

# Odontogenic keratocyst: Analysis of recurrence by AgNOR, p53 and MDM2 profiling

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## Abstract

**Background:** Odontogenic keratocyst (OKC) is a clinical entity with characteristic microscopic features, high growth potential and propensity to recur. Aggressive behavior and higher tendency for recurrence have been attributed to greater proliferative activity of epithelial lining. The incidence of recurrence in various reported series ranges from 2.5% to 62%.

**Objectives:** The objective of the study was to investigate the clinical behavior of OKC by evaluating p53, MDM2 expression, AgNOR staining and to ascertain if the expression of these markers correlate with the clinical outcome and tendency for recurrence.

**Materials and Methods:** All recurrent and nonrecurrent OKCs from the archives were included, and sections were subjected to AgNOR staining, p53 and MDM2 immunohistochemical staining.

**Results and Conclusion:** There was a significant difference in the staining pattern of MDM2 and AgNOR in the recurrent group as compared to the nonrecurrent group. The higher expression of these markers in recurrent lesions may be important in order to consider additional surgical interventions to improve prognosis.

**Keywords:** AgNOR, Odontogenic keratocyst, recurrence

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## INTRODUCTION

Odontogenic keratocyst (OKC), a developmental odontogenic cyst first described by Philipsen, had been designated as keratocystic odontogenic tumor, a benign cystic neoplasm in 2003 by the WHO.<sup>[1]</sup> However, the 4<sup>th</sup> edition of WHO Classification of odontogenic lesions has redesignated it as OKC, a cyst that exhibits features reflecting aggressiveness, infiltrative behavior and potential for recurrence.<sup>[2,3]</sup> OKC is the third-most common jaw cyst,

having a frequency of 11.6%,<sup>[4]</sup> with the most common site of presentation being the posterior mandible.<sup>[5]</sup> The histological features are characteristic with uniform thin lining of parakeratinized stratified squamous epithelium which is usually separated from the underlying connective tissue capsule with the palisaded basal cells showing reversal of polarity.<sup>[3,6]</sup> Though the epithelium lacks rete ridges, irregularity and basal budding may be seen in few cases especially those associated with nevoid basal cell carcinoma syndrome (NBCCS).<sup>[5,7]</sup> Cyst capsule usually

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consists of thin loose uninfamed connective tissue with few showing presence of satellite cysts/daughter cysts.<sup>[3,8]</sup> Epithelial islands, dental lamina rests and hyalinization of collagen subepithelially has also been reported.<sup>[7,9,10]</sup>

OKCs have a propensity to recur following surgical removal with rates varying from 2.5%–62.5%,<sup>[2,9]</sup> the recurrence rate being higher in patients with NBCCS.<sup>[9,11]</sup> An increased frequency for recurrence associated with OKC has been attributed to various clinical predictors that include age, location, size, shape and most importantly differences in surgical techniques.<sup>[12]</sup> Also, an increased recurrence rate was earlier reported in parakeratinized variant than orthokeratinized variant, which is now considered a distinct entity, orthokeratinized odontogenic cyst.<sup>[13]</sup> Along with other histological features as predictors of recurrence like parakeratinization, sub epithelial splitting, satellite cysts, dental lamina rests, basal cell budding, subepithelial hyalinization of the capsule is also implicated as a predictor of recurrence.<sup>[7,8,11,14-16]</sup>

The p53 protein, a product of the *TP53* tumor suppressor gene, is expressed in the G1 phase of the cell cycle to allow repair of possible DNA damage and arrest cell cycle progression to S phase or alternatively, to induce apoptosis of cells that cannot be repaired. A low concentration of wild type P53 is usually found in cells because of its relatively short half-life which is about 20 min. Its concentration increases as its half-life is extended, which may occur due to *TP53* gene mutation, association of wild type P53 with other proteins, or disruption of its degradation pathway.<sup>[17,18]</sup> Under normal conditions the P53 protein is synthesized continuously. In the nucleus, it binds to the mouse double minute, MDM2 protein, and the MDM2/P53 complex is exported to the cytoplasm, where it is degraded by proteosomes. This process keeps the cell concentration of P53 low. Thus the increase in p53 protein concentration does not depend on gene activation, transcription and translation, but rather on inhibition of its degradation. Under stress, certain types of protein are released from the nucleus to the nucleoplasm, where it binds to MDM2 or to the MDM/P53 complex, blocking P53 export to the cytoplasm and later preventing its degradation, which results in the accumulation of the P53 protein in the nucleus.<sup>[19,20]</sup> Abnormal expression of tumor suppressor gene and oncogenes have been described in OKCs.<sup>[21]</sup>

Nucleolar organizer regions (NORs) are loops of DNA which transcribes to ribosomal RNA. The NOR-related protein becomes visible in nucleus by a silver-staining technique under a light microscope, and it has been named argyrophilic protein of NOR (AgNOR). Silver

staining of AgNORs is considered to be the best and most cost-effective marker to assess the proliferative behavior of a lesion. The rapidity of the cell turnover is evaluated by speed of the cell cycle, so as to assess the growth rate of the lesion, which is easily assessed by AgNOR count per nucleus. The amount of AgNOR represents a cell kinetics parameter and can be used for prognostic purposes.<sup>[22,23]</sup>

The objective of the study was to investigate the clinical behavior of OKC by evaluating p53, MDM2 expression, AgNOR staining and to ascertain if the expression of these markers correlates with the clinical outcome and tendency for recurrence.

## MATERIALS AND METHODS

This retrospective study used formalin-fixed, paraffin embedded tissue specimens obtained from the departmental archives. The study group composed of 21 histologically confirmed recurrent and nonrecurrent cases of OKC with a minimum follow up of 2 years.

All the slides stained with hematoxylin and eosin were reexamined and histological assessment was done and correlated with the clinical data including recurrence. For AgNOR staining, 5- $\mu$ m-thick sections were deparaffinized with xylene for 30 min and washed with distilled water through grades of alcohol. The 50% silver nitrate solution was mixed in 2 g/dL gelatin solution dissolved in 1 g/dL formic acid in a 1:2 proportion and the sections were incubated in a dark room for 30 min. After washing off the distilled water, the sections were dehydrated with ethanol, cleared with xylene and coverslipped and evaluated by a light microscope.

Immunohistochemistry was performed with an avidin-biotin technique and 4  $\mu$ m sections placed on slides coated with 3-aminopropyltriethoxysilane, utilizing commercially prepared Leica Novocastra (Newcastle upon Tyne, UK) antibodies for p53 and MDM2 (RTU-P53-D07, NCL-MDM2, respectively). The clone and Ig class of p53 and MDM2 were D07 and IgG2b and IB10 and IgM, respectively. Briefly, the sections were deparaffinized, rehydrated and quenched for endogenous peroxidase activity. Epitope retrieval in sodium citrate buffer (pH 6.0) under pressure, nonspecific antigen blocking and incubation with primary antibody (prediluted P53 and 1:100 dilution MDM2). The sections were covered with a polymer penetration enhancer (i.e., postprimary block), which was followed by incubation with secondary antibody. Antigen antibody binding was detected using DAB chromogen system and sections were counterstained with Mayer's hematoxylin. The recommended

positive control included were colon carcinoma for p53 and osteosarcoma for MDM2.

The number of AgNORs in the nucleus was counted in 250 cells for each case under oil immersion. Black dots/aggregated clusters within cellular nucleoli were counted as one dot. The average number of AgNORs was calculated. P53 and MDM2 positivity was evaluated in the nucleus, and the labeling index (LI) was calculated by using Image Analysis software (Image J- free download version) as follows.  $LI = (\text{No of positive cells}/\text{no of cells}) \times 100$ . For P53 and MDM2, the sections were examined at  $\times 400$ . The percentage of positive epithelial cells (nuclear staining) in ten high-power fields was determined. Also, the intensity of staining with P53 and MDM2 antigen was evaluated based on the following method: (0) when cells had not been staining and (+1), (+2), (+3) for low, moderate and intense staining. The H score was calculated as: % of intense staining cells  $\times 3$  + % of moderate staining  $\times 2$  + % mild staining  $\times 1$ . The results of the recurrent and nonrecurrent groups were analyzed by means of Mann-Whitney U-test.  $P < 0.005$  was considered to indicate statistical significance.

## RESULTS

Out of the 21 cases of OKCs, 16 were nonrecurrent and 5 were recurrent cases. Majority of the cases were seen to occur in the posterior mandible with a male predominance. 2 of the recurrent cases were multiple OKC's and associated with NBCCS. The histopathological characteristics are as show in Table 1.

Rete ridges showed the presence of budding in all the cases of recurrent OKCs whereas most of the cases of nonrecurrent OKCs had blunt rete ridges. The epithelial and connective tissue interface was predominantly intact in majority of the nonrecurrent cases and all cases of recurrent group showed subbasal/suprabasal split. Features of epithelial dysplasia were noted in 2 cases in the recurrent group. 3 of the recurrent cases showed the presence of odontogenic rests and satellite cysts. Interestingly, all cases of recurrent OKCs showed subepithelial hyalinization with 60% showing a diffuse pattern. Only 3 cases of nonrecurrent OKC's exhibited subepithelial hyalinization in focal areas.

The average AgNOR/nucleus among the recurrent and nonrecurrent group was 2.41 and 1.49, respectively. P53 and MDM2 expression was seen to be more in the suprabasal cells in the recurrent group when compared to the nonrecurrent group [Table 2 and Figures 1-3].

**Table 1: Histopathological characteristics of odontogenic keratocyst**

Histologic features	Total (21)	Recurrent (5)	Nonrecurrent (16)
Lining			
Complete	0	0	0
Incomplete	21	5	16
Type			
Para keratinized	17	4	13
Ortho keratinized	0	0	0
Mixed	4	1	3
Thickness			
Thick	2	1	3
Thin	17	3	10
Mixed	2	1	3
Corrugations	18	5	13
Intracellular edema	18	4	14
Basal cells			
Columnar	16	4	12
Cuboidal	5	1	4
Reversal of polarity	16	4	12
Rete ridges			
Blunt	13	0	13
Budding	8	5	3
Epithelia-CT interface			
Intact	10	0	10
Subbasal	5	4	1
Suprabasal	6	1	5
Epithelial islands	6	4	2
Capsule-Collagen			
Loose	15	3	12
Dense	2	0	2
Mixed	4	2	2
Inflammation			
Localized	12	2	10
Generalized	7	3	4
Calcifications	4	3	1
Odontogenic rests	5	3	2
Satellite cysts	4	3	1
Cholesterol clefts	3	1	2
Hyalinization			
Focal	6	3	3
Diffuse	10	2	8
Keratin			
Empty	4	1	3
Filled	15	4	11

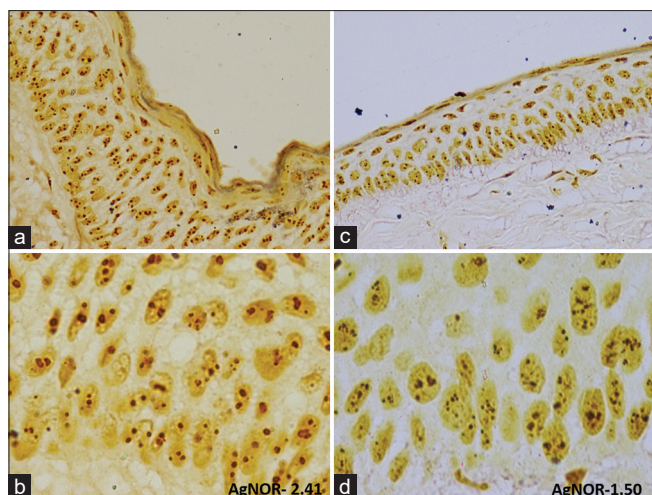
CT: Connective tissue

**Table 2: AgNOR, p53 and MDM2 staining characteristics in odontogenic keratocyst**

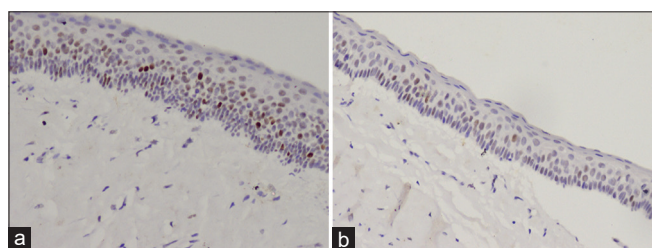
Group	Mean	SD	P
AgNOR			
Nonrecurrent (16)	1.50685	0.13927	0.001
Recurrent (5)	2.41	0.1839	
p53			
Nonrecurrent (16)	99.9375	23.6346	0.017
Recurrent (5)	139.6	29.54 31	
MDM2			
Nonrecurrent (16)	53.25	14.3457	0.001
Recurrent (5)	116.2	10.917	

SD: Standard deviation, AgNOR: Argyrophilic protein of nucleolar organizer region, MDM2: Mouse double minute 2

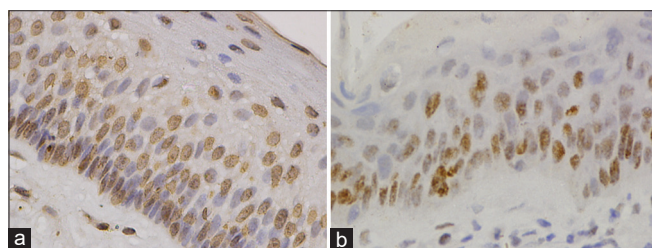
AgNOR values and MDM2 scores of recurrent group were found to be statistically higher than the nonrecurrent group ( $P = 0.001$ ), and no significant difference in p53 scores was noted between the recurrent and nonrecurrent



**Figure 1:** AgNOR staining in recurrent odontogenic keratocyst (a and b) and nonrecurrent odontogenic keratocyst (c and d)



**Figure 2:** P53 nuclear staining in recurrent (a) and nonrecurrent odontogenic keratocyst (b)



**Figure 3:** MDM2 nuclear staining in recurrent (a) and nonrecurrent odontogenic keratocyst (b)

group. A significant positive correlation (Spearman rank correlation) was noted between p53 and MDM2 and AgNOR and MDM2.

## DISCUSSION

The increased P53 expression in OKC in the present study was similar to the findings of Piatelli *et al.*<sup>[21]</sup> The high expression of P53 in the epithelial lining of OKC's indicates a greater proliferative activity of epithelium. The expression pattern of P53 protein was similar to previously reported studies.<sup>[18,23,24,25]</sup> The findings of the present study indicate that expression of P53 protein in the parabasal layer of recurrent OKC is more than that of nonrecurrent group, indicating a higher proliferative activity. Slootweg

believes that the increase in proliferation is not necessarily related to the mutation of the P53 gene. This phenomenon cannot be determined through immunohistochemical studies, because stabilization of P53 protein can occur due to the increase in protein production or protection of P53 protein against damage by bonding to other cell proteins.<sup>[26]</sup> The stability of the p53 molecule is also dependent on degree of phosphorylation and therefore the detection of phosphorylated p53 by specific antibody could be useful to evaluate the functional status of this protein. Carvalhais *et al.*<sup>[27]</sup> described only a higher positivity for MDM2 protein, which is member of the p53 induced family of proteins with regulatory effect against p53. In the present study, MDM2 expression was higher in recurrent group suggesting that these upstream regulators of p53 are involved in the aggressive behavior of the lesion. In the present study, all recurrent cases showed co-expression of both p53 and MDM2. p53 aberrant expression as an early event probably leads to the activation of MDM2 feedback loop for proteasome degradation of activated p53. Despite the activation of MDM2, degradation of p53 do not takes place. One explanation of this argument is that MDM2 has two apparently opposite functions, a tumorigenic function and a growth arrest one. This dual role of MDM2 could depend on the level of MDM2 expressed in the cell and also the balance of positive and negative regulators of cell cycle which is critical for the control of cell proliferation.<sup>[28]</sup>

Limited number of studies are published on the evaluation of AgNORs in odontogenic cysts and tumors with conflicting results. The mean AgNOR counts for all odontogenic cysts ranged between 2.02 and 2.65, and for ameloblastomas was 2.24.<sup>[29]</sup> In the present study, the AgNOR dots were higher in the nucleus of suprabasal cells than basal cells in recurrent OKC's. Increased AgNOR proteins probably indicates increased demand for ribosomal biogenesis reflecting high metabolic activity.<sup>[29]</sup>

## CONCLUSION

Though surgical management is the most influential factor for recurrences following any surgical intervention, studies on a group of OKCs undergoing a single surgical procedure thus reducing the influence of confounding factors are limited. Furthermore, little is known about any relationship between expression of cell proliferative markers and recurrence. The higher expression of these markers in recurrent lesions may indicate a difference in their behavior and may be valuable for clinician to consider additional surgical interventions to improve the prognosis. The limitation of study being the small sample size, further

studies with a larger sample size in both the recurrent and nonrecurrent groups are required to confirm the present findings.

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### Conflicts for interest

There are no conflicts for interest.

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