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# PROLIFERATIVE CAPACITY OF KCOT, DENTIGEROUS CYST AND AMELOBLASTOMA

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## ABSTRACT:

**Aim:** In view of the current designation of KCOT as benign cystic neoplasm, the aim of this study was to assess the proliferative activity of KCOT and compare the same with that of an acknowledged indolent odontogenic cyst (like dentigerous cyst) and an aggressive tumour (like ameloblastoma) using image analysis of AgNORs.

**Materials And Methods:** Histological sections were prepared from formalin-fixed, paraffin-embedded tissue blocks of 15 cases each of KCOT, dentigerous cyst and conventional ameloblastoma and 10 cases of unicystic ameloblastoma to be stained with silver stain for visualization of AgNORs. The number of AgNORs per nucleus was counted manually whereas AgNOR area and perimeter were determined using computerized image analysis.

**Results:**The results obtained were statistically analysed. The results obtained for mean AgNOR area and perimeter suggested the mean AgNOR area and perimeter to be unreliable parameters to assess the proliferative potential of the odontogenic lesions studied. The results obtained for mean AgNOR number suggested that mean AgNOR number is a reliable marker of the proliferative activity of a cell.

**Conclusion:**Based AgNOR count of 55 archival samples, we conclude that KCOT is comparable to conventional ameloblastoma in its biological behaviour and behaves as an odontogenic tumour.

**Key words:** OKC, Benign cystic neoplasm, AgNORs count, AgNORs area, AgNORs Perimeter, proliferative marker, laboratory research

## INTRODUCTION:

Keratocystic odontogenic tumors (KCOTs) are cystic tumors originating from the dental lamina of the maxilla and mandible that are lined with keratinized epithelium. While benign, they can be locally destructive and have a high recurrence rate despite treatment [1]. Its aggressive nature and invasive behavior have been substantiated by various molecular studies determined by expression of PCNA, p53, PTCH gene mutation etc. [2-4]

This lesion was previously termed odontogenic keratocysts (OKC) and was believed to be a developmental odontogenic cyst. However, in view of its clinical progress and aggression, it has been re-classified by the World Health Organization as a benign cystic neoplasm of odontogenic origin. The currently favored terminology is that of a keratocystic odontogenic tumor (KCOT). [5]

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Ameloblastoma is a true neoplasm of enamel organ origin that has been described by Robinson as being 'usually unicentric, non-functional, intermittent in growth, anatomically benign and clinically persistent'. Ameloblastoma is an aggressive lesion that has a high incidence rate combined with a high recurrence rate and persistence, thereby necessitating aggressive treatment. The unicystic variant of ameloblastoma is thought to be less aggressive. The dentigerous cyst is a developmental odontogenic cyst that presents as an indolent lesion that is slow growing and rarely recurs and may be managed relatively easily by enucleation or marsupialization. [6, 7]

Nucleolar Organizing Regions (NORs) are loops of DNA that code for ribosomal RNA. They are situated in the nucleolus and are thought to reflect the proliferative activity of the cell. Their relationship to the DNA, the cell cycle and protein transcription make them ideal as markers to detect cellular proliferative activity [8]. NORs are best visualized using a silver-staining technique that enables qualitative and quantitative assessment of NORs. The silver-stained NORs (AgNORs) can then be analyzed based on their number and size [9-10].

The proliferation activity of various lesions has been successfully assessed by counting the number of AgNORs. Measurement of their area and perimeter has been performed using digital image analysis. The application of computerized image analysis enables standardization and reproducibility of results [9, 10].

However, image analysis of AgNORs (area and perimeter) has not been used much to compare the proliferation of odontogenic cysts and tumors. The aim of this study was to assess the proliferative activity of KCOT and compare the same with that of an acknowledged indolent odontogenic cyst (like dentigerous cyst) and an aggressive tumor (like ameloblastoma), using the various parameters enabled by visualization of AgNORs like count, area and perimeter with Image J, an image analysis software.

## MATERIALS AND METHODS:

For the present study formalin-fixed, paraffin-embedded blocks of 55 cases were selected. Clinically and histologically diagnosed, 15 cases each of KCOT, dentigerous cyst and conventional ameloblastoma, and 10 cases of unicystic ameloblastoma were taken from the archives (year 2000 to 2009) of the Department of Oral Pathology, MCODS, Mangalore. The cases which showed inflamed cystic lining were excluded.

Two sections of 4µm thickness each were taken from formalin-fixed, paraffin-embedded tissues of aforementioned lesions. One section was stained with routine Hematoxylin and Eosin (H&E) and other tissue section was stained with silver solution for the visualization of NORs. Sections were routinely

deparaffinised in xylene, dehydrated through descending grades of alcohol (all dilutions were done in deionized water) and brought to deionized water. Sections were then incubated in working solution at 40°C for 30 minutes (hot air oven). Sections were washed in deionized water and rehydrated through ascending grades of alcohol, cleared in xylene, and mounted in DPX [11]. Figure I shows the sections stained for AgNORs. In each case, AgNORs in 100 nuclei were counted using x1000 magnification. The protocol followed was in accordance to Li et al [2]. The mean number of AgNORs per cell (N) was calculated. The mean AgNOR area and perimeter per cell were calculated using IMAGE J version 1.40g/ Java 1.6.0 [12, 13].

ANOVA test analysis was performed to compare the mean AgNOR count, area and perimeter per nucleus of KCOT with those of dentigerous cyst, unicystic ameloblastoma and conventional ameloblastoma. Bonferroni test was performed for the multiple comparisons between the mean AgNOR number, area, and perimeter per nucleus, of the various lesions included in the study. Finally a regression analysis was done to analyze which of the three AgNORs parameter is most valuable in estimating the aggressiveness of any lesion.

## RESULTS:

The ANOVA test analysis for the mean AgNOR number per nucleus showed the mean value obtained for AgNOR number in ameloblastoma was 2.453 (SD=.4068, SE=.1050). The dentigerous cyst had the mean AgNOR number value of 1.320 (SD=2042, SE=.0527), while KCOT was 2.240 (SD=.2874, SE=.0742) and unicystic ameloblastoma was 1.920 (SD=.4894, SE=.1548). For all the lesions studied, the 'F' value was 29.933, which is a highly significant value (P=0.000) (Table I). Since the P value was statistically highly significant (P=0.000) for mean AgNOR number per nucleus, multiple comparisons were performed using Bonferroni test.

The Bonferroni test was used for the multiple comparisons of mean AgNOR number per nucleus among the various lesions studied. The difference was statistically highly significant between ameloblastoma and dentigerous cyst (P=.000), ameloblastoma and unicystic ameloblastoma (P=.003), dentigerous cyst and KCOT (P=.000) and unicystic ameloblastoma and dentigerous cyst (P=.001), whereas the difference between the mean AgNOR number per nucleus was statistically not significant for ameloblastoma and KCOT (P=.602) and odontogenic cyst and unicystic ameloblastoma (P=.175) (Table II).

The ANOVA test analysis was made for the mean AgNOR area per nucleus. The mean value for AgNOR area in ameloblastoma was 2.163 (SD=.5005, SE=.1292), for dentigerous cyst was 1.982 (SD=.3585, SE=.0925), for KCOT was 2.009 (SD=.3926, SE=.1013) and unicystic ameloblastoma was 1.765 (SD=.4050, SE=.1280). For all the lesions studied, the 'F' value was 1.822. P value was statistically non-significant (P=.155)

(Table III). Since the P value obtained from ANOVA was statistically not significant, there was no need for the Bonferroni test for multiple comparisons for mean AgNOR area per nucleus among the various lesions.

The ANOVA test analysis was made for mean AgNOR perimeter per nucleus. The mean value for AgNOR perimeter in ameloblastoma was 8.244 (SD=.1.219, SE=.3149), for dentigerous cyst was 5.988 (SD=.7581, SE=.1957), for KCOT was 6.787 (SD=.1.578, SE=.4096) and unicystic ameloblastoma was 7.138 (SD=.9113, SE=.2882). For all the lesions studied the 'F' value was 9.403, with highly significant P value (P=.000) (Table IV). The statistically highly significant p value obtained for mean AgNOR perimeter per nucleus necessitated performance of multiple comparisons using Bonferroni test. The p-value for the Bonferroni test for the mean AgNOR perimeter per nucleus was statistically highly significant for ameloblastoma and dentigerous cyst (P=.000) and ameloblastoma and KCOT (P=.008) and was insignificant among all other lesions (Table V).

All the AgNOR parameters in KCOT were compared with those in ameloblastoma using t-test. A comparison of the mean AgNOR number, area and perimeter per nucleus between KCOT and ameloblastoma was made using the T-test. The difference in the mean value of the mean AgNOR perimeter per nucleus was statistically highly significant (P=.009) but was not significant for AgNOR number and AgNOR area.

(Table VI).

All the AgNOR parameters in KCOT were compared with those in dentigerous cyst using t-test. This table shows the comparison of the mean AgNOR number, AgNOR area and AgNOR perimeter per nucleus between KCOT and dentigerous cyst. The difference in the mean value of mean AgNOR number per nucleus was statistically highly significant (P=.000) but a statistical significance was not obtained for AgNOR area and AgNOR perimeter (Table VII).

A comparison of the mean AgNOR number, area and perimeter per nucleus between KCOT and unicystic ameloblastoma was performed using the T-test. All the parameters in KCOT were compared with those in unicystic ameloblastoma and the difference was not found to be statistically significant for all the three parameters (i.e. AgNOR number, AgNOR area and AgNOR perimeter) (Table VIII).

Regression Analysis for the three parameters was also performed using the values of dentigerous cyst and ameloblastoma as standardized coefficients and KCOT as un-standardized coefficient. The statistical significance of the mean AgNOR number in analyzing the aggressiveness of a lesion was found to be very high (t =.5.767), followed by perimeter (t =1.646). It was least for mean AgNOR area (t =-1.421).

## DISCUSSION:

KCOT was known to be a distinctive form of developmental odontogenic cyst but has recently been reclassified as an odontogenic tumor by W.H.O. The high recurrence rate associated with the KCOT has been a subject of considerable study. Voorsmit RA et al (1981) [14] suggested that recurrences might develop due to any or a combination of the following three factors: (1) incomplete removal of the original cyst lining, (2) retention of microcysts or epithelial islands in the wall of the original cyst or (3) development of new keratocysts from epithelial off-shoots of the basal layer of the oral epithelium. The aggressive nature of KCOT has also been studied using proliferative IHC markers and chromosomal studies [2, 15]. The aggressive nature, high rate of recurrence and unresponsiveness to conservative treatment has made surgeons continuously review the treatment of KCOT [16].

Nucleolar organizer regions (NORs) are loops of DNA that code for ribosomal RNA. They are situated in the nucleolus and are known to reflect the proliferative activity of the cell. Various studies have used AgNORs to understand the proliferative behavior of various lesions and have suggested it to be a reliable proliferative marker [17, 18]. In the present study, the difference in the mean AgNOR count was found to be statistically significant (p= 0.000) between KCOT and dentigerous cyst. The difference in the mean AgNORs count was insignificant for KCOT and unicystic ameloblastoma (p= 0.175) as well as for KCOT and conventional ameloblastoma (p= 0.602). It can be inferred from the present study that the proliferative activity of the lining epithelium of KCOT is similar to that of conventional ameloblastoma as well as unicystic ameloblastoma, but is very high when compared to dentigerous cyst.

The difference in the mean AgNORs area gave highly variable results and the difference between the various groups was not found to be statistically significant (p=0.155). This is in accordance with the results of Paula et al where the AgNOR area did not give statistically significant results [19]. In the present study, the difference in the mean AgNOR perimeter was statistically significant (p=0.008) between ameloblastoma and KCOT. If the AgNOR perimeter is to be considered as truly indicative of the proliferative potential of the epithelial lining, the results of the present study show that the proliferative potential of the epithelial lining of KCOT is lower (p- value =0.008)) from that of conventional ameloblastoma (p=0.602). This contradicts the results obtained from the difference in mean AgNOR count of KCOT from conventional ameloblastoma, which were not statistically significant (p=0.602). This would mean that either the perimeter is a more sensitive index, which shows significant difference in values with little difference in the biological activity, or, that perimeter is an insignificant AgNOR parameter. The latter opinion is in

accordance with that stated by Paula et al. [19]

To determine the validity of the NOR perimeter as a more sensitive index, or to validly comment on the NOR perimeter as an insignificant parameter, a study similar to the present study has to be paralleled with the assessment of proliferative index using other markers, such as Ki-67 and PCNA.

Certain studies [19, 20] have used image analysis to study the measurements of nuclei and other nuclear features such as shape, perimeter, diameter etc. to determine various nuclear parameters. The use of image analysis enabled the calculation of area and perimeter of the AgNORs which could have been highly tedious if routine micrometry were to be used. It also eliminated the bias due to variation in the depth of focus i.e., with the use of photomicrograph only those structures could be assessed which lay in the same depth of focus as the objective.

Additionally, at times, inflammation can mask the typical histological features of a cystic lining so that it becomes difficult to make a confirmatory diagnosis, especially if the clinical features are not very specific. The statistically significant difference obtained between the mean AgNORs count in the lining of KCOT and dentigerous cyst could be of value under such circumstances, though further studies are required to compare the inflamed parts of the cyst with those of un-inflamed parts.

## CONCLUSION:

The present study determined that the mean AgNOR number (count) per nucleus was statistically significant parameter in assessing the proliferative potential of the KCOT versus the proliferative capacity of unicystic ameloblastoma, conventional ameloblastoma and dentigerous cyst. This suggests that KCOT is comparable to conventional ameloblastoma in its biological behaviour i.e. it behaves as an odontogenic tumor. Therefore, the treatment plan of KCOT should be similar to a neoplasm and not that of odontogenic cysts. This is likely to reduce the recurrence rate of KCOT. With a larger sample size, it may be possible to establish a cut-off for the mean AgNOR number per nucleus that would definitively designate a particular keratinizing lesion as being cystic or neoplastic. This could have a bearing on the approach and treatment plan that the surgeon would use to best manage such lesions.

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## TABLES:

Table I: ANOVA test analysis for the mean AgNOR count in various lesions

| | Cases | Mean | Standard Deviation | Standard Error | 95%Confidence Interval for Mean | | F value | P=.000 |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower boundary | Upper boundary | | |
| Ameloblastoma | 15 | 2.453 | .4068 | .1050 | 2.228 | 2.679 | 29.933 | Highly significant |
| Dentigerous cyst | 15 | 1.320 | .2042 | .0527 | 1.207 | 1.433 | | |
| Odontogenic keratocyst | 15 | 2.240 | .2874 | .0742 | 2.081 | 2.399 | | |
| Unicystic ameloblastoma | 10 | 1.920 | .4894 | .1548 | 1.570 | 2.270 | | |

Table II: Bonferroni test for multiple comparisons for mean AgNOR number per nucleus

| Dependent variable | Type (I) | Type (J) | Mean difference (I-J) | Std Error | P value | |
|---|---|---|---|---|---|---|
| AgNOR Number | Ameloblastoma | Dentigerous cyst | 1.1333 | .1275 | .000 | HS |
| | | Odontogenic keratocyst | .2133 | .1275 | .602 | NS |
| | | Unicystic Ameloblastoma | .5333 | .1425 | .003 | HS |
| | Dentigerous cyst | Odontogenic keratocyst | -.9200 | .1275 | .000 | HS |
| | | Unicystic Ameloblastoma | -.6000 | .1425 | .001 | HS |
| | Odontogenic keratocyst | Unicystic Ameloblastoma | .3200 | .1425 | .175 | NS |

Table III: ANOVA test analysis for the mean AgNOR area in various lesions

| | Cases | Mean | Standard Deviation | Standard Error | 95%Confidence Interval for Mean | | F value | P=.155 |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower boundary | Upper boundary | | |
| Ameloblastoma | 15 | 2.163 | .5005 | .1292 | 1.886 | 2.440 | 1.822 | Non-significant |
| Dentigerous cyst | 15 | 1.982 | .3585 | .0925 | 1.783 | 2.180 | | |
| Odontogenic keratocyst | 15 | 2.009 | .3926 | .1013 | 1.791 | 2.226 | | |
| Unicystic ameloblastoma | 10 | 1.765 | .4050 | .1280 | 1.475 | 2.054 | | |

Table IV: ANOVA test analysis for the mean AgNOR perimeter in various lesions

| | Cases | Mean | Standard Deviation | Standard Error | 95%Confidence Interval for Mean | | F value | P=.000 |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower boundary | Upper boundary | | |
| Ameloblastoma | 15 | 8.244 | 1.219 | .3149 | 7.569 | 8.919 | 9.403 | Highly significant |
| Dentigerous cyst | 15 | 5.988 | .7581 | .1957 | 5.568 | 6.408 | | |
| Odontogenic keratocyst | 15 | 6.787 | 1.578 | .4076 | 5.913 | 7.661 | | |
| Unicystic ameloblastoma | 10 | 7.138 | .9113 | .2882 | 6.486 | 7.790 | | |

Table V: Bonferroni test for multiple comparisons for mean AgNOR perimeter per nucleus

| Dependent variable | Type (I) | Type (J) | Mean difference (I-J) | Std Error | P value | |
|---|---|---|---|---|---|---|
| AgNOR Perimeter | Ameloblastoma | Dentigerous cyst | 2.256 | .4315 | .000 | HS |
| | | Odontogenic keratocyst | 1.456 | .4315 | .008 | HS |
| | | Unicystic Ameloblastoma | 1.1060 | .4825 | .156 | NS |
| | Dentigerous cyst | Odontogenic keratocyst | -.7993 | .4315 | .419 | NS |
| | | Unicystic Ameloblastoma | -1.150 | .4825 | .125 | NS |
| | Odontogenic keratocyst | Unicystic Ameloblastoma | -3.507 | .4825 | 1.00 | NS |

Table VI: T-test for comparison of all three AgNOR parameters between odontogenic keratocyst and ameloblastoma

Table VII: T-test for comparison of all three parameters between odontogenic keratocyst and dentigerous cyst

| | | Mean | Std Deviation | T | Mean Difference | interval of the difference | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| Number | Odontogenic keratocyst | 2.240 | .2874 | T(28)= 1.659; p=.108; NS | -.2133 | -.4768 | .0501 |
| | Ameloblastoma | 2.453 | .4068 | | -.2133 | -.4781 | .0514 |
| Area | Odontogenic keratocyst | 2.009 | .3926 | T(28)= 0.938; p=.356; NS | -.1540 | -.4904 | .1824 |
| | Ameloblastoma | 2.163 | .5005 | | -.1540 | -.4913 | .1833 |
| Perimeter | Odontogenic keratocyst | 6.787 | 1.578 | T(28)= 2.828; p= .009; HS | -1.456 | -2.511 | -.4017 |
| | Ameloblastoma | 8.244 | 1.219 | | -1.456 | -2.514 | -.3986 |

| | | Mean | Std Deviation | T | Mean Difference | 95% confidence interval of the difference | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| Number | Odontogenic keratocyst<br>Dentigerous cyst | 2.240<br>1.320 | .379 | T(28)= 10.107;<br>p=.000; HS | .9200<br>.9200 | .7335<br>.7326 | 1.1065<br>1.1074 |
| Area | Odontogenic keratocyst<br>Dentigerous cyst | 2.009<br>1.982 | .894 | T(28)= 0.199;<br>p=.884; NS | .0273<br>.0273 | -.2539<br>-.2540 | .3085<br>.3086 |
| Perimeter | Odontogenic keratocyst<br>Dentigerous cyst | 6.787<br>5.988 | .125 | T(28)= 1.768;<br>p= .088; NS | .7993<br>.7993 | -.1268<br>-.1434 | 1.725<br>1.742 |

Table VIII: T-test for comparison of all three AgNOR parameters between odontogenic keratocyst and unicystic ameloblastoma

| | | Mean | Std Deviation | T | Mean Difference | 95% confidence interval of the difference | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| Number | Odontogenic keratocyst<br>Unicystic Ameloblastoma | 2.240<br>1.920 | .2874<br>.4894 | T(23)= 2.066;<br>p=.085; NS | .3200<br>.3200 | -.0005<br>-.0503 | .6405<br>.6903 |
| Area | Odontogenic keratocyst<br>Unicystic Ameloblastoma | 2.009<br>1.765 | .3926<br>.4050 | T(23)= 1.505;<br>p=.146; NS | .2443<br>.2443 | -.0914<br>-.0975 | .5800<br>.5862 |
| Perimeter | Odontogenic keratocyst<br>Unicystic Ameloblastoma | 6.787<br>7.138 | 1.578<br>.9113 | T(23)= 1.578;<br>p= .533; NS | -.3507<br>-.3507 | -1.496<br>-1.384 | .7954<br>.6827 |

## FIGURE:

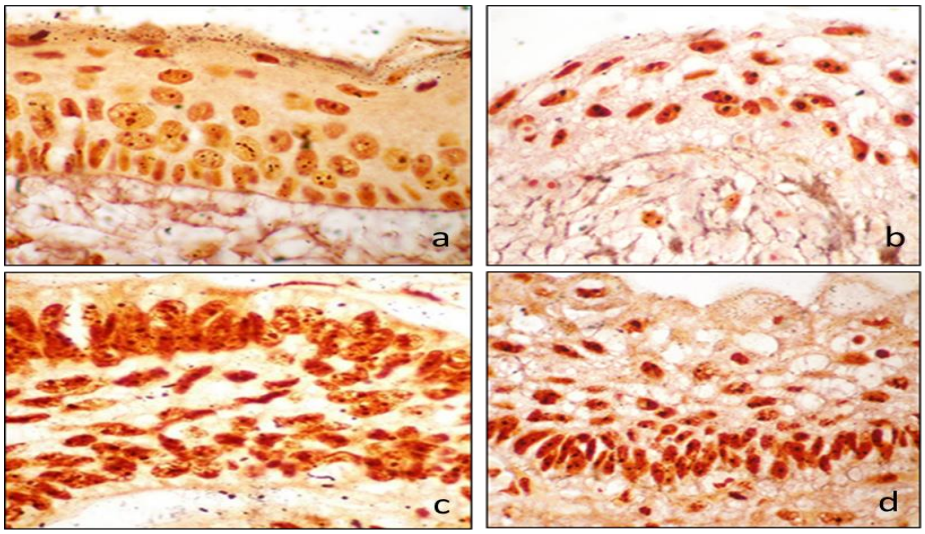


Figure I: Photomicrograph showing AgNORs in the nuclei of the epithelial lining of a. Odontogenic keratocyts , b. Dentigerous cyst, c. Conventional ameloblastoma, and d. Unicystic ameloblastoma (100x)