the same parameters using p63 as a marker, similar studies have been done by Sudiono *et al.*,^[18] using other proliferative markers such as proliferating cell nuclear antigen (PCNA). They found that PCNA expression in Unicystic ameloblastoma with cystic tumor lining showed a low index when compared to OKC and mural Ameloblastoma. Thus they concluded that OKC is the most aggressive type of odontogenic cysts and that Unicystic Ameloblastoma Type III is more aggressive as compared to the other types of Unicystic Ameloblastomas.

Comparison of p63 expression between Solid Ameloblastoma and Unicystic Ameloblastomas (Type I and III) in the basal and stellate reticulum-like cells showed that the labeling index was higher in Solid Ameloblastoma. The difference was highly significant statistically when compared between the stellate reticulum-like cells of Solid Ameloblastoma and suprabasal cells of Unicystic Ameloblastoma Type I [Table 2 and Figure 2].

No studies have been done comparing the expression of p63 in solid Ameloblastoma and Unicystic Ameloblastoma Type I and Type III. However the results obtained are in accordance with similar studies done in the past using other proliferative markers by Li *et al.*,^[19] Funaoka *et al.*,^[20] Piattelli *et al.*,^[21] Sandra *et al.*,^[22] and Santos *et al.*^[23] stated that differences in the proliferative indices explain the biologic behavior of these lesions. Similar proliferative indices between Solid Ameloblastoma and Unicystic Ameloblastoma Type III corroborate the pattern of higher aggressive clinical behavior in these tumor variants.

The labeling indices for p63 in OKC, Ameloblastoma, Unicystic Ameloblastoma Type I and Type III were compared to that of Follicular tissue, which acted as a negative control for the study [Figures 1 and 2 and Table 2]. Logically the follicular tissue should not show any staining. But some of the cases which did show positivity could be associated with pathological changes, especially those associated with the Dentigerous Cyst (DC). The labeling index was more in the basal and suprabasal layers of all the lesions i.e. in OKC, Ameloblastoma and Unicystic Ameloblastoma Type I and Type III when compared to follicular tissue, though the increase was not statistically significant.

This is in accordance with previous studies on p63 by Brkić *et al.*,^[24] who found that the immunoreactivity for p63 was

stronger in the dental follicles associated with completely impacted teeth, than those associated with partially impacted teeth, and concluded that these results might be associated with the follicular stem cells.

According to Oliveira *et al.*, follicular tissue could have low proliferation potential but changes such as squamous metaplasia, hyperplasia of the epithelial lining and presence of proliferative odontogenic epithelial rests in the connective tissue could be associated with pathological changes in the follicle and may be early signs of developing lesions of odontogenic origin.^[25]

Thus the results of our study suggest that OKC and Solid Ameloblastoma have similar proliferation indices in the basal compartment whereas in the suprabasal compartment, OKC has a higher proliferative capacity. This indicates that the nature of OKC is at least on par if not higher than that of Solid Ameloblastoma thus lending support to the new nomenclature for this lesion as 'Keratocystic odontogenic tumor'. Solid Ameloblastomas and Mural Ameloblastomas well-established for their aggressive behavior are treated by radical resection. Our study shows that the indices in case of OKC are similar to that of Solid Ameloblastoma and Unicystic Ameloblastoma Type III, it upholds the opinion that OKCs also exhibit aggressive behavior. This calls for an aggressive and more radical treatment for OKC too, than just a simple enucleation as is usually performed.

Thus evaluation of the expression of p63 in different lesions can help us in determining the biological behavior of these lesions and help us in determining the treatment modality accordingly. Also attempts need to be made to improve surgical techniques in treating these lesions or improvise the existing ones.

CONCLUSION

The present study was undertaken to study the expression of p63 in OKC, Solid Ameloblastoma and Unicystic Ameloblastoma Type I and III and compare them with each other in order to assess the biologic behavior of these lesions.

From the study, it was concluded that:

- Expression of p63 in OKC was comparable with that of Solid Ameloblastoma and Unicystic Ameloblastoma Type III and significantly higher than that of Unicystic Ameloblastoma Type I
- Expression of p63 in Solid Ameloblastoma was comparable to Unicystic Ameloblastoma Type III and was significantly higher than Unicystic Ameloblastoma Type I.

Based on the biological behavior of OKC, its recognition as a neoplasm by the WHO and other microscopic, molecular and genetic evidences, our study upholds the neoplastic nature of

KCOT. Accordingly this lesion should not be managed just as a simple cyst but a more aggressive mode of treatment along with a mandatory long term follow up of patients is required to reduce the risk of recurrence. Also Unicystic Ameloblastoma Type I is clinically less aggressive compared to its solid and mural variants, behaving more or less like a cyst than a tumor while Unicystic Ameloblastoma Type III is more aggressive and behaves like a tumor.

Unicystic Ameloblastoma Type I should be treated conservatively while Type III needs to be treated more radically.

Thus the expression of p63 in cysts and tumors can be used to assess the proliferative potential of the lesions and thereby identify the more aggressive lesion and accordingly the treatment modalities can be reviewed.

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