Original Article

Quantitative analysis of Argyrophilic Nucleolar organizer regions in odontogenic cysts and tumor – A comparative study

Bhavana Gupta^{a,*}, Shaleen Chandra^b, Vineet Raj^c, Vivek Gupta^d^a Senior Resident, Department of oral pathology, King George's Medical University, Lucknow, India^b Professor and Head, Department of oral pathology, King George's Medical University, Lucknow, India^c Professor and Head, Department of oral pathology, Chandra Dental College and Hospital, Lucknow, India^d Associate professor, Department of Periodontology, RIMS, Ranchi (Jharkhand), India

ARTICLE INFO

Article history:

Received 19 September 2016

Accepted 7 November 2016

Available online 17 November 2016

Keywords:

Argyrophilic Nucleolar organizer regions (AgNOR)

Keratocystic odontogenic tumor (KCOT)

ABSTRACT

Background: The nucleolar organizer region (NOR) is by definition part of a chromosome, and nucleolus is a structure containing this chromosomal part and in addition the material which accumulate around the NOR, mostly rRNAs and their precursors as well as specific ribosomal proteins. Argyrophilic Nucleolar organizing region (AgNOR) are silver binding NORs often used to study cell proliferation in various types of tumors.

Aim: Quantitative assessment of Argyrophilic Nucleolar organizing region count and its comparison among dentigerous cyst, keratocystic odontogenic tumor and ameloblastoma.

Material and method: Forty-five histologically confirmed cases, 15 cases each of keratocystic odontogenic tumor, dentigerous cysts and ameloblastomas were examined for Argyrophilic Nucleolar organizing region. The sections were obtained and Argyrophilic Nucleolar organizer regions staining was done for comparing the proliferative capacity among these lesions.

Result: Post hoc analysis for inter-group comparison and one way ANOVA were done in all three groups in this study. $P < 0.001$ was considered significant. The results of AgNOR counts were higher in KCOTs as compared to ameloblastoma and least in dentigerous cysts. The mean AgNOR counts between the study groups were compared using one way ANOVA test and the differences were found to be significant ($P < 0.001$).

Conclusion: AgNOR counts were significantly higher in KCOT and ameloblastoma as compared to dentigerous cyst suggesting that these lesions have a higher proliferative capacity than dentigerous cyst. The finding of a significantly higher AgNOR counts in KCOT as compared to ameloblastoma represent a difference in proliferative activity and greater growth potential between these two lesions.

© 2016

1. Introduction

Odontogenic cysts are more commonly encountered in dental practice, in contrast to odontogenic tumors.¹ Nucleolar organizing region (NORs) are loops of DNA containing ribosomal RNA (r-RNA) genes. Silver-stained NORs (AgNORs) are argyrophilic proteins of the NORs. NORs are argyrophilic due to their association with acidic proteins (C23, B23 and possibly RNA Polymerase I), which contain abundant sulfhydryl and carboxyl groups that precipitate the silver ions. They transcribe for ribosomal RNA which are

located on the short arm of chromosomes 13, 14, 15, 21 and 22.² Studies suggest usefulness of AgNOR count as maker of proliferation and may be helpful in diagnosis and prognosis of various neoplastic lesions, including oral squamous cell carcinoma and odontogenic lesions.^{3,4} This study was conducted to access the role of Argyrophilic Nucleolar organizing region in the biological behavior of cysts and tumors.

The rationale of the current study was quantitative assessment of Argyrophilic Nucleolar organizing region count and its comparison among dentigerous cyst, keratocystic odontogenic tumor and ameloblastoma.

2. Material and methods

The present retrospective cross sectional study was carried out on biopsy tissues obtained from the archives of Department of Oral

* Corresponding author at: C-2/600 Sector C, Jankipuram, Lucknow, UP, India.

E-mail addresses: drbhavanagupta@gmail.com (B. Gupta),

shal_nam@hotmail.com (S. Chandra), venetraj@gmail.com (V. Raj),

drvivek10feb@gmail.com (V. Gupta).

and Maxillofacial Pathology, of our college. The study sample included 45 cases of odontogenic cysts and tumors, 15 cases each of KCOTs, dentigerous cysts and ameloblastomas. The diagnoses were reviewed using routine hematoxylin and eosin stained sections. The inclusion criteria were all sub types of ameloblastomas, dentigerous cyst and KCOT were taken from both male and female patients of age between 16 and 60 years without any inflammation. Exclusion criteria were patients having any other oral lesions along with cysts and tumors were not included in the study sample. Patients having systemic diseases, pregnancy and tobacco habits were also not included in the study samples.

2.1. Equipment and armamentarium

Microscopic fields, representative of the lesion, were identified and photographed using Olympus BX51 light microscope, Live View Digital SLR Camera E-330. The photographs were analyzed using Image Pro Express 6.0, for windows (Media Cybernetics), USA.

AgNOR staining solutions: Two solutions were taken, solution A and B.

Solution A		
Silver nitrate	–	50 g
De-ionized Water	–	100 ml
Solution B		
Gelatin Powder	–	2 g
Formic acid	–	1 ml
De-ionized water	–	100 ml

Each time the final working solution was freshly prepared by mixing one volume of solution A and two volume of solution B. The staining procedure was standardized after a pilot study was carried out in the department, using internal controls such as the development of silver dots on lymphocytes and oral squamous cell carcinoma (OSCC) tissue was taken as a positive control.

2.2. Staining procedure

The slides were subjected to AgNOR (Fisher Scientific, New Hampshire) staining according to the method of Ploton et al.⁵ and counting was done as recommended by Crocker et al.⁶ The slides were progressively rehydrated through descending grades of alcohol and dewaxed in xylene. Finally the slides were washed with de-ionized water. Excess water was shaken off from the slides and the freshly prepared working solution was poured over the slides. These were then placed in the incubator at 37 °C for 30 min. After staining the slides were washed in de-ionized water, followed by sequential dehydration in ascending grades of alcohol. The slides were cleaned in xylene and mounted in synthetic medium (DPX).

2.3. Counting

All sections were examined under 1000× magnifications in oil immersion using light microscope and AgNOR dots were counted in 100 randomly selected cells using point counting tool from the basal and parabasal layers.

AgNORs were seen as individually discernible and separate dark brown to black dots or “blebs” of varying size, observed in a light brown stained nucleus within a pale yellow cytoplasm.

2.4. Statistics

Post hoc analysis for inter-group comparison and one way ANOVA were done to compare among three groups.

$P < 0.001$ was considered significant.

3. Results

In dentigerous cyst, the AgNOR count ranged from 1.0 to 1.25 (mean 1.11) (Fig. 1). AgNOR dots/nuclei. In KCOT, AgNOR count ranged from 1.32 to 2.92 (mean 2.39) (Fig. 2). AgNOR dots/nuclei. In ameloblastoma AgNOR count ranged from 1.43 to 2.84 (mean 1.86) AgNOR dots/nuclei (Fig. 3) (Table 1 and Graph 1). The mean

Fig. 1. Photomicrograph showing AgNOR expression in dentigerous cysts (1000×).

Fig. 2. Photomicrograph showing AgNOR expression in KCOT (1000×).

Fig. 3. Photomicrograph showing AgNOR expression in ameloblastoma (1000×).

Table 1

Showing comparison of AgNOR along with the mean AgNOR counts/nuclei between study groups.

	GROUPS	N	Mean	Std. deviation	P value
AgNOR dots/nuclei	Dentigerous cyst	15	1.112	0.091745	<0.001
	KCOT	15	2.306667	0.535666	
	Ameloblastoma	15	1.884	0.537452	
	Total	45	1.767556	0.660376	

Table 2Showing post hoc analysis for inter-group comparison of AgNOR counts. The results showed that AgNOR counts were significantly lower in dentigerous cyst as compared to KCOT ($P < 0.001$) as well as ameloblastoma ($P < 0.001$).

Dependent variable	(I) V7	(J) V7	Mean difference (I – J)	Sig.
AgNOR dots/nuclei	Dentigerous cysts	KCOT	–1.1946667	<0.001
		Ameloblastoma	–0.7720000	<0.001
	KCOT	Ameloblastoma	0.4226667	0.032

AgNOR counts between the study groups were compared using one way ANOVA test and the differences were found to be significant ($P < 0.001$) (Table 1). The AgNOR count of KCOT was found to be higher than ameloblastoma and this difference was also statistically significant ($P < 0.001$) (Table 2). Our study showed higher VEGF expression in KCOTs and ameloblastoma as compared to dentigerous cysts similarly higher AgNOR count in seen in KCOTs and ameloblastoma and least in dentigerous cysts (Fig. 4).

4. Discussion

Odontogenic keratocyst is one of the more commonly encountered jaw cysts which differ from other cysts by its aggressive growth potential and higher recurrence rate.¹ WHO 2005 classification considers this lesion under the group of benign odontogenic epithelial neoplasm and has suggested renaming it as “keratocystic odontogenic tumor” (KCOT).⁷ Dentigerous cyst is one of the developmental odontogenic cysts that enclose the crown of an unerupted tooth.⁸ It is a slow growing, usually asymptomatic lesion presenting as unilocular pericoronal radiolucent area.⁹ Ameloblastoma is a true neoplasm of enamel organ type tissue which does not undergo differentiation to the point of enamel formation. The lesion can occur as a solid multicystic mass or as a unicystic lesion with neoplastic changes in the cystic lining with or without luminal or mural proliferations.^{7,10}

Nucleolar organizing region (NORs) are loops of DNA containing ribosomal RNA (r-RNA) genes. NORs vary in size and shape according to nucleolar transcription. They are intimately related to cell cycle and may be related to proliferation or ploidy in some circumstances. During prophase the components of the fibrillar center disperse and in metaphase these structures exist in constant positions on the short arms of the five acrocentric chromosomes (namely 13, 14, 15, 21, 22).¹¹ Argyrophilic Nucleolar organizing

regions (AgNOR) are silver binding NORs often used to study cell proliferation in various types of tumors. Studies suggest potential usefulness of AgNOR count as maker of proliferation and it may be helpful in diagnosis and prognosis of various neoplastic lesions, including oral squamous cell carcinoma and odontogenic lesions.^{3,12}

Our results show that mean AgNOR counts per nuclei were significantly higher in KCOT and ameloblastoma as compared to dentigerous cyst (Table 1 and Graph 1). This is consistent with the results of Eslami et al.,¹³ who emphasized significantly higher AgNOR count in KCOT and ameloblastoma than dentigerous cysts, suggesting that these lesions have a higher proliferative capacity than dentigerous cyst. Santos et al.,¹⁴ concluded a higher AgNOR count in ameloblastoma which exhibits due to higher proliferative activity and higher aggressive behavior of these tumors. These findings are similar to previous studies which have shown higher AgNOR index in KCOT and ameloblastoma as compared to other cystic odontogenic lesions¹² which correlates with the greater growth potential of these lesions. Our results are not in accordance with Coleman et al.¹⁵ who suggested that AgNOR counts are not of diagnostic significance and cannot be used to distinguish the various odontogenic cysts from ameloblastoma. Seifi et al.¹⁶ studied AgNOR on ameloblastoma, KCOT, dentigerous cysts and evaluated that there is a significant statistical difference in NORs expression between ameloblastoma, dentigerous cyst and KCOT ($P < 0.001$) and concluded that number of NORs in ameloblastoma is higher than odontogenic cysts which contradicts our study.

Graph 1. Showing AgNOR counts between study groups. **Fig. 4.** Photomicrograph showing counting of AgNOR dots using Image Pro Express (1000×).

Thus our study showed a unique finding, of significantly higher AgNOR counts in KCOT as compared to ameloblastoma and dentigerous cyst as compared to other studies which evaluated that ameloblastoma showed higher AgNOR count as compared to KCOT and other odontogenic cysts including dentigerous cysts. This result may be either be incidental, given the small size of the sample, or may actually represent a difference in proliferative activity between these two lesions. A higher proliferative activity in KCOT as compared to unicystic ameloblastoma has been reported in studies with the help of other proliferative markers like PCNA, Ki67 and the difference were statistically significant.¹⁷

5. Conclusion

AgNORs in KCOT were numerous and more widespread than in ameloblastoma with statistically significant differences in AgNOR patterns that reflect differences in cellular and nuclear activities. This finding of a significantly higher AgNOR counts between these lesions represent a difference in proliferative activity and greater growth potential. A prospective studies with larger sample size of these lesions and by using different proliferative markers and comparing the solid and cystic variants (of ameloblastoma) separately, will help in assessing the biological behavior of these cysts and tumors. And thus will guide us to know about the difference in growth potential of these lesions so as to reach at an equivocal conclusion.

Conflicts of interest

The authors have none to declare.

References

1. Neville BW, Damm DD, Allen CM, Bouquot JE. *Oral and Maxillofacial Pathology*. 3rd ed. Philadelphia: Saunders; 2009:213–221.
2. Derenzini M, Tere D. Importance of interphase nucleolar organizer regions in tumor pathology. *Virchow Arch B Cell Pathol*. 1991;61(1):1–8.
3. Prabhu VD, Vidya M, Ray JG, Prabhu R. Quantitative and qualitative evaluation of silver staining nuclear organizer regions in normal odontogenic epithelium and odontogenic cysts – a comparative study. *J Oral Health Res*. 2010;1:141–145.
4. Fonseca LMS, Carmo MAV. AgNORs in hyperplasia, papilloma and squamous cell carcinoma. *Braz Dent J*. 2000;11:105–110.
5. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon J, Adnet J. Improvement in the staining and in visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. *Histochem J*. 1986;18:5–14.
6. Crocker J, Boldy DAR, Egan MJ. How should we count AgNORs? Proposals for a standardized approach. *J Pathol*. 1989;158(3):185–188.
7. Rajendran R, Sivapathasundharam B. *Shafer's Textbook of Oral Pathology*. 6th ed. New Delhi: Elsevier; 2009. [chapter 4].
8. Shear M, Speight P. *Cysts of the Oral and Maxillofacial Regions*. 4th ed. Australia: Blackwell Munksgaard; 2007. Classification and frequency of cysts of the oral and maxillofacial tissues [chapter 1].
9. Shear M. Cysts of the jaws: recent advances. *J Oral Pathol*. 1985;14:43–59.
10. Kumamoto H, Ohki K, Ooya K. Association between vascular endothelial growth factor (VEGF) expression and tumor angiogenesis in ameloblastomas. *J Oral Pathol Med*. 2002;31:28–34.
11. Egan MJ, Crocker J. Nucleolar organizer regions in pathology. *Br J Cancer*. 1992;65(1):1–7.
12. Carmo MAV, Silva EC. AgNORs in ameloblastomas and adenomatoid odontogenic tumours. *J Oral Pathol Med*. 1998;27(4):153–156.
13. Eslami B, Yaghmaei M, Firoozi M, Saffar AS. NORs in selected odontogenic lesions. *Oral Surg Oral Path Oral Med*. 2003;95(2):187–192.
14. Santos AC, Tarquinio SBC, Rivero ERC, Araujo LMA, Krause CI. Quantitative AgNORs study in ameloblastomas. *Rev odontol ciênc*. 2009;24(1):10–14.
15. Coleman HG, Altini M. AgNORs in odontogenic cyst and ameloblastomas. *J Oral Pathol Med*. 1996;25(8):436–440.
16. Seifi S, Shafagh E, Alaei A. Qualitative and quantitative evaluation of AgNOR staining in odontogenic cysts and ameloblastoma. *Sci Res J Shahed Univ*. 2010;17(88):37–44.
17. Song MY, Lee SS, Lee JK, et al. Relation of the radiologic findings and labeling index of Ki-67, PCNA and cytokeratin in unicystic ameloblastoma, dentigerous cyst and odontogenic keratocyst. *Korean J Oral Maxillofac Radiol*. 2004;34(2):75–79.