Original Article

Immunohistochemical evaluation of inducible nitric oxide synthase in the epithelial lining of odontogenic cysts: A qualitative and quantitative analysis

B K Akshatha, Karpagaselvi Karuppiah[1], G S Manjunath[2], Jayalakshmi Kumarswamy[1], Lokesh Papaiah[1], Jyothi Rao[1]

Departments of Oral Pathology and [2]Oral and Maxillofacial Surgery, MR Ambedkar Dental College and Hospital, [1]Department of Oral Pathology, Vydehi Institute of Dental Sciences and Research Center, Bengaluru, Karnataka, India

Abstract

Introduction: The three common odontogenic cysts include radicular cysts (RCs), dentigerous cysts (DCs), and odontogenic keratocysts (OKCs). Among these 3 cysts, OKC is recently been classified as benign keratocystic odontogenic tumor attributing to its aggressive behavior, recurrence rate, and malignant potential. The present study involved qualitative and quantitative analysis of inducible nitric oxide synthase (iNOS) expression in epithelial lining of RCs, DCs, and OKCs, compare iNOS expression in epithelial linings of all the 3 cysts and determined overexpression of iNOS in OKCs which might contribute to its aggressive behavior and malignant potential.

Aims: The present study is to investigate the role of iNOS in the pathogenesis of OKCs, DCs, and RCs by evaluating the iNOS expression in the epithelial lining of these cysts.

Subjects and Methods: Analysis of iNOS expression in epithelial lining cells of 20 RCs, 20 DCs, and 20 OKCs using immunohistochemistry done.

Statistical Analysis Used: The percentage of positive cells and intensity of stain was assessed and compared among all the 3 cysts using contingency coefficient. Kappa statistics for the two observers were computed for finding interobserver agreement.

Results: The percentage of iNOS-positive cells was found to be remarkably high in OKCs (12/20) –57.1% as compared to RCs (6/20) – 28.6% and DCs (3/20) – 14.3%. The interobserver agreement for iNOS-positive percentage cells was arrived with kappa values with OKCs → Statistically significant ($P > 0.000$), RCs → statistically significant ($P > 0.001$) with no significant values for DCs. No statistical difference exists among 3 study samples in regard to the intensity of staining with iNOS.

Conclusions: Increased iNOS expression in OKCs may contribute to bone resorption and accumulation of wild-type p53, hence, making OKCs more aggressive.

Keywords: Dentigerous cyst, immunohistochemistry, inducible nitric oxide synthase, odontogenic keratocyst, radicular cyst

Address for correspondence: Dr. GS Manjunath, Department of Oral and Maxillofacial Surgery, MR Ambedkar Dental College and Hospital, 1/36 Cline Road, Cookes Town, Bengaluru - 560 005, Karnataka, India.
E-mail: manjunathmaxfac@gmail.com

Received: 25.05.2017, **Accepted:** 09.11.2017

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/jomfp.JOMFP_96_17

How to cite this article: Akshatha BK, Karuppiah K, Manjunath GS, Kumarswamy J, Papaiah L, Rao J. Immunohistochemical evaluation of inducible nitric oxide synthase in the epithelial lining of odontogenic cysts: A qualitative and quantitative analysis. J Oral Maxillofac Pathol 2017;21:375-81.

INTRODUCTION

Cyst is defined as "A pathological cavity having fluid, semifluid, or gaseous contents and which is not created by the accumulation of pus."[1] Odontogenic cystic lesions present with distinct histopathologic features and biologic behavior, which are derived from odontogenic apparatus or its remnants.[2] Among the various odontogenic cysts, the three most common are radicular cysts (RCs), dentigerous cysts (DCs), and odontogenic keratocysts (OKCs). RCs are inflammatory in origin, were as OKCs and DCs are developmental in origin.[3]

Most of the RCs are slow growing without achieving a large size. Takeichi *et al*. reported inducible nitric oxide synthase (iNOS) production in RCs and found iNOS presence increased the size of RCs thereby prolonging their pathologic conditions.[4]

In the year 1956, Philipsen introduced the term OKC which accounts for 3%–11% of all odontogenic cysts. Because of the aggressive growth pattern and neoplastic nature of OKC, it is now designated by the World Health Organization as Kerato Cystic Odontogenic Tumor. The actively proliferating cells are known to express PCNA, Ki-67, p53, and to a lesser extent AgNORs particularly in neoplasms, being expressed more potent in OKCs than any other odontogenic cysts which has provided supportive evidence that OKC is a benign neoplasm.[5]

p53 is a tumor suppressor gene known to play a pivotal role in the regulation of cell proliferation. p53 protein attained from mutation of p53 gene has increased half-life, which can be detected immunohistochemically. However, wild-type p53 protein can be observed in case of overproduction or stabilization of this protein. Many studies have shown increased expression of p53 in OKCs then DCs and RCs been correlated with ki-67, suggesting p53 protein is related to cell proliferative activities in OKCs.[3]

The relationship between iNOS and tumor suppressor gene p53 was extensively studied. At sites of inflammation, iNOS is released, which is a calcium-independent cytosolic enzyme induced mainly by cytokines such as interleukin-1ß, tumor necrosis factor and interferon-γ.

Nitric oxide is a product of conversion of L-arginine to L-citrulline by nitric oxide synthase which has 3 isoforms. The 3 isoforms are NOS I (nNOS) neuronal form, NOS II iNOS present in several cell types upon inflammatory stimulation and NOS III (eNOS) constitutive enzyme primarily discovered in endothelium.[6] With intact p53 in murine model pathway, increased the concentration of nitric oxide results by iNOS inducing accumulation of wild-type p53 protein which promotes apoptosis. This effect is absent in mutant form of p53 protein. Increased expression of iNOS is seen in p53 knockout mice, missing the gene for p53 resulting in the formation of multiple tumors thereby leading to early death.[6]

Currently, molecules showing a close relation to angiogenesis and carcinogenesis includes various gene products such as iNOS, vascular endothelial growth factor, and cyclooxygenase-2(COX-2).[7]

According to various studies increased expression of p53 is documented in OKCs, thereby correlation of p53 and iNOS expression may be responsible for the aggressive behavior of OKCs. Cytokines such as Interleukin-α and Interleukin-6 are produced by the epithelial lining cells of OKCs, DCs, and RCs which may activate iNOS expression of the epithelial cells in autocrine fashion.[3] OKCs, DCs, and RCs may participate in bone resorption and cystic enlargement due to increased nitric oxide production. This is because matrix metalloproteinases are known to play key role in the breakdown of bone matrix which can be activated by nitric oxide. The epithelial lining of OKC appears to have intrinsic growth potential not present in other types of odontogenic cysts. Hence, the present study is to investigate role of iNOS in the pathogenesis of OKCs, DCs, and RCs by evaluating the iNOS expression in the epithelial lining of these cysts.

SUBJECTS AND METHODS

The present study was conducted on archived paraffin-embedded tissue specimens of RCs, DCs and OKCs received at the Department of Oral Pathology and Microbiology, Bengaluru. A total of 60 cases which were previously diagnosed as RCs, DCs, and OKCs were retrieved. The H and E stained sections were reviewed, and 20 cases of RCs [Figure 1], DCs [Figure 2] and OKCs [Figure 3] were selected..

Two Tissue sections of 3.5 μ thickness were cut and transferred on to APES coated slides were one marked as case and other as control. The sections were deparaffinized and rehydrated. Then the slides were transferred to TRIS-Citrate buffer and antigen retrieval was done using pressure cooker for 15 min. The slides were allowed to cool and then washed in cold TRIS buffer (TBS) solution for 5 min. Slides were treated with 3% hydrogen peroxide for quenching of endogenous peroxidase activity of cells to avoid nonspecific

staining. The slides were then dipped in three changes of TBS buffer for 5 min each. The protein block reagent was added on to sections for 10 min and washed in three changes of TRIS buffer. Excess TBS was removed by blotting. Anti-iNOS antibody (1:40 dilution in TBS) was added to section marked as case, and TBS was added to section marked as control. The slides were incubated at room temperature for 1 h 20 min. The slides taken out were washed in cold TBS for 5 min each to remove excess antibody. Then, the slides were blotted dry without touching tissue sections. Then, a drop of biotinylated secondary antibody anti-INOS added on both the sections and the slides were incubated for 30 min and then washed in 3 changes of cold TRIS buffer for 5 min each. Then the slides were blotted dry without touching tissue sections. Then, a drop of streptavidin was added on to both the sections on slide and was incubated for 30 min. The sections were washed in 3 changes of cold TRIS buffer for 5 min each. Then, the slides were blotted dry without touching tissue sections. Then, a drop of freshly prepared DAB (3'diaminobenzidine–tetrahydrochloride a substrate chromogen) was added on both sections. Excess DAB was removed by dipping in TBS and then counterstained with hematoxylin.

A known positive tissue was stained with each batch of slides to serve as positive control. The control tissue on each slide served as negative control.

Evaluation of tissue sections

The stained sections were scanned under low power and brown cytoplasmic staining was termed as positive for iNOS. The sections were visualized by 2 observers and 4 random areas with 40x magnification were chosen and 100 cells were analyzed. Percentage of positive cells was calculated and then categorized as

0 = No staining in any field, 1+ = ≤25% of tissue stained, 2+ = between 25% and 50% stained, 3+ = between 50% and 75% stained, 4+ = More than 75% stained.

Intensity was documented by comparing study samples at scanner view (4x) with the positive control sections by 2 observers independently, according to following scale,

0 = none, 1+ = Weak staining, 2+ = Moderate staining, 3+ = Intense staining. In OKCs, intensity of staining was evaluated separately in both basal layer and suprabasal layer of epithelium.

Statistical analysis

The results were tabulated and analyzed using statistical software SPSS 16.0. The evaluation of iNOS-positive cells between 2 study groups at a time was done using Mann–Whitney test. Comparison of percentage positivity of cells for iNOS and intensity of stain among 3 study groups was done using Contingency coefficient. Kappa statistics for the two observers were computed for finding interobserver agreement.

RESULTS

Tissue localization of iNOS stain

iNOS staining was limited to basal and parabasal layers of the epithelium or seen throughout all layers of the epithelium in OKCs. In 5 of the OKC, sections staining were seen in all layers of epithelium. Whereas 11/20 (55%) showed staining limited to only basal and parabasal layers of the epithelium, 3/20 (15%) presented staining involving only parabasal layer without involving surface portion of the epithelium and only 1/20 (5%) showed staining limited to surface layer of the epithelium. Besides the expression of iNOS in the epithelial lining cells of all the 3 study groups, the reactivity of iNOS was detected in many cells of fibrous connective tissue walls which includes fibroblasts, endothelial cells of blood vessels, macrophages, and some plasma cells.

Comparison of intensity of stain:

- Based on the scale of score for intensity
- 1+ → mild intensity
- 2+→ Moderate intensity
- 3+→ Severe intensity
- RCs (6/20) 31.6% – showed severe intensity
- DCs (4/20) 21.1% – showed severe intensity
- OKCs (9/20) 47.4% – showed severe intensity.

($P < 0.233$) – Not significant.

DISCUSSION

One of the most common osseous destructive lesions is odontogenic cysts affecting the jaws arising from the epithelial components of the odontogenic apparatus or its remnants.[8]

RCs being inflammatory in origin whereas OKCs and DCs are developmental cysts. Among these, RCs are the common cystic lesions affecting the jaws. Of these cysts, OKC has clinical importance of aggressive behavior, recurrence risk, and malignant potential. Recently, OKC has been designated by the WHO as KeratoCystic Odontogenic Tumor. However, the neoplastic nature of OKC is still controversial.[3]

In an attempt to strengthen the clause that OKC is a tumor, we analyzed that iNOS staining in OKC in comparison to DC and RC. Nitric oxide (NO) is a short-lived,

endogenously produced gas, suggested to modulate different events such as angiogenesis, apoptosis, cell cycle, invasion, and metastasis.[9] NO is synthesized by a complex family of enzymes called NO synthases (NOS). Among the 3 isoforms NOS under inductive conditions, iNOS produces NO, which contributes to a variety of pathological phenomena associated with inflammatory processes and cancer formation.[3]

The present study was a preliminary study being carried out to examine the expression of iNOS in epithelium of all 3 cysts which includes OKCs, DCs, and RCs.

In our study, 20 samples of OKCs were considered among which 13 were noninfected and 7 were infected OKCs. Intensity of iNOS staining in all layers of epithelium in OKCs samples varied. Severe iNOS staining was found in all layers of epithelium in (4/20) cases with moderate staining seen involving basal and parabasal layers of epithelium in (12/20) cases and weak intensity was found involving basal and parabasal layers of epithelium in (4/20) cases. In all infected OKCs staining involved both basal and parabasal layers of epithelium. In noninfected OKCs, (4/20) cases showed staining in all layers of epithelium, with (5/20) of them showing staining involving only basal and parabasal layers and (4/20) cases showing staining involving only parabasal layer of epithelium.

According to a study carried out by Poomswat *et al*., cytoplasmic staining was found in epithelial lining cells in all 20 samples of OKCs. Nuclear staining was also detected, which was likely to be the overlayer of strong intensity of the cytoplasm. Most of OKCs showed strong intensity of iNOS staining.

In the present study, 20 samples of DC were considered. Among which 7 cases were infected DC with 13 cases of noninfected DCs. (4/20) cases showed severe intensity involving all the epithelial lining cells with (9/20) cases showing moderate intensity and (7/20) cases presented with weak intensity.

In the present study, 20 samples of RCs were considered. Variation of iNOS reactivity was seen among all the cases.(7/20) cases expressed severe intensity of staining with (8/20) cases presented with moderate intensity and (5/20) cases showed weak intensity. Apart from epithelial lining cells connective tissue components such as inflammatory cells and endothelial lined blood vessels, fibroblasts showed iNOS reactivity.

In the present study, about 11 cases of OKCs showed 40.7% of severe intensity, with 8 cases of DCs showed 29.6% of severe intensity and 8 cases of RCs showing 29.6% of severe intensity [Figures 4-6].

No statistical difference exists among 3 study samples in regard to intensity of staining with iNOS [Table 1].

In our study, mean rank of iNOS-positive cells in epithelial lining in 20 cases of RCs was compared with 20 cases of DCs. RCs showed mean rank of 22.73 compared to DCs with mean rank of 18.27 concluding there is no significant difference among these 2 samples [Table 2].

The present study revealed mean rank of 16.88 for iNOS-positive cells in epithelial lining of 20 cases of RCs when compared to mean rank of 24.13 of OKCs. There was a statistical difference in mean rank with the $P > 0.049$ [Table 3].

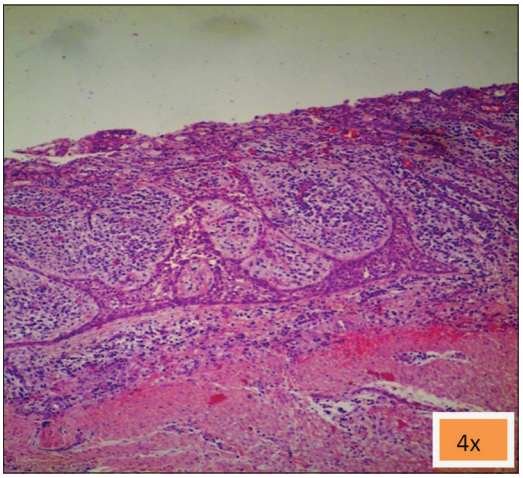

Figure 1: H&E, section of radicular cyst

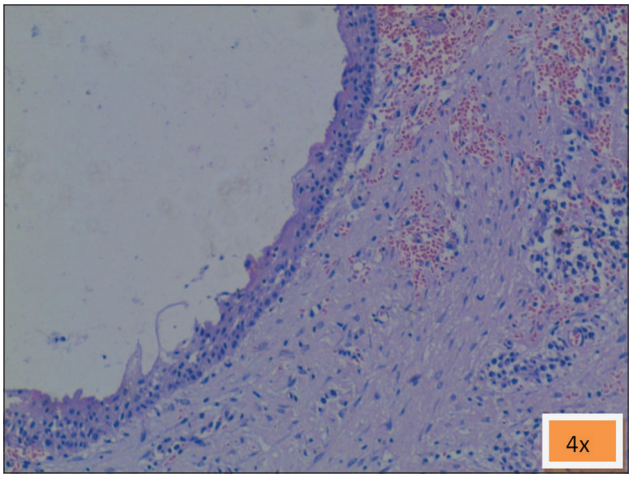

Figure 2: H&E, section of dentigerous cyst

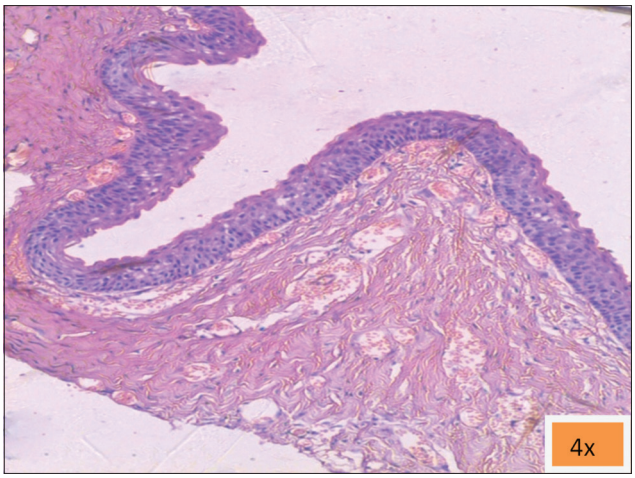

Figure 3: H&E, stained tissue section of odontogenic keratocyst

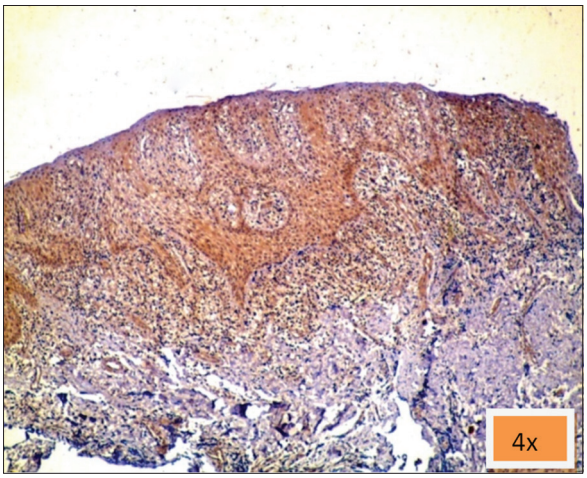

Figure 4: IHC of radicular cyst

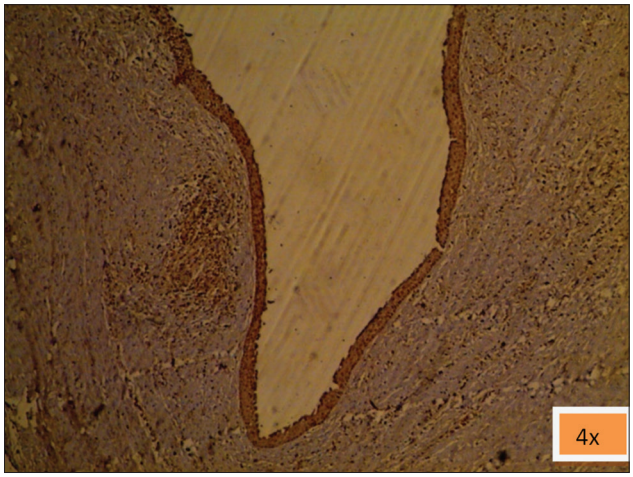

Figure 5: IHC of dentigerous cyst

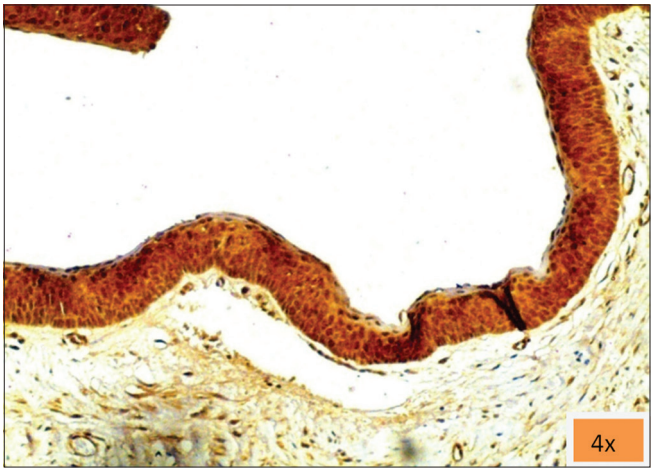

Figure 6: IHC of odontogenic keratocyst

Table 1: Distribution of intensity of stain in study groups

Crosstab					
Scales of scoring of intensity		Cyst			Total
		RC	DC	OKC	
Intensity					
1+	Count	3	5	2	10
	Percentage of intensity	30.0	50.0	20.0	100.0
2+	Count	9	7	7	23
	Percentage of intensity	39.1	30.4	30.4	100.0
3+	Count	8	8	11	27
	Percentage of intensity	29.6	29.6	40.7	100.0
Total	Count	20	20	20	60
	Percentage of intensity	33.3	33.3	33.3	100.0

Symmetric measures			
Nominal	Contigency coefficient	Value	Approximate significance
Nominal by nominal	Contingency coefficient	0.197	0.660
Number of valid cases		60	

No statistical difference exists in intensity of stain among 3 study groups.OKC,DC and RC. OKC: Odontogenic keratocyst, DC: Dentigerous cyst, RC: Radicular cyst

Table 2: Comparison of mean rank of inducible nitric oxide synthase-positive cells between radicular cysts and dentigerous cysts

Ranks				
	Cyst	*n*	Mean rank	Sum of ranks
AVTOT	RC	20	22.73	454.50
	DC	20	18.27	365.50
	Total	40		

Test statistics[b]	
	AVTOT
Mann-Whitney U-test	155.500
Wilcoxon W	365.500
Z	−1.216
Asymptotic significance (two-tailed)	0.224
Exact significance (2 × [one-tailed significance])	0.231[a]

[a]Not corrected for ties, [b]grouping variable;No statistical siginificant results exists between RCs and DCs. AVTOT: Average total, RC: Radicular cyst, DC: Dentigerous cyst

Our study analyzed mean rank of 13.90 for iNOS-positive cells in epithelial lining of 20 cases of DCs when compared to 27.10 to OKCs with mean rank of 27.10 concluding a statistical difference in mean rank with the $P > 0.000$ [Table 4].

The interobserver agreement for percentage of iNOS-positive cells among RCs showed statistically significant

Table 3: Comparison of mean rank of inducible nitric oxide synthase-positive cells between radicular cysts and odontogenic keratocysts

Ranks				
	Cyst	*n*	Mean rank	Sum of ranks
AVTOT	RC	20	16.88	337.50
	OKC	20	24.13	482.50
	Total	40		

Test statistics[b]	
	AVTOT
Mann-Whitney U-test	127.500
Wilcoxon W	337.500
Z	−2.014
Asymptotic significance (two-tailed)	0.044
Exact significance (2 × [one-tailed significance])	0.049[a]

[a]Not corrected for ties, [b]Grouping variable: Cyst. *P*>0.049 (statistically significant). AVTOT: Average total, RC: Radicular cyst, OKC: Odontogenic keratocyst

Table 4: Comparison of mean rank of inducible nitric oxide synthase-positive cells between dentigerous cysts and odontogenic keratocysts

Ranks				
	Cyst	*n*	Mean rank	Sum of ranks
AVTOT	DC	20	13.90	278.00
	OKC	20	27.10	542.00
	Total	40		

Test statistics[b]	
	AVTOT
Mann-Whitney U-test	68.000
Wilcoxon W	278.000
Z	−3.641
Asymptotic significance (two-tailed)	0.000
Exact significance (2 × [one-tailed significance])	0.000[a]

[a]Not corrected for ties, [b]Grouping variable: Cyst. *P*>0.000 (statistically significant). AVTOT: Average total, DC: Dentigerous cyst, OKC: Odontogenic keratocyst

results with $P > 0.001$. No agreement among interobservers for DCs exists. The inter-observer agreement among OKCs showed significant results with $P > 0.021$ [Table 5].

In general, cells with DNA damage could be arrested or apoptotic by mediation of wild-type p53 protein. Since mutation of p53 gene exists in some OKCs, not in Dentigerous and RCs, it is, therefore, possible that OKCs with mutated p53 gene have a chance to accumulate cells with DNA damage induced by high concentration of nitric oxide.[3] The present study may contribute to the clinical behavior and neoplastic nature found in some OKCs. The increased percentage of iNOS-positive cells in OKCs when compared to RCs and DCs suggests nitric oxide produced by iNOS may cause DNA damage.

CONCLUSIONS

In the present study, 57.1% of OKCs showed high percentage of iNOS-positive cells when compared to 28.6% of RCs and 14.3% of DCs [Table 6].

Table 5: Inter-observer agreement for inducible nitric oxide synthase-positive cells in odontogenic keratocysts

Crosstabulation for OKCs					
Examiner 1	Count percentage for OKCs	Examiner 2			Total
		2+	3+	4+	
Examiner 1					
2+	Count	1	1	0	2
	Percentage of OKC E1	50.0	50.0	0.0	100.0
3+	Count	1	2	2	5
	Percentage of OKC E1	20.0	40.0	40.0	100.0
4+	Count	3	0	10	13
	Percentage of OKC E1	23.1	.0	76.9	100.0
Total	Count	5	3	12	20
	Percentage of OKCE1	25.0	15.0	60.0	100.0

Symmetric measures				
	Value	Asymptotic SE	Approximate *t*	Approximate significance
Measure of agreement (κ)	0.361	0.165	2.312	0.021

P>0.021 (statistically significant). OKCs: Odontogenic keratocysts, SE: Standard error

Table 6: Mean percentage of inducible nitric oxide synthase-positive cells in study groups

Crosstab					
Scales of scoring		Cyst			Total
		RC	DC	OKC	
1+	Count	4	8	0	12
	Percentage	33.3	66.7	0.0	100.0
2+	Count	8	6	5	19
	Percentage	42.1	31.6	26.3	100.0
3+	Count	2	3	3	8
	Percentage	25.0	37.5	37.5	100.0
4+	Count	6	3	12	21
	Percentage	28.6	14.3	57.1	100.0
Total	Count	20	20	20	60
	Percentage	33.3	33.3	33.3	100.0

Symmetric measures			
Nominal	Contigency coefficient	Value	Approximate significance
Nominal by nominal	Contingency coefficient	0.447	0.020
Number of valid cases		60	

P>0.020 (statistically significant). RC: Radicular cyst, DC: Dentigerous cyst, OKC: Odontogenic keratocyst

In conclusion, the overexpression of iNOS in OKCs might contribute to the aggressive behavior and malignant potential. Further studies using immunohistochemistry on larger sample size will be helpful in substantiating the neoplastic potential of the epithelium of OKCs.

Acknowledgments

The authors would like to acknowledge the support extended by Mrs. Dorthy Anita, Senior technician, Vydehi Institute of Dental Sciences, Ms. Priyadarshini, Junior Technician, Vydehi Institute of Dental Sciences.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Shear M. Speight's Cysts of Oral and maxillofacial regions.4th edition, Blackwell Munksgaard; 2007.
2. Nadalin MR, Fregnani ER, Silva-Sousa YT, Perez DE. Syndecan-1 (CD138) and KI-67 expression in odontogenic cystic lesions. Braz Dent J 2011;22:223-9.
3. Poomsawat S, Punyasingh J, Vejchapipat P. Immuno-histochemical expression of p53 protein and iNOS in odontogenic cysts. J Med Assoc Thai 2009;92:952-60.
4. Shear M. The aggressive nature of the odontogenic keratocyst: Is it a benign cystic neoplasm? Part 2. Proliferation and genetic studies. Oral Oncol 2002;38:323-31.
5. Hemavathy S, Roy S Follicular odontogenic keratocyst mimicking dentigerous cyst-report of 2 cases. Arch Oral Sci Res 2011;1;100-3.
6. Korde Choudhari S, Sridharan G, Gadbail A, Poornima V. Nitric oxide and oral cancer: A review. Oral Oncol 2012;48:475-83.
7. Sappayatosok K, Maneerat Y, Swasdison S, Viriyavejakul P, Dhanuthai K, Zwang J, *et al*. Expression of pro-inflammatory protein, iNOS, VEGF and COX-2 in oral squamous cell carcinoma (OSCC), relationship with angiogenesis and their clinico-pathological correlation. Med Oral Patol Oral Cir Bucal 2009;14:E319-24.
8. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer 2006;6:521-34.
9. de Souza LB, Gordón-Núñez MA, Nonaka CF, de Medeiros MC, Torres TF, Emiliano GB, *et al*. Odontogenic cysts: Demographic profile in a Brazilian population over a 38-year period. Med Oral Patol Oral Cir Bucal 2010;15:e583-90.