Original Article

Comparative evaluation of podoplanin in odontogenic cysts and tumours to determine their proliferative potential—An immunohistochemical study

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Abstract

Context: Odontogenic cysts and tumours are a wide array of complex pathological entities ranging from mild indolent to aggressive detrimental in nature, which occur as a result of anomalous alterations in normal odontogenesis. Hence, these odontogenic lesions need to be evaluated extensively by using potential immunohistochemical markers.

Aim: To evaluate and compare the expression of podoplanin, a lymphoendothelial IHC marker in odontogenic cysts and odontogenic tumours to determine their proliferative potential.

Settings and Design: All the study samples were retrieved from the archives of the Department of Oral Pathology and Microbiology, PIDS&RC, Hyderabad. The study samples were selected as per the standard histopathological diagnostic criteria and subjected for IHC analysis using podoplanin.

Method and Materials: Seventy paraffin-embedded tissue specimens of OKC, OOC, dentigerous cyst (DC) and ameloblastoma (AM) include study sample, which were stained with podoplanin IHC marker and staining properties were evaluated. All the cases were categorized as high, moderate, weak or negatively reactive on the basis of the composite scoring.

Statistical Analysis Used: Statistical analysis was done using SPSS version 14, and then results were compared by ANOVA post hoc test and Kruskal Wallis Test.

Results: In the comparison of composite scores of OKCs and AM, there was no significant statistical difference.

Conclusion: The present study contributes to the significant association of podoplanin expression with cellular proliferation, cystic expansion and local invasiveness of odontogenic cysts and tumours through cytoskeletal reorganization and cell migration.

Keywords: Odontogenic cysts, odontogenic keratocyst, odontogenic tumours, podoplanin

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INTRODUCTION

Odontogenic cysts and odontogenic tumours are myriad of lesions ranging from an innocuous lesion to catastrophic and disastrous lesions that may cause extraoral disfigurement. These lesions arise resultant of some alteration in the normal pattern of odontogenesis reflecting the multiformity and complex developmental pattern of dental apparatus.[1] Odontogenic keratocyst (OKC) is a most aggressive lesion belonging to aforementioned aggressive and destructive group of odontogenic cysts. There has always been metamorphosis in the nomenclature and categorization of this unique odontogenic lesion since 1887 till 2017.[2] Orthokeratinized odontogenic cyst (OOC) is a rare, developmental odontogenic cyst, which was considered in the past to be a variant of OKC, but in 1981, Wright identified it as distinct entity owing to its different histology and relatively low recurrence rate.[3,4] In 1992, Philipsen and Reichart reclassified OKC as tumour, and in 2005, WHO classified it as keratocystic odontogenic tumour in 2005 due to several factors like local aggressive behaviour, high recurrence rate, epithelial budding, high mitotic rate, genetic mutations in PTCH-1 gene and dysregulation of Hedgehog signalling pathway.[5,6] However, in 2017, the WHO reclassified OKC back into the cystic category as the evidence supporting hypothesis like clonality is considered insufficient and remained unchanged in 2022 WHO classification.[7] Dentigerous cyst (DC) is the second most frequently encountered odontogenic cyst possessing malignant potential transforming into ameloblastoma (AM), epidermoid carcinoma and mucoepidermoid carcinoma, which reflects the pluripotentiality of its lining epithelium.[8] However, the existing clinical criteria cannot predict the potential for neoplastic behaviour or aggressive, localized expansion and infiltration, in such lesions. Unfortunately, there is no much research done to find an appropriate IHC marker that can assess the proliferative potential and aggressiveness of various odontogenic cysts and tumours. A long arduous search for such efficient marker had brought researchers' attention towards podoplanin, an emerging IHC marker. Podoplanin (PDPN), is a mucin-type 38 kDa type-1 transmembrane glycoprotein known as a lymphatic endothelial marker is expressed in oral squamous cell carcinoma (OSCC) demonstrating its role in tumorigenesis.[9] Studies have also shown enhanced expression of PDPN in various odontogenic tumours suggesting its role in odontogenic tumorigenesis.[10,11] Hence, from the accumulated evidence the present study aims to evaluate and compare PDPN expression in the odontogenic cysts like DC, OOC and OKCs with odontogenic tumour like AMs which are locally aggressive odontogenic neoplasms of the jaws and additionally, to elucidate and emphasize the role and importance of PDPN molecule in their varied behaviour as well as the proliferative potential by comparing the expression pattern.

MATERIALS AND METHOD

The present study includes 70 histopathologically confirmed paraffin-embedded tissue blocks of OKC, OOC, AM and DC. The keratocysts were classified according to Philipsen criteria and Wright's criteria for OKCs (parakeratinized) and OOCs (orthokeratinized), respectively. The research sample consists of 10 cases of AM, 10 cases of DC, 13 cases of OOC and 37 cases of OKC obtained from archives of Department of Oral & Maxillofacial Pathology. The study was approved by the institutional Ethical Committee of Panineeya Institute of Dental Sciences & Research Centre, Hyderabad, Telangana with approval No. PMVIDS/OP/0009/2018. Paraffin-embedded tissues were sectioned to 4-μm thickness and mounted on poly-lysine-coated slides stained with the immunohistochemical monoclonal antibody podoplanin D2-40 (DAKO) following the standard procedure. The underlying lymphoendothelial structures, osteoblasts and osteocytes served as internal positive controls and negative tissue control consisted stromal collagen fibres. The immune expression of PDPN was assessed using binocular light microscope under 10X and 40X magnifications. For scoring purposes, five high power fields (HPFs) representative areas from each section were evaluated. Cytoplasm and/or membrane immunoreactivity was considered to indicate positive PDPN expression. The intensity of immunopositivity, per cent of positive cells and number of positive strata were evaluated. Immunostaining results were scored as given by Krajewska *et al.*[12] The percentage of positive tumour cells was graded into the following: none or no expression as grade 0, 1 to 25% positive cells grade 1, 26 to 50% as grade 2, 51 to 75% as grade 3 and 76 to 100% as grade 4. Immunostaining intensity was rated as follows: none-0, weak-1, moderate-2 and intense-3. Specimens were considered positive when >1% of the tumour cells had clear evidence of immunostaining. As there was heterogenicity in staining of tumours for IHC marker, the intensity of staining(A) and percentage of positive cells(B) were scored independently for each HPF and were multiplied to obtain a total score (A X B=C). The total scores were then summed up for 5 HPF to obtain composite score (C1+C2+C3+C4+C5=D), and the average of composite scores(D/5) was taken as final composite score(S). As suggested by Krajewska *et al.*,[12] theoretically, the final composite scores could range from 0 to 12. A final composite score of 9 or higher was considered as strong, 5–8 moderate, 2–4 mild and 0–1 as

negative expression on the basis of final composite scoring criteria. Statistical analysis was done using SPSS version 14. A P value of $P < 0.05$ was considered to be statistically significant, and the results were compared by performing ANOVA post hoc test and Kruskal Wallis Test.

RESULTS

Followed by immunohistochemical analysis, various scores (total, composite and final composite) of AM, DCs, OKCs and OOCs were compared statistically [Table 1] and revealed statistical significance was seen. When the final composite scores were compared among all the lesions, statistical difference was found in most of the groups with AMs showing a greater mean value followed by OKCs, OOCs and DCs [Table 2]. Hence, the final composite score was used for evaluation and comparison of PDPN expression among odontogenic tumours and cysts due to heterogeneity in the staining expression among the lesions.

DISCUSSION

A multitude of odontogenic tumours and cysts arise through aberration from the normal process of odontogenesis involving epithelial, ectomesenchymal or mesenchymal components of the tooth-forming apparatus or their remnants entrapped either within the jawbones or into the adjacent soft tissues reflecting their complex multiformity.[12] Among odontogenic cysts, OKC had drawn the attention of oral pathologists and dental community due to its complex behaviour and multiformity. It was called 'primordial cyst' back then ascertaining to its origin from the primitive odontogenic cells of the dental lamina but over time it was recognized as a distinct entity and the term primordial cyst was discontinued for its ambiguity and uncertain behaviour. It was dated back to 1980s when Ahlfors *et al.* considered OKC as a benign cystic tumour due to high recurrence rate and its growth mechanisms.[13] Later then in 2005, WHO reclassified OKC to KCOT as a benign odontogenic neoplasm and not as a cyst. According to research, OKC exhibits high reactivity to Ki67 index, AgNOR, CD44v6, VEGF, MMP-9 immune profile, which stands as markers of local invasiveness and certain molecular genetic alterations that are associated with other neoplasms as well.[14] Even though it has been reclassified as KCOT in 2005, still many authors in later years continued to use the name of OKC due to the unavailability of sufficient evidence-based data to justify the reclassification but later in 2017, it was reclassified under odontogenic cysts by WHO.[15] Unfortunately as there is lack of research in other odontogenic cysts, it is imperative to determine whether these alterations are unique to the OKC. The clinical, radiographic features, clinical behaviour, few of the molecular properties of OKC are similar to those of AM but the exact local invasive phenomenon in OKC is not elucidated yet.[16] Though the occurrence of DC is more common in odontogenic region, the fact of great clinical significance is its potential to transform into a malignant lesion de novo or due to its long-standing presence.[17] Hence, we aimed to compare the mechanism of molecular changes and proliferative potential in odontogenic cysts and odontogenic tumour. As per research, PDPN is one such marker preferably used as potential marker for predicting the risk of oral cancer in pre-malignant lesion like oral leukoplakia and served as a lymphatic endothelial marker in OSCCs, reflecting its potential role in modulation of actin cytoskeleton, thereby leading to tumorigenesis, tumour invasion and metastasis.[18] Recent literature also provided evidence about its expression in odontogenic tissues like secretory ameloblasts, developing and mature odontoblasts, Tomes' fibres, pulp cells and neoplastic lesions originating from odontogenic apparatus. Sawa *et al.* suggested an association of podoplanin in cellular proliferative activity due to its expression in tooth germ, which is present in cells with high mitotic activity such as in dental lamina, terminal portion of Hertwig's sheath and pre-ameloblasts.[19] Findings from the present study demonstrated that the expression of PDPN was more evident in AMs than in DCs. The studies conducted by Gonzalez-Alva *et al.*, and Zustin *et al.*, where predominant heterogeneous expression was seen on the surfaces of peripheral columnar cells and decreased expression in stellate reticulum-like central

Table 1: Comparison of different scores for all the groups (ANOVA post-hoc test)

	Dentigerous Cyst DC		Ameloblastoma AM		Odontogenic Keratocyst OKC		Orthokeratinized Odontogenic Cyst OOC		*P*	Post-hoc test
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Total score	1.90	3.14	35.90	15.60	25.49	14.65	13.54	12.50	<0.001; Sig	AM, OKC , OOC > DC AM > DC, OOC OKC > D, OOC
Composite score	0	0	2.00	0.94	1.57	0.77	0.77	0.73	<0.001; Sig	AM, OKC, OOC > DC AM, OKC > OOC
Final Composite Score	0.30	0.48	7.10	3.11	4.97	2.85	2.54	2.37	<0.001; Sig	AM >OKC > OOC > DC

*Statistical Significance was found when different scores were compared among various groups of lesions. (Significant $P<0.05$)

Table 2: Mean comparison of composite score between groups (Kruskal Wallis Test)

Groups	Mean	SD	Test statistic	*P*
DC	0.0000	0.00000	31.116	0.000*
AM	2.0000	0.94281		
KCOT	1.5676	0.76524		
OOC	0.7692	0.72501		
Post HOC analysis				
Comparison between		**Mean difference**		*P*
DC	AM	-2.00000		0.000*
	KCOT	-1.56757		0.000*
	OOC	-0.76923		0.298
AM	KCOT	0.43243		1.000
	OOC	1.23077		0.015*
KCOT	OOC	0.79834		0.053

(Significant $P<0.05$). *On comparison of Composite Score among various groups of lesions. *DC Vs AM & KCOT-Statistical Significance was found. *AM Vs OOC- Statistical Significance was found

cells.[20,21] Similar findings were observed in the present study except for the varied expression in central cells among the variants of AM, with increased expression in plexiform type [Figure 1] and focal positivity to completely negative expression in follicular variant. This particular variation in PDPN expression might be due to the resemblance of plexiform variant to the tooth germ in the dental lamina stage, when the differentiation process of the odontogenic epithelium has not initiated which may reflect the homogeneity of PDPN expression found in these tumours. Another possible explanation for under expression of PDPN may be due to low mitotic activity in central stellate cells, keratinized acanthomatous cells or granular cells.[21] Evidence from studies also showed that increased proliferative activity within odontogenic cells increases PDPN expression, thereby suggesting role of this protein in cell proliferation and local invasion.[20,21] Moreover, Gonzalez-Alva *et al*.[20] also proposed that this pattern of distribution of PDPN immunostaining according to histological variants of AMs may also be helpful to the classification of odontogenic tumours. Hence, the present study compared PDPN expression between AMs and OKCs and revealed no statistically significant difference in expression suggesting that PDPN expression in OKCs is on par with that of AMs. In this study, OKCs showed strong expression in one-fourth of cases, moderate and weak expression with nearly equal frequency followed by negligible negative expression. These findings were contradictory to the results of Okamoto *et al*., where strong expression was seen in majority of their cases followed by weak to moderate and negative expression in few cases.[9] This difference in intensity of PDPN expression might be attributed to contrast in evaluation methodology, where only intensity was recorded in later study, but in the present study, both intensity and percentage of cells were evaluated. The present study revealed PDPN immunoreactivity in

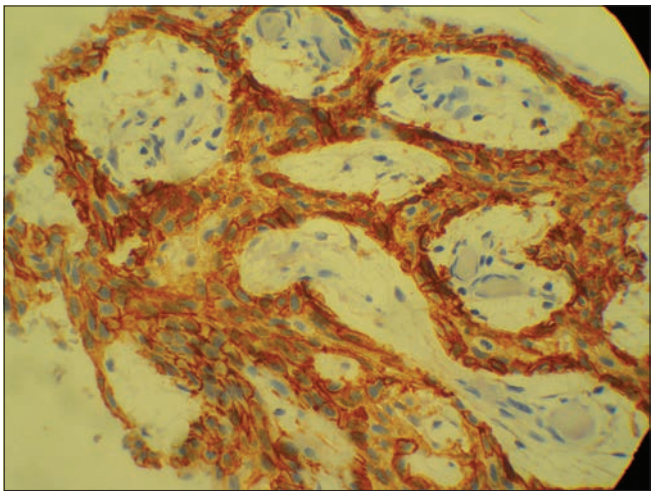

Figure 1: Immunohistochemically stained image shows plexiform ameloblastoma with strong PDPN expression in peripheral epithelial cells and negative to focal positive expression in central cells (Podoplanin marker 10x40X)

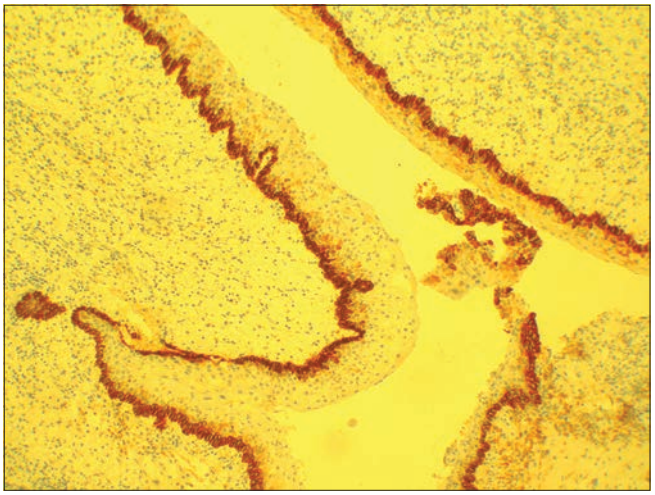

Figure 2: Immunohistochemically stained image shows OKC with positive PDPN expression in basal cell hamartias (Podoplanin marker 10x 10X)

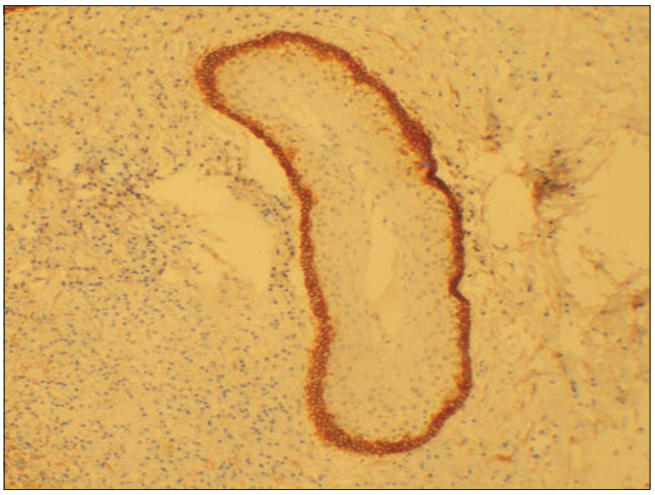

Figure 3: Immunohistochemically stained image shows OKC with positive PDPN expression in the peripheral cells of the daughter cyst (Podoplanin marker 10x 40X)

basal cell hamartias [Figure 2], epithelial cell rests and peripheral cells of daughter cysts [Figure 3], which was corroborating with studies done by Gonzalez-Alva *et al*.,[20] Zustin *et al*.,[21] Singhal *et al*.[22] Our results also reveal PDPN expression in multiple epithelial strata of OKCs ranging from positivity in only basal cells in few cases, suprabasal (2 to 3) layers in majority cases, followed by cases showing positivity till upper suprabasal layers also extending to entire thickness of lining epithelium except superficial parakeratinized layer [Figure 4]. Moreover, gradual decrease in the staining intensity from the basal to superficial layers and shift in membranous to cytoplasmic expression was noticed. To the best of our knowledge, the present study is unique demonstrating the expression of PDPN in terms of strata of lining epithelium. Friedrich and co-workers, in their study of OKCs demonstrated strong expression only in basal layer which is in contrast to the present study.[23] Moreover, its expression in peripheral cells of AMs speculated the possibility of PDPN may be associated with remodelling of cytoskeleton of neoplastic cells. Since PDPN is unable to bind itself to the actin filaments, the ezrin, radixin and myosin (ERM) membrane proteins aid in the organization of cytoskeleton, thereby promoting linkage of filamentous actin to the apical membrane of the cells. It was also found that possible association of PDPN and ezrin might mediate the cellular motility by developing filopodia-like protrusions in process of tumour invasion via epithelial-mesenchymal transition.[24] In comparison between OOCs to AMs, the PDPN expression was statistically significant as the AMs are the locally aggressive benign odontogenic neoplasms showed higher expression. In comparison between the OKCs and OOCs, strong PDPN expression was showed in OKCs and none seen among OOCs. Moderate expression was observed in most of the cases OKCs but only few cases of the OOCs, with almost similar frequency of weakly expressed cases. Statistical analysis also revealed significant difference in the PDPN over expression between these two variants, suggesting that OOCs are comparatively less aggressive lesions than OKCs. Further evaluation of extent of PDPN expression in lining epithelium of OKCs and OOCs showed stronger and higher expression extending even up to the upper suprabasal layers seen in OKCs, whereas only two cases of OOCs showed single suprabasal layer and six cases with linear expression in basal cells. Okamoto *et al*. and Andriana dos Santos Caestino *et al*. also compared the PDPN expression in OKCs and OOCs qualitatively and concluded that the PDPN was higher in OKCs than in OOCs, probably because OKCs have more of an aggressive nature with high proliferative, mitotic rate and local invasiveness which was similar to the present study.[9,25] Keratin profiling studies in OOCs have indicated alteration in differentiation mechanisms of keratinocytes with constant process of keratinization in OOC when compared to OKC.[24] As proposed by Sawa *et al*. and Imaizumi *et al*., the podoplanin-negative structures in odontogenesis, such as in ameloblasts in crown stage and odontoblasts at root formation stage, might represent the matured quiescent cells.[19,26] Thus, this pattern of expression in OOC might reflect more mature cells in OOC than OKC as also described in relation to central cells of AM. In the comparison of expression of PDPN between the DCs and OOCs, though both are developmental cysts, there was a significant difference in the over expression in OOC but both had showed a similar kind of response in the presence of inflammation [Figures 5 and 6] which was also reported

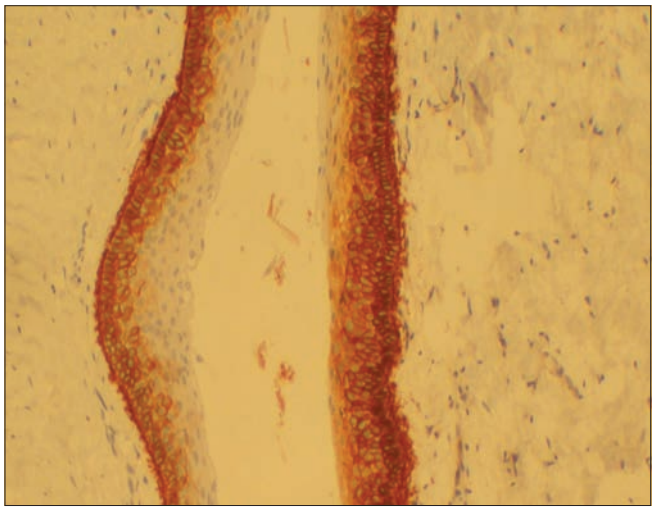

Figure 4: Immunohistochemically stained image shows positive PDPN expression multiple layers of cystic lining epithelium (Podoplanin marker 10x 40X)

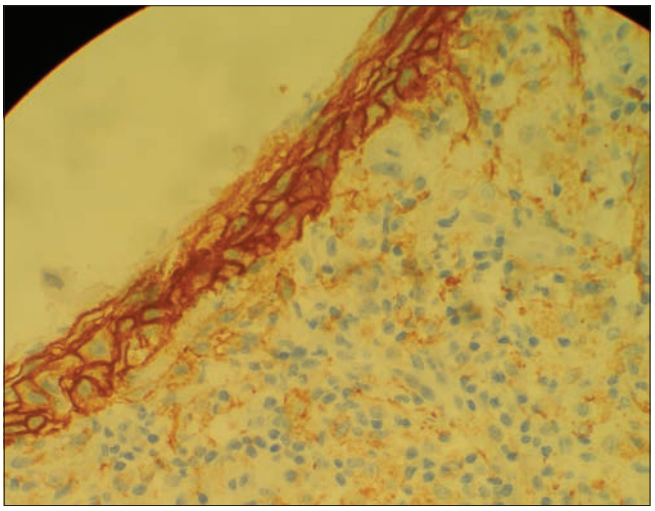

Figure 5: Immunohistochemically stained image shows dentigerous cyst lining with strong expression for PDPN in the presence of severe juxtaepithelial inflammation (Podoplanin marker 10×10X)

by Okamoto *et al*.[9] With strong expression in areas of inflammation, it indicates a probable role of inflammatory reaction in expansion and growth of cysts. No PDPN expression in non-inflammatory areas of cystic epithelium was evident in DCs [Figure 7], and these findings were in line with studies of Gonzalez-Alva *et al*., and Okamoto *et al*.[9,20] In the present study, the expression by the epithelium varied according to the amount of inflammatory changes in connective tissue wall of DCs, OOCs and OKCs and this expression was found to be extended up to the suprabasal cell layers and areas with mild to moderate inflammation showed weak to moderate PDPN expression limiting to basal cells. These findings were contrary to other studies, where PDPN expression is restricted to basal layers even in areas of severe inflammation, thereby indicating that morphologic changes such as regeneration and reparative process may have an impact over proliferative activity of lining epithelium.[27,28] In the present study, there is a rare finding observed satellite cysts associated with OOC being positive for the marker reflecting the active cellular proliferative mechanism in daughter cysts [Figure 8]. As mentioned earlier that association between PDPN and ezrin and their role in regulating actin cytoskeleton dynamics, interaction with CD44 and its role in cellular migration and adhesion, CD9 in tumour suppression and from all these findings it is understood that PDPN may have many associations with other cellular proteins for its expression. These interactions further need to be explored for better understanding of the pathophysiology. Agaram NP *et al*. observed that a significant number of OKCs showed clonal loss of heterozygosity of tumour suppressor genes like p16, p53, PTCH.[29] Hence, they also supported the hypothesis that OKCs are neoplastic rather than developmental in origin. Our observation of strong expression of podoplanin in basal and suprabasal layers in KCOTs suggests the proliferative activity of these cells, increasing their potential for intrinsic growth and making them locally invasive and aggressive. We, therefore, believe that podoplanin probably plays a role along with other proteins and growth factors, in increasing the proliferative activity of the lining epithelium in odontogenic cyst and tumours. Hence, we suggest that podoplanin can be used as a potential proliferative marker to indicate the differential behaviour and proliferative potential of odontogenic cysts and tumours in our cohort, and it may be a useful adjunct to measure the local aggressive potential and plan appropriate treatment regime.

CONCLUSION

The present study contributes to the significant association of PDPN expression with local invasiveness of OKC

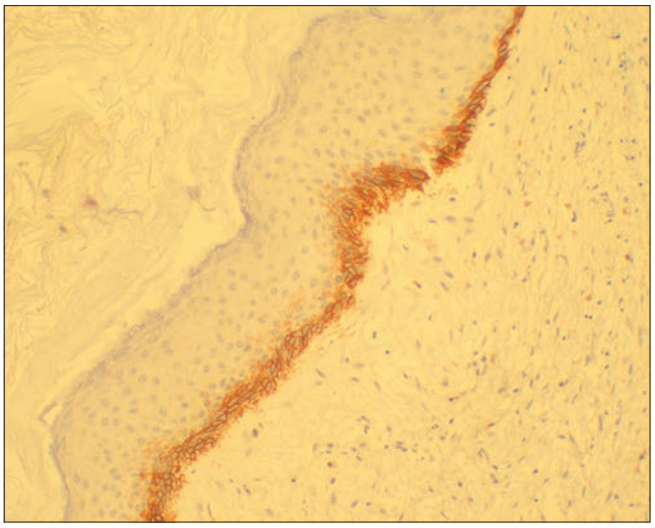

Figure 6: Immunohistochemically stained image shows OOC showing increase in the extent of PDPN expression in lining epithelium in the presence of severe juxtaepithelial inflammation (Podoplanin marker 10x4X)

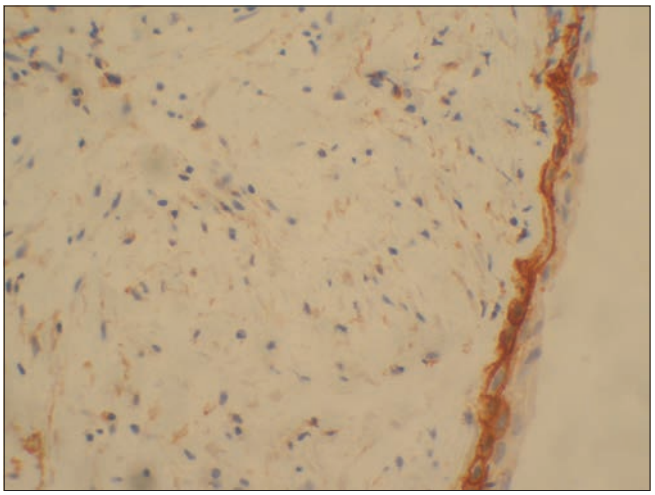

Figure 7: Immunohistochemically stained image shows dentigerous cyst lining with diminished expression for PDPN in the absence of juxtaepithelial inflammation (Podoplanin marker 10x10X)

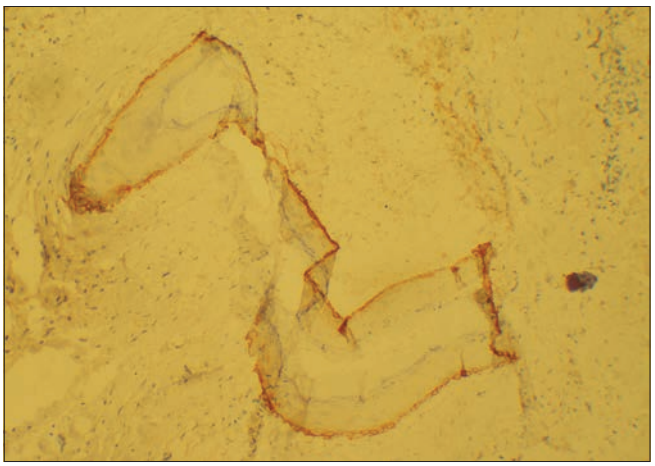

Figure 8: Immunohistochemically stained image shows OOC with PDPN expression in the peripheral cells of daughter cyst (Podoplanin marker 10x10X)

previously considered as KCOT, which was on par with AMs, and there was statistically significant difference in the PDPN overexpression in OKCs compared to OOCs and DCs reflecting their differential aggressiveness. This suggests that the podoplanin influences the proliferative activity of these cells increasing their potential for intrinsic growth and making them locally invasive and aggressive. Nevertheless, it has been topic of dispute within the field of dentistry regarding the true nature of OKCs, and further studies should be carried out along with its downstream and upstream regulators to establish the exact molecular mechanism for the local invasiveness and also to evaluate potential of podoplanin as a composite marker of maturation, proliferation, cell motility and aggressiveness.

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Department of Oral Pathology and Microbiology, Panineeya Institute of Dental Sciences Sciences and Research Centre, Hyderabad.

Department of Oral and Maxillofacial Surgery, Panineeya Institute of Dental Sciences Sciences and Research Centre, Hyderabad.

Conflicts of interest

There are no conflicts of interest.

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