Original Article

Comparative immunohistochemical analysis of WT-1, Syndecan and Snail in Ameloblastoma and odontogenic keratocyst: A retrospective study

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Abstract

Background: The purpose of this experimental study was to evaluate and compare the degree of expression of Wilm's Tumor Gene-1 (WT-1), Syndecan (CD 138) and Snail in Ameloblastoma and odontogenic keratocyst (OKC) and to analyse their potential role in pathogenesis.

Methods and Material: Immunohistochemical analysis was performed to evaluate WT-1, Syndecan and Snail expression in Ameloblastoma ($n = 20$) and OKC ($n = 20$). Topographical immunoexpression pattern of Ameloblast-like cells, Stellate Reticulum-like cells in Ameloblastoma and basal layer as well as suprabasal layer of cells of OKC were also compared. The results obtained were subjected to ANOVA test and Tukey HSD test through SPSS software 20.0 for Microsoft Windows.

Results: WT-1 and Snail overexpression was seen in both Ameloblastoma and OKCs. Syndecan, responsible for maintaining normal cellular morphology, cell–cell adhesion and differentiation was significantly downregulated in both the lesions. The Ameloblasts-like cells and the basal cells showed significantly higher immunopositivity for WT-1 and Syndecan as compared to that of basal cells. An inverse relation was noted for Snail protein. The ANOVA test predicted a statistically significant difference of expression across the lesions with a P value <0.0001 for Syndecan and Snail.

Conclusions: The under-expression of epithelial membrane protein Syndecan-1 and upregulation of EMT transcription factor Snail can promote local invasion and is indicative of poor prognosis of these lesions. The overexpression of WT-1 results in tumorigenesis, proliferation and localized aggressiveness of Ameloblastoma and intrabony growth of OKC. Further investigation on the biologic behaviour of OKC is still recommended to arrive at more specific conclusions regarding its nature.

Keywords: Ameloblastoma, molecular pathogenesis, odontogenic keratocysts, Snail, Syndecan, WT-1

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INTRODUCTION

Odontogenesis or tooth development is the result of numerous genetic and epigenetic events that develops from interaction of epithelial cells with the ectomesenchymal cells. Disruptions in the control mechanisms of these orderly events can result in an array of odontogenic cysts and tumours.[1,2] Ameloblastoma is one of the most frequently occurring benign epithelial odontogenic tumor after odontomas. It is considered as a benign but locally aggressive neoplasm with variable clinico-pathologic expression, whose controversial aetiopathogenesis has baffled scholars and clinicians alike.[3] Odontogenic keratocyst (OKC) is a controversial lesion that was reclassified as a tumor with the name "keratocystic odontogenic tumor" in 2005 due to its locally aggressive behavior, relatively high recurrence rate, and molecular mechanisms involved in its development and progression. In the latest 2017 WHO classification, the lack of evidence supporting the neoplastic nature of OKC was taken into account and the assumption that this lesion actually does not constitute a tumor was discussed.[4] OKCs are known to show locally aggressive behavior with a tendency to recur following excision.[5]

The molecular mechanisms that are involved in the local invasion of this aggressive lesions are currently unknown, despite the fact that various theories have been proposed in this respect.[6,7] There are some proteins responsible for maintaining the intracellular adhesion as well as their adhesion with their extracellular matrix (ECM). Studies have shown that a reduction in its expression might be related with the ability of epithelial invasion to the capsule and adjacent structures.[8] Among these, CD138 (Syndecan), a protein encoded by the SDC1 gene, is in charge of mediating the adhesion between cells and between the cells and the ECM. It acts as an integral part of the membrane proteins which participate in cell proliferation, cell migration and cell-ECM interactions.[8,9] Only a handful of literature have determined the altered expression of Syndecan in Ameloblastomas and OKCs.[10,11]

One of the important phenomenon involved in progression and metastasis of various neoplasms is epithelial-mesenchymal transition.[12] This process is mediated by EMT-inducing transcription factors (EMT-TFs), such as Twist, Snail, and ZEB families.[13] Recent immunohistochemical studies on Ameloblastoma samples have identified the expression of Snail, suggesting its role in the progression and development of Ameloblastoma.[14] None of the studies using Snail have been conducted in OKC yet.

Numerous oncogenes along with their immunotherapy have been identified that are responsible for the aggressive nature in Ameloblastomas and OKCs.[6,15] Wilms Tumor Gene-1 (WT-1) is one of such gene which was originally described as a tumor suppressor of gene, but subsequent research have indicated that it also plays an oncologic role in variety of solid tumors.[16] Also, many clinical trials examining WT1 peptide-based cancer immunotherapy on various types of tumors have proven its clinical efficacy and safety.[17] To date, none of the studies have investigated WT-1 expression in OKCs.

Furthermore, no studies have compared the expression patterns of the aforementioned proteins to see if there is a correlation between them. As a result, the current study was designed to assess and correlate the expression patterns of Wilm's Tumor gene-1 (WT-1), Syndecan (CD-138) and Snail in Ameloblastoma and OKC.

SUBJECTS AND METHODS

Patients and tissue samples

The present study was conducted using 20 formalin-fixed paraffin-embedded (FFPE) blocks, each of previously diagnosed cases of Ameloblastoma and OKC, from the archives of the Department of Oral and Maxillofacial Pathology and Microbiology, Kalinga Institute of Dental Sciences, Bhubaneswar, Odisha. Biopsies with tissue insufficient for histopathological evaluation, questionable diagnosis and autolyzed samples were excluded from the study. Approval from the Institutional Ethics Committee was obtained (ref no. KIMS/KIIT/IEC/200/2018 dated 28/09/2018) before the commencement of the study.

Immunohistochemistry with WT-1, Syndecan, Snail

- Three tissue sections of three-micron thickness were obtained from each block and were placed on poly-l-lysine-coated slides for immunohistochemical analyses. Immunohistochemistry was performed using antibodies WT-1 (Pre-diluted, pathnsitu), Syndecan (Concentrated, Novus USA), Snail (Concentrated, Novus USA). The Syndecan and Snail were subjected to IHC after dilution to 1:200 ratio. Ovarian carcinoma, multiple myeloma, gall bladder carcinoma was taken as positive control for WT-1, CD-138, and Snail, respectively.

Assessment of immunoscoring

Assessment was done by an individual observer in accordance to the morphometric analysis conducted by Bologna-Molina *et al.* 2017.[18,19] Five high power fields were observed for each of the slides stained,

respectively, with WT-1, Syndecan and Snail where 1 field contained nearly about 50 cells. Topographical analysis of immunoexpression pattern of the cell types were designated as AM for Ameloblast-like cells and SR for Stellate Reticulum-like cells in Ameloblastoma cases. In OKC samples, basal layer of cells were designated as BS and superficial or suprabasal layer of cells as SP.

Statistics

The results obtained were subjected to ANOVA test and Tukey HSD test using software SPSS (IBM, SPSS Statistics, version 20). The confidential interval was kept as 95% and *P* value was considered significant if $P < 0.05$.

RESULTS

As per the procedure laid down in the section "Materials and Methods", 20 cases of Ameloblastoma and OKC were considered and stained individually for WT-1, Syndecan (CD-138) and Snail [Figures 1 and 2].

In Ameloblastoma, the expression of ameloblast-like cells were seen to be the highest in case of Snail and WT-1 with 55.2% and 51.6%, respectively, followed by that of stellate reticulum-like cells for WT-1 and Snail at 19.3% and 16.5%, respectively. The lowest expressions were seen for Syndecan with 12.6% for ameloblast-like cells and 1.9% for stellate reticulum-like cells [Figure 3].

On the other hand, Figure 4 depicts that the highest expression was seen in the basal cells of OKCs for WT-1 at 45.3%, followed by that of the superficial cells for Snail at 28.4%. Basal cells for Syndecan and Snail showed 18.2% and 11.4%, respectively, with the lowest expressions seen in superficial cells for WT-1 and Syndecan at 6.5% and 3.4%, respectively.

Figure 5 depicts the expression of WT-1, Syndecan-1 and Snail in different types of cell. The ANOVA test predicted a statistically significant difference across the groups with a *P* value of 0.001.

For multiple comparisons, a Tukey HSD test was performed for each antibodies to identify the honestly significant difference when one group is compared to another [Tables 1–3]. It was noted that for WT-1, statistically significant difference was present between the expression levels when compared between four different types of cells. But the difference in the expression level between the Ameloblast-like cells and basal cells were not statistically significant [Table 1]. For Syndecan expression, no statistically significant differences was found for AM cells when compared to all other cell types [Table 2], and for Snail expression, statistically significant difference was found between all the cell types. But no statistically significant difference was noted in the expression levels of Snail for BS cells when compared to SR cells [Table 3].

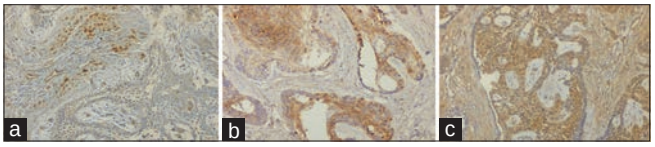

Figure 1: Immunostaining of WT-1 (a), Syndecan (b) and Snail (c) in cases of Ameloblastoma

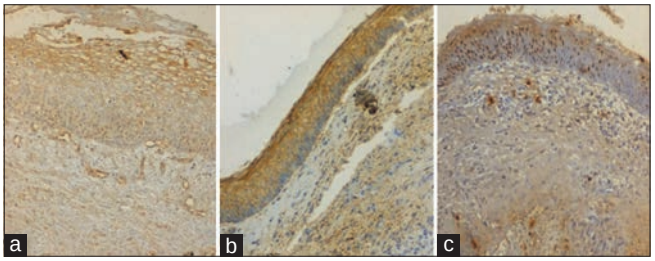

Figure 2: Immunostaining of WT-1 (a), Syndecan (b) and Snail (c) in cases of odontogenic keratocysts

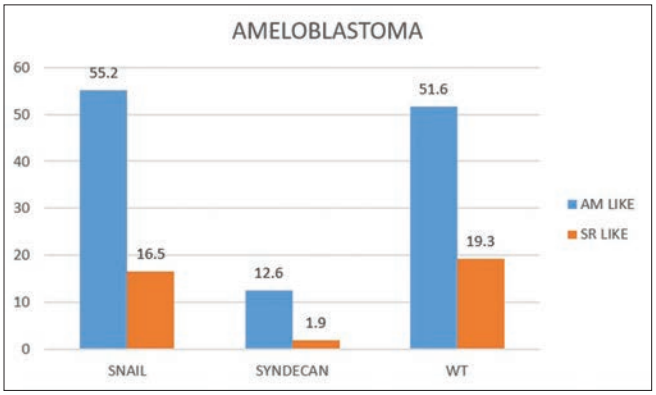

Figure 3: Ameloblastoma cell types as compared between Snail, Syndecan and WT-1

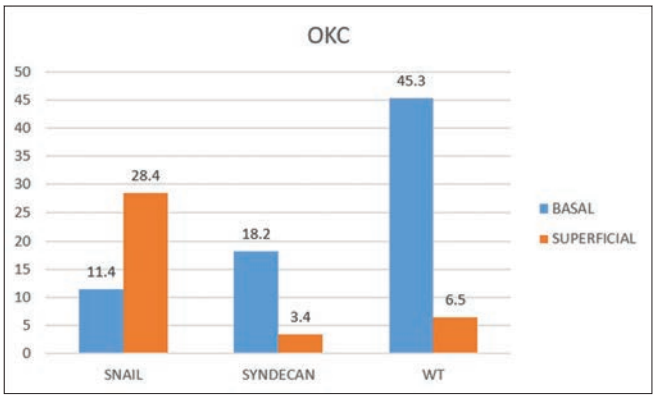

Figure 4: Odontogenic keratocyst cell types as compared between Snail, Syndecan and WT-1

Table 1: Difference of expression of WT-1 across various cell types. Tukey HSD (*P*<0.05) statistically significant

Multiple comparisons								
Dependent variable: PERCENT								
Stain		(I) Cell	(J) Cell	Mean difference (I-J)	Std. error	Sig	95% Confidence interval	
							Lower bound	Upper bound
WT-1	Tukey HSD	AM	SR	32.300*	5.170	<0.0001*	18.72	45.88
			B	6.300	5.170	0.617	−7.28	19.88
			S	45.100*	5.170	<0.0001*	31.52	58.68
		SR	AM	−32.300*	5.170	<0.0001*	−45.88	−18.72
			BS	−26.000*	5.170	<0.0001*	−39.58	−12.42
			SP	12.800	5.170	0.072	−.78	26.38
		BS	AM	−6.300	5.170	0.617	−19.88	7.28
			SR	26.000*	5.170	<0.0001*	12.42	39.58
			SP	38.800*	5.170	<0.0001*	25.22	52.38
		SP	AM	−45.100*	5.170	<0.0001*	−58.68	−31.52
			SR	−12.800	5.170	0.072	−26.38	0.78
			BS	−38.800*	5.170	<0.0001*	−52.38	−25.22

*The mean difference is significant at the 0.05 level

Table 2: Difference of expression of Syndecan-1 across various cell types. Tukey HSD (*P*<0.05) statistically significant

Multiple comparisons								
Dependent variable: PERCENT								
Stain		(I) Cell	(J) Cell	Mean difference (I-J)	Std. error	Sig	95% Confidence interval	
							Lower bound	Upper bound
Syndecan	Tukey HSD	AM	SR	10.700	4.531	0.093	−1.20	22.60
			BS	−5.600	4.531	0.606	−17.50	6.30
			SP	9.200	4.531	0.186	−2.70	21.10
		SR	AM	−10.700	4.531	0.093	−22.60	1.20
			BS	−16.300*	4.531	0.003*	−28.20	−4.40
			SP	−1.500	4.531	0.987	−13.40	10.40
		BA	AM	5.600	4.531	0.606	−6.30	17.50
			SR	16.300*	4.531	0.003*	4.40	28.20
			SP	14.800*	4.531	0.009*	2.90	26.70
		SP	AM	−9.200	4.531	0.186	−21.10	2.70
			SR	1.500	4.531	0.987	−10.40	13.40
			BS	−14.800*	4.531	0.009*	−26.70	−2.90

*Statistically significant. The mean difference is significant at the 0.05 level

Table 3: Difference of expression of Snail across various cell types. Tukey HSD (*P*<0.05) statistically significant

Multiple comparisons among the cells for Snail stain								
Dependent variable: PERCENT								
Stain		(I) Cell	(J) Cell	Mean difference (I-J)	Std. error	Sig	95% Confidence interval	
							Lower bound	Upper bound
SNAIL	Tukey HSD	AM	SR	38.700*	3.900	<0.0001*	28.46	48.94
			BS	43.800*	3.900	<0.0001*	33.56	54.04
			SP	26.800*	3.900	<0.0001*	16.56	37.04
		SR	AM	−38.700*	3.900	<0.0001*	−48.94	−28.46
			BS	5.100	3.900	0.561	−5.14	15.34
			SP	−11.900*	3.900	0.016*	−22.14	−1.66
		BS	AM	−43.800*	3.900	<0.0001*	−54.04	−33.56
			SR	−5.100	3.900	0.561	−15.34	5.14
			SP	−17.000*	3.900	<0.0001*	−27.24	−6.76
		SP	AM	−26.800*	3.900	<0.0001*	−37.04	−16.56
			SR	11.900*	3.900	0.016*	1.66	22.14
			BS	17.000*	3.900	<0.0001*	6.76	27.24

*Statistically significant. The mean difference is significant at the 0.05 level

We also compared the expression levels of WT-1, Syndecan and Snail between Ameloblastoma and OKC [Table 4]. The ANOVA test predicted a statistically significant difference of expression across the lesions with a *P* value <0.0001 for Syndecan and Snail.

DISCUSSION

Odontogenic cysts and tumors are a diverse group of lesions spanning from hamartomatous or non-neoplastic tissue proliferations to benign and malignant neoplasms with

Table 4: Comparison of Ameloblastoma and OKC across the expression levels of WT-1, Syndecan-1 and Snail. ANOVA test ($P \leq 0.05$) statistically significant

Stain	Lesion	*n*	Mean	Std. deviation	Std. error	95% Confidence interval for mean		Min	Max	*F*	*P*
						Lower bound	Upper bound				
Snail	AM	20	35.85	8.312	1.859	31.96	39.74	20	51	39.695	<0.0001*
	OKC	20	20.30	7.263	1.624	16.90	23.70	6	34		
	Total	40	28.08	11.016	1.742	24.55	31.60	6	51		
Syndecan-1	AM	20	7.25	9.514	2.127	2.80	11.70	0	40	27.504	<0.0001*
	OKC	20	25.90	12.744	2.850	19.94	31.86	3	42		
	Total	40	16.58	14.574	2.304	11.91	21.24	0	42		
WT-1	AM	20	35.45	8.519	1.905	31.46	39.44	20	50	7.762	0.008*
	OKC	20	25.90	12.744	2.850	19.94	31.86	3	42		
	Total	40	30.68	11.742	1.857	26.92	34.43	3	50		

*Statistically significant

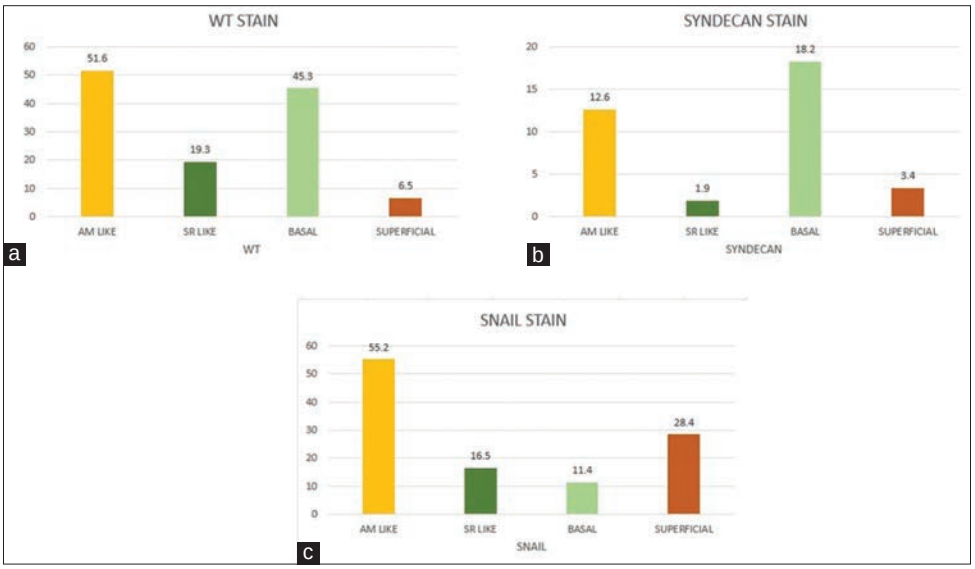

Figure 5: Percentage scores across various types of cells for WT-1 (a), Syndecan (b), Snail (c)

unpredictable aggressiveness and invasiveness. They can originate from the odontogenic apparatus and its remnants at any stage of life in an individual.[2,20] The cellular changes involved in proliferation, differentiation, senescence, and local invasion of these lesions can be identified through immunohistochemical studies. In this study, WT-1, Syndecan and Snail were analyzed and compared in Ameloblastomas and OKCs which provides an insight to their tumoriogenesis.

The Basal cell layer of the oral epithelium has been rightfully regarded as a potential source of odontogenic cysts and tumors but without substantial evidence.[21,22] Ameloblastoma, microscopically, resembles enamel organ of a developing tooth which lacks dental hard tissue formation. It has two types of cells with different proliferative activity, and these activities are based on their cytological pattern and histological variant. The peripheral ameloblast-like cells are known for their anti-apoptotic character, while the central stellate reticulum-like cells are known for their pro-apoptotic activity.[23] In our study, all the three aforementioned markers are upregulated in AM-like cells when compared to SR-like cells. These suggests that the oncogenesis, loss of cell adhesion and gain of migratory ability causing local invasion is predominantly due to the peripheral AM-like cells. The marked underexpression in SR cells might indicate their limited role in inducing oncogenesis and defined role in pro-apoptotic activity of this neoplasm. Compared to WT-1 and Snail, the expression of Syndecan has shown low immunoreactivity. Siar *et al.*were among the earliest to demonstrate overexpression of Snail in 94% of Ameloblastoma.[14] Studies have shown that under-expression of epithelial membrane protein Syndecan and upregulation of EMT transcription factor Snail can promote local invasion and is indicative of poor prognosis of these lesions.[10] These findings are in concurrent with our study.

In a study by Bologna-Molina *et al.*[18], it was seen that all the tooth germs were negative for WT-1, whereas more than half of the Ameloblastoma tissue samples showed WT-1 overexpression. These findings suggests that WT-1 might play an oncogenic role in these lesions. Syndecan immunostaining was also strongly depicted in stromal cells,

ECM and basement membranes of Ameloblastomas in various studies.[10] Immunohistochemistry have demonstrated decreased expression of Syndecan in solid Ameloblastomas and supports the increased localized aggressive behaviour of solid subtype over unicystic variant. Numerous studies have concluded that the role of Syndecan is to regulate cellular morphology, adhesion and differentiation, hence its downregulation may lead to localized aggressive behaviour, uncontrolled proliferation and potential invasiveness.[23] Hammad *et al*.[24] on the other hand refuted this by immunohistochemical analyses that the expression of Syndecan in Ameloblastoma and OKC as inconclusive under-reflecting the biologic behaviour of the lesions.

Previous WHO classification of head and neck tumors have aroused extensive discussion around the nature of OKCs.[2] The expression pattern of WT-1 and Snail in OKC has not yet been previously documented. This study may throw light on the pathogenesis of this highly recurrent lesion. Otaibi *et al*.[10] had shown that Syndecan is expressed in upper layers of keratinocytes in 92.3% cases of OKC. The satellite cysts did not differ from their main cysts in the expression of Syndecan, while the epithelial budding showed decreased Syndecan expression. On contrary, in our study, 18.2% of basal cells and only 3.4% of SP cells of OKC were immunopositive. This was in accordance with a study where the distribution patterns of Syndecan in the epithelia of DC, OKC, and AB were compared and no significant differences was seen between the three study groups, although the expression was lower in AB and OKC compared to DC.[10]

In our study, WT-1 was highly expressed in the basal cells of OKC. The basal budding of the epithelium lining the parent cyst produces basal cell hamartias which multiply and form satellite cysts. According to research, the remnants of these satellite cysts are responsible for the recurrence of OKCs.[5] These findings highlights a correlation between basal cell, recurrence and and WT-1 overexpression. Hence targeting WT-1 genes may help to reduce the recurrence of this aggressive cyst.

For the first time, we demonstrated overexpression of Snail in OKC. But to our surprise, it was noted that the SP cells were highly immunopositive compared to that of basal cells. Overexpression of Snail in both subsets suggests that this molecule is most likely the prototype transcription factor involved in inducing EMT.[13] Snail may be a part of a larger comprehensive pathway, yet to be uncovered, whose principal role has been seen to promote both invasion as well as aggressiveness of Ameloblastomas as opined by various authors.[25] The experimental evidences found in this study highlights the highest positivity for Snail antibody in Ameloblastoma when compared to all the other markers. In OKC, a marked decrease in expression was seen suggesting that the lesion is less aggressive than Ameloblastoma. Snail may affect the epithelial-mesenchymal transition in Ameloblastic carcinoma and be involved in recurrence of Ameloblastoma.[14] Thus, it may be promising proliferative as well as prognostic marker for Ameloblastoma.

When the comparative expression of WT-1 in the four cell types mentioned above are carried out, the AM cells showed the highest immunoreactivity. BA cells of OKC showed immunoreactivity comparable to AM cells as evident by Tukey HSD test which showed no statistically significant difference between the expression level of WT-1. The SR cells of Ameloblastoma and SP Cells of OKC showed less immunoreactivity in comparison to AM and BA cells. WT-1 expression was seen mostly in the nucleus and nucleolus of ameloblast-like cells. The basal cells of OKC also showed a significant number of nuclear and nucleolar staining with mild to moderate intensity. This is in line with previous evidences where ameloblast-like cells show very strong positivity towards WT-1 expression with only mild expression in stellate reticulum-like cells.[22] However the expression of WT-1 was cytoplasmic in the one study[22], whereas our study finds experimental evidences of both nuclear and nucleolar expressions along with cytoplasmic expression. It may be opined that the promotion in local invasiveness of Ameloblastoma as well as the intrabony growth and proliferation of basal cells and superficial cells of OKC may be attributed to the WT-1 expression.

Syndecan was found to have the least expression in both Ameloblastoma and OKC, when compared to WT-1 and Snail, which is in line with the aforementioned results from experimental evidences.[10] But when compared across cell types, the basal cells of OKC and ameloblast-like cells in Ameloblastoma showed the greatest expression with very weak expression in case of both superficial cells and stellate reticulum-like cells.

Snail upregulates the MMP activity, resulting in ECM degradation and thereby promoting invasion.[14] In our study, AM cells showed highest Snail immunoreactivity followed by SP cells of OKC, followed by SR cells of Ameloblastoma. The basal cells of OKC showed least expression of Snail which implies that this particular cell presents another molecular pathway responsible for conserving its neoplastic behavior. Also higher immmunopositivity of Snail of superficial layers indicates the retained progenitor characteristics of tooth bud presenting an EMT-like phenotype. These difference in pattern of expression is still unclear, and further studies are required for a conclusive explanation.

From the available scientific literature, it is seen that the overexpression of WT-1 and Snail in Ameloblastoma and OKC contribute to its proliferative activity and local aggressiveness.[26] The downregulation of Syndecan deviates the tissue from normalcy and in lieu promotes the loss of cell–cell adhesion, uncontrolled proliferation and local aggressiveness and invasiveness of Ameloblastoma and OKC.[10]

Several notable findings have also been unearthed in the course of this immunohistochemical analyses which require further insight in regard to expression pattern of WT-1 which being a cytoplasmic marker shows both nuclear and nucleolar positivity in almost all the cell types especially in ameloblast-like cells and basal cells. The expression of WT-1 in OKC has never been previously documented and more studies are warranted in the future in this regard. Thus in locally aggressive and invasive lesions like Ameloblastoma and OKC, the tumor proliferation and aggressiveness is promoted by the upregulation or overexpression of WT-1 and Snail and the overall downregulation of Syndecan deviates the tissue from normalcy, further promoting tumor progression, invasion and aggressiveness. The expression patterns of the three markers also shows a probable association between the behaviour of ameloblast-like cells when compared to basal cells and that of stellate reticulum-like cells when compared to superficial cells. Understanding the pathogenesis and nature of these lesions is important not only to clarify its aggressive nature but also to propose new treatment modalities.

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Conflicts of interest
There are no conflicts of interest.

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