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Original Article

Dentistry Section

Comparison of β-Catenin Protein Expression in Calcifying Odontogenic Cyst and Dentinogenic Ghost Cell Tumour

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ABSTRACT

Introduction: Calcifying Cystic Odontogenic Tumour (CCOT)/ Calcifying Odontogenic Cyst (COC) display a varying tissue morphology, while exhibiting different biological progression also at the same time. Attempts at classifying COC have largely been unsuccessful due to the present lack of knowledge about the development of these tumours and their underlying molecular changes. Wingless-beta catenin (Wnt–β catenin) signalling pathway has been found to be a cornerstone in the ectodermal development and tumour initiation-progression to malignant tumours, but its specific role in the pathogenesis of odontogenic ghost cell lesions is unknown.

Aim: To elucidate the participation and comparison of β-catenin protein expression in pathogenesis of benign odontogenic ghost cell lesions, CCOT and Dentinogenic Ghost Cell Tumour (DGCT).

Materials and Methods: A cross-sectional Immunohistochemical (IHC) study was performed in the Department of Oral and Maxillofacial Pathology, AECS Maaruti College of Dental Sciences and Research Centre, Bengaluru, Karnataka, India, from December 2019 to June 2021. Research was conducted on tissue sections of centrally located 16 cases of CCOT categorised as group 1 and four cases of DGCT categorised as group 2 using β-catenin tumour marker. The study samples were retrieved from the archives. IHC stained slides were subjected for histopathological analysis, where labelling index of tumour cells were assessed in three high power fields. Resultant β-catenin expression was compared between Benign Odontogenic Ghost Cell Lesions (BOGCL). Results were subjected to statistical analysis, Statistical Package for Social Sciences for Windows 17.0 (SPSS, Philadelphia, IL) software to analyse the data.

Results: β-catenin positivity was assessed in tumour cells of both the groups, 16 CCOT (group 1) and 4 DGCT (group 2). In each case, number of cells in three high power field i.e., under 40X magnification were evaluated. Both the groups expressed membranous, cytoplasmic and nuclear positivity in the basaloid tumour cells. Whereas, ghost cells showed no reactivity to the biomarker, β-catenin. On comparison using Mann Whitney U and Wilcoxon W test, there was no statistically significant difference in β-catenin expression between CCOT and DGCT.

Conclusion: β-catenin plays an important role in the tumourigenesis of benign odontogenic ghost cell lesions. Immunohistochemically CCOT and DGCT showed no significant difference in the β-catenin expression. Hence, the results suggest that CCOT and DGCT may show variation in clinical behaviour but share similar histogenesis.

Keywords: Canonical pathway, Benign odontogenic ghost cell lesions, Basaloid tumour cells, Ghost cells, Transcription factors, Labelling index

INTRODUCTION

Odontogenic ghost cell lesions are rare odontogenic tumours exhibiting heterogeneity with wide neoplastic potential. This varied biologic behaviour was first demonstrated in Calcifying Cystic Odontogenic Tumour (CCOT) [1]. There has been a lot of argument about their terminology and classification. Till date seven classifications have been tabulated by various authors and still no single classification is accepted universally and is yet debatable. Calcifying Odontogenic Cyst (COC) comprises of 0.37-2.1% of all odontogenic tumours. About 86-98% of cases demonstrate cystic architecture [2]. While histology is unique, exhibiting ameloblastic lining with the formation of characteristic ghost cells. Some lesions show solid and infiltrative growth which displays extensive diversity posing it a topic of controversy and always in scope to unveil the facts still not known since its discovery by Rywkind [3,4]. Also referred to as Gorlin's cyst after he coined the term in 1962 [5]. Praetorius (1981) believed in dualistic concept and categorised CCOT as two entities, a cyst and a neoplasm [4]. Further Buchner E (1991), Hong SP et al., (1991,1992) and Makoto Toida (2008) also classified CCOT based on the dualistic concept due to its variability in biological behavior [3,6]. Whereas, World Health Organisation (WHO) classification 1971,1992, 2005, 2017 had classified CCOT based on Monistic concept calling it either a tumour or a cyst, even though most of them appeared to be simple, and someneoplastic [7-10]. The WHO 1992 categorised it as a cyst while WHO 2005 considered it as a tumour and renamed it as CCOT for the cystic lesions and Dentinogenic Ghost Cell Tumour (DGCT) for the solid variant and Odontogenic Ghost Cell Carcinoma (OGCC) for frank malignant counterpart [1,8,9]. Recently WHO 2017 classified CCOT as a cyst, as studies were insufficient to prove that the cystic CCOTs were neoplastic. The solid form was retained as DGCT and the termodontogenic ghost cell carcinoma for malignant counterpart [10]. This study will be an addition to the existing insufficient literature on this controversial topic. And CCOT/COC is considered as a synonym here in our study. Wingless-beta catenin (Wnt-β catenin) signalling cascade also known as canonical pathway greatly contributes to the development of craniofacial structures by coordinating with neural crest cells. It has been a cornerstone in embryogenesis that determines cell proliferation, cell polarity and fate of the cell [11]. It acts as a primary morphogenetic signalling pathway throughout the development of tooth and its morphogenesis by influencing its pattern of development in dental lamina and regulating the shape of every single tooth [5,12]. Hence, molecular aberrations causing activation of the Wnt/β-catenin pathway during odontogenesis have been associated with the development of cysts and odontogenic tumours. CCOT is found to be one such tumour which has consistently shown elevated β-catenin expression [5].

The literature has enormous studies on role of Wnt/β-catenin signalling pathway in development of human malignancies like adrenocortical carcinomas, hepatocellular carcinoma, ovarian cancer, colon cancer and melanoma [13-15]. The relation between Wnt pathway and disease first came to light during 1990 [14]. Germ line mutations in the Wnt pathway molecules have shown to result in hereditary diseases and somatic mutations involved in development of tumours in various tissues [12]. β-catenin mutations in Wnt signalling pathway is known to increase malignant potential in tumours thus playing major role in oncogenesis. Over last few years the oncogenic role of Wnt/β-catenin signalling in ghost cell lesions, such as Adamantinomatous craniopharyngioma, Pilomatrixoma, CCOT and Ameloblastoma has caught attention [16,17]. These tumours are often compared due to their structural analogy to enamel organ during their development and also histological resemblance of the tumours [16,18].

Few studies with immunohistochemical analysis of CCOT showed atypical β-catenin expression in the tumour cells and no reactivity in ghost cells. Since β-catenin positivity was marked in cystic variant, these findings prompted us to examine a few cases of DGCT for β-catenin activity to study the β-catenin expression pattern in DGCT and its comparison with cystic variant (CCOT). DGCT is a very rare tumour which accounts for 2-14% of all CCOTs [19]. Considering that there are no similar reports, the objective of this novel study was to compare β-catenin protein expression in CCOT and DGCT and to explore its role in the tumourigenesis of the same.

MATERIALS AND METHODS

A cross-sectional Immunohistochemical (IHC) study was performed in the Department of Oral and Maxillofacial Pathology, AECS Maaruti College of Dental Sciences and Research Centre, Bengaluru, Karnataka, India, from December 2019 to June 2021.

Total 20 tissue sections of histopathologically diagnosed centrally located benign odontogenic ghost cell lesions were retrieved from the archives. They were divided into two groups:

- Group 1: 16 CCOTs
- Group 2: 4 DGCTs
- Control: 5 adenocarcinoma of colon cases

All the sections were subjected to immunohistochemical study using β-catenin antibody.

Peripherally located CCOTs and CCOTs associated with hybrid and malignant CCOTs were excluded from the study. The lesions were classified according to WHO 2005 classification [9]. The sample size was calculated using G* power software version 3.1.9.4, keeping the statistical significance level at 0.05.

Study Procedure

For immunohistochemical reactions 3 μm tissue sections were mounted on Poly-L-lysine coated microscope l slides. The sections were then subjected to series of procedures according to the manufacturer's protocol. Antigen retrieval was carried out in a microwave followed by peroxide block and protein block to inhibit enzyme activity, non specific antibody binding and to eliminate background staining, respectively. Tissue was then incubated with anti β-catenin primary antibody, (Monoclonal- BIOGENEX- Leica Biosystems) continued with secondary antibody (Novolink-Mini polymer detection system) (NOVOCASTRA-UK). Visualisation was acquired with the chromogen liquid Diaminobenzidine solution (DAB) prepared according to the manufacturer's protocol. Mayers haematoxylin provided in kit was used for nuclear stain. Slides were interpreted for the intensity of the β-catenin antibody concentration (brown coloured end product) and graded as strong, moderate, weak and absent [20,21]. In each case, total number of cells were counted in three high power fields (40X) using research microscope (Olympus BX41). Cells positive for β-catenin biomarker were counted in the same three high power fields and the percentage of positively stained cells is presented using the labelling index. The labelling index is the ratio of number of positively stained cells (X) divided by total number of cells (n) multiplied by 100 i.e., X/n x 100 [17,18]. Grading is tabulated based on the labelling index as strong (>90%) moderate (80-90%), weak (<80%) and absent [20,22].

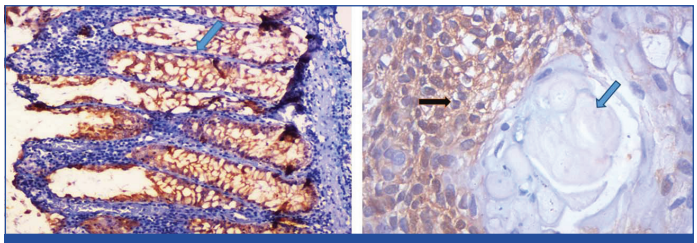
STATISTICAL ANALYSIS

Statistical Package for Social Sciences for Windows 17.0 (SPSS, Philadelphia, IL) software was used to analyse the data. Based on the mean labelling index values between group 1 and group 2, quantitative analysis of data using Mann-Whitney U and Wilcoxon W test was performed. A p-value >0.05 was considered significant to reject null hypothesis.

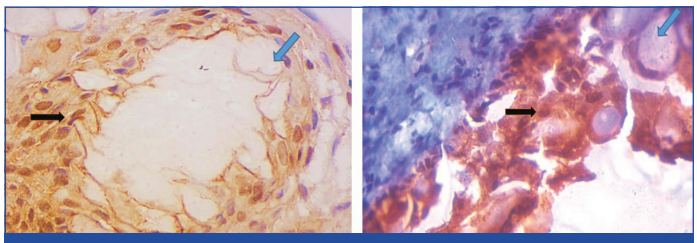
RESULTS

Current study included histopathologically diagnosed 16 cases of CCOT (group 1) with equal proportion of males and females with mean age of 39.8±6.8 years (mean±standard deviation). Four cases of DGCT (group 2) with 3:1 male to female ratioand mean age of 54±6 years were included. In the control group 3:2 male to female ratio and age distribution of 62±4 years was included. All the cases subjected to immunohistochemistry showed immunoreactivity to β-catenin. All the positive controls showed cytoplasmic and membrane positivity for β-catenin in glandular cells of colon [Table/Fig-1].

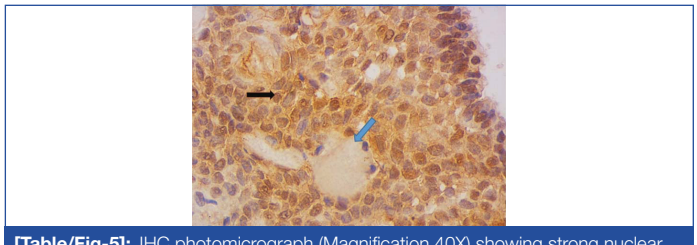
In the study groups, group1 [Table/Fig-2,3] and group 2 [Table/Fig-4,5] exhibited strong membranous, cytoplasmic and nuclear β-catenin expression in the basaloid cells around the ghost cells and basaloid cells in focal aggregation. Positive expression was seen



[Table/Fig-1]: IHC photomicrograph (Magnification 20X) showing Cytoplasmic and membrane positivity of β-catenin in adenocarcinoma of colon (control).
[Table/Fig-2]: IHC photomicrograph (Magnification 40X) showing strong membranous, and cytoplasmic β-catenin expression in basaloid cells surrounding ghost cells (black arrow). Ghost cells (blue arrow) are negative for the stain in CCOT (Group 1). (Images from left to right)



[Table/Fig-3]: IHC photomicrograph (Magnification 40X) showing strong membranous, cytoplasmic and nuclear β-catenin expression in basaloid cells surrounding ghost cells (black arrow). Ghost cells are negative for the stain in CCOT (blue arrow) (Group 1).
[Table/Fig-4]: IHC photomicrograph (Magnification 40X) showing strong cytoplasmic, membranous and nuclear β-catenin expression in basaloid cells surrounding-ghost cells (black arrow). Ghost cells are negative for the stain in DGCT (blue arrow) (Group 2). (Images from left to right)



[Table/Fig-5]: IHC photomicrograph (Magnification 40X) showing strong nuclear, membranous and cytoplasmic β-catenin expression in basaloid cells (black arrow) surrounding ghost cells and Ghost cells are negative for the stain in DGCT (Group 2) (blue arrow).

in the cell membrane of peripheral palisading epithelial cell lining and stellate reticulum like cells, owing to the presence of β-catenin at cell junctions. Ghost cells were negative in all the cases. Based on the mean labelling index values between group 1 and group 2 and their comparison using Mann Whitney U and Wilcoxon W test, p-value obtained was 0.156 [Table/Fig-6,7]. Thus, it was interpreted that there was no statistically significant difference in the β-catenin expression between CCOT and DGCT. Results are represented in the form of Box-Plot graph [Table/Fig-8].

Case no.	Total no. of cells per 3 hpf	No. of cells showing β-catenin expression	β-catenin (total) percentage positivity (labelling index)	Grading	Diagnosis
1	201	154	76.6	Weak	Group 1
2	276	265	96	Diffuse	Group 2
3	158	156	98.7	Diffuse	Group 2
4	216	194	89.8	Moderate	Group 1
5	557	550	98.7	Diffuse	Group 1
6	283	263	92.9	Moderate	Group 1
7	242	202	83.4	Moderate	Group 2
8	361	310	85.8	Weak	Group 1
9	362	328	90.6	Moderate	Group 1
10	252	214	85	Moderate	Group 1
11	451	442	98	Moderate	Group 1
12	249	225	90.3	Moderate	Group 1
13	200	169	84.5	Moderate	Group 1
14	411	161	39	Weak	Group 1
15	236	205	86.8	Weak	Group 1
16	271	200	73.8	Weak	Group 1
17	339	315	92.9	Moderate	Group 2
18	201	168	84.5	Moderate	Group 1
19	128	104	81.5	Weak	Group 1
20	164	152	92.6	Moderate	Group 1

[Table/Fig-6]: β- Catenin percentage positivity and grading in group 1 and 2 samples.

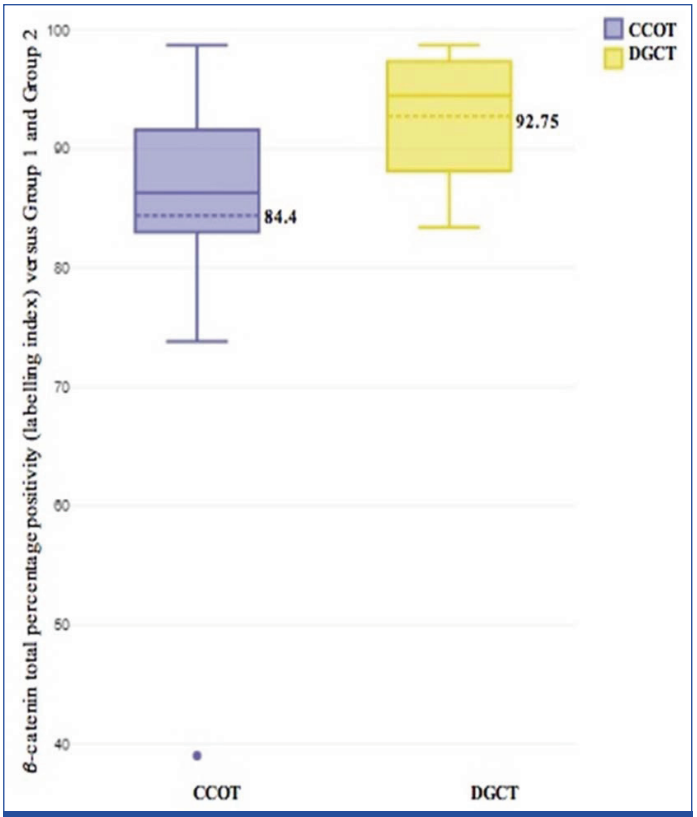
Group	No. of cases	Mean rank of labelling index	Sum of ranks	Mann-Whitney U	Wilcoxon W	Z	p-value (2 tailed)	Significance
1	16	9.56	153.00	17.000	153.00	-1.41	0.156	Not significant
2	4	14.25	57.00					

[Table/Fig-7]: Values of Mean labelling index, Mann Whitney U, Wilcoxon W test.

DISCUSSION

β-catenin plays multiple roles, the most important being behaviour protein of canonical/Wnt pathway. It functions as a structural molecule in adherens junction forming an integral part of the cell membrane-bound cadherin-catenin complex. With the centrosome it plays a crucial role in cell division. Under physiological conditions, that is in absence of Wnt signal, any free cytosolic β-catenin is constantly regulated by the scaffolding proteins APC/Axin and GSK3B (degradation complex). Phosphorylation of the amino terminal of β-catenin by GSK3B leads to ubiquitination and proteosomal degradation of β-catenin by linking it to β-trcp (transduction repeat containing protein [5,11,23].

The key switch in the process of odontogenesis is the Wnt/β-catenin/TCF behaviour pathway. During embryogenesis or homeostatic renewal, activated Wnt forms the Wnt-FZ-LRP6 (frizzled-LDL Related Protein) ligand complex along with Dvl (Dishevelled) protein. This, in turn, dissociates β-catenin from the degradation complex, inhibiting its phosphorylation. Active β-catenin can now reach the nucleus, where it modulates Wnt target genes using the transcription factors TCF/LEF causing cell proliferation [5,23,24].



[Table/Fig-8]: Box- Plot showing difference in mean labelling index among group1 and group 2. The mean positivity in CCOT is 84.4 and for DGCT it is 92.75 (dotted line). *As 39 is the lowest positivity value shown it's not included in the graph.

The aberrations in this pathway during the process of odontogenesis leads to hyperactivation of β-catenin and oncogenic transformation of Wnt target genes causing uncontrolled cell multiplication forming the classical ghost cell lesions. There might have been multiple genetic mutations implicated in the abnormal activation of β-catenin like APC, β-TRCP, long deletion of exon 3 and Axin1/2 [24,25]. Positive cytoplasmic and nuclear expression observed in this study conveys β-catenin has got a role in tumorigenesis of CCOT and DGCT but the exact molecular pathogenesis in emergence of these tumours remains unresolved.

To support the role of β-catenin, Ahn SG et al., Sekine S et al., and Kim SA et al., also evaluated genetic mutation of β-catenin in CCOT (multiple), DGCT (single case) and OGCC (single case) in addition to immunohistochemistry [25-27].

Molecular analysis was implemented using a pair of primers of the GSK3B phosphorylation (degradation complex) sites of β-catenin. A combined mutation at codons 4 and 5 resulted in substitution of glutamine for histidine and alanine for valine, respectively in simple cystic CCOT. A paired mutation at codons 3 and 57 resulted in substitution of threonine for serine and valine for alanine, respectively in ameloblastomatous CCOT. A missense mutation in codon 5 substituting alanine for valine was seen in odontoma-associated CCOT while a similar mutation in codon 3 substituting threonine for serine was seen in neoplastic-type DGCT [25-27].

All these somatic mutations of β-catenin at codons 3,4,5 and 57 increase the β-catenin concentration by inhibiting degradation (ubiquitin-proteosome pathway). This excessive β-catenin reaches the nucleus and its interaction with TCF/LEF transcription factors results in hyper proliferation of stem cells causing BOGCL. A similar mutation in codon 33 (TCT-TAT) of β-catenin gene resulting in substitution of serine for tyrosine was consistently seen in malignant ghost cell lesion, OGCC. The mutations of β-catenin gene at codons 33,37,41 and 45 are considered "mutational hotspots" for many human cancers notably ovarian and colorectal cancers [25,27,28].Interestingly, one of the CCOTs studied by Sekine S et al., did not yield anything on mutational

analysis despite having β-catenin expression [26]. To summarise, β-catenin expression and β-catenin mutation do not go hand in hand, instead can be considered that the mutation and atypical accumulation may play a characteristic role in tumorigenesis of CCOT and DGCT.

Similarly, ameloblastomas (bearing striking resemblance to ghost cell lesions) showed no β-catenin mutation despite having a mild to moderate β-catenin expression. The only exception was single follicular ameloblastoma which showed these mutations suggesting their heterogeneous genetic origin [29]. All the benign odontogenic ghost lesions subjected to immunohistochemical analysis were heterogeneously positive for β-catenin in the present study. Both the groups consisting of CCOT and DGCT, expressed nuclear and cytoplasmic β-catenin positivity in the basaloid cells in focal aggregates and around the ghost cells, lining and stellate reticulum like cells.

Ghost/shadow cells are dyskeratotic cells with the membrane outline preserved and intracellular keratin replacing the nucleus along with cytoplasm. There formation from odontogenic epithelium remains unclear. It could be a process of aberrant keratinisation, necrosis or defective enamel formation [30-32]. However, the role of ghost cells in the biologic nature of the seodontogenic ghost cell lesions is yet to be proved. None showed positivity in the ghost cells. These results were similar to previously reported study implying the role of β-catenin in the histogenesis [25-27,29]. Another important finding was that there was no significant difference in β-catenin expression pattern between DGCT and CCOT. Regardless of the subtype, all CCOTs showed positivity for β-catenin. These results reinforce the fact that abnormal activation of β-catenin protein acts as a central key for the tumorigenesis in all types of CCCOT and DGCT [26,29].

Furthermore, it is seen that at a similar level to β-catenin expression, there is a wide variation in clinical presentation, radiological changes and local destruction (aggressive DGCT to low-grade neoplasia of CCOT lesions). The level of expression may reflect the histological origin of these tumours but genetic mutational variability (as yet inconclusive) decides the tumour behaviour (less aggressive to destructive). Though this may be attributable to a limited number of DGCT cases studied. Also, the deciding factor may depend on initial β-catenin stimulus and degree of odontogenesis prior to the stimulus [31,33].

The expression of Wnt proteins and their possible association in CCOT and ameloblastoma was recently studied by Dutra SN et al., [34]. The expression of Wnt1 (potent activator of canonical pathway) and Wnt5 (inhibitor of canonical pathway) in CCOT epithelium including ghost cells was found to be variable across the spectrum of these tumours. Most of the ameloblastomas were positive for Wnt1 explaining their aggressive behaviour [34]. Odontogenic keratocysts were also observed displaying a similar phenomenon [35]. Interestingly, the epithelium and ghost cells of CCOTs expressed Wnt5a along with Wnt1 which may testify their benign nature in comparison with ameloblastomas. On the basis of these results the authors inferred that Wnt proteins are instrumental in the histogenesis of CCOT and ghost cells in ghost cell lesions [34].

There are few studies in the literature in relation to β-catenin and odontogenic ghost cell lesions, briefly mentioned in the tabulated form [Table/Fig-9] [25-27,29,34]. Similar studies on DGCT may help in uncovering their role in the aggressive behaviour of these tumours.

Author and year of publication	Place of study	Lesions	No. of cases studied	No of cases positive	β-catenin expression			β-catenin mutation
					Cells that are positive	Location of positivity	Cells that are negative	
Hassanein AM et al., 2003 [29]	Florida, USA	Pilomatricoma	10	10	Basaloid cells surrounding ghost cells (strong)	Cytoplasm + Nucleus	Ghost cells	-
		CCOT	6	6				
		Craniopharyngioma	9	9				
Sekine S et al., 2003 [26]	Japan	CCOT	11	11	-Cells around ghost cells -Follicular -Plexiform (weak)	Cytoplasm + Nucleus (strong)	Ghost cells	9
		Ameloblastoma	20	20		Cytoplasm + Nucleus membranous (weak)	-	1
Kim SA et al., 2007 [27]	South Korea	DGCT	1	1	Cells around ghost cells (Strong)	Cytoplasm + Nucleus	Ghost cells	1
Ahn SG et al., 2008 [25]	South Korea	CCOT	3	3	Cells around ghost cells (Strong)	Cytoplasm + Nucleus	Ghost cells	3
		GCCOT	1	1				1
Dutra SN et al., 2017 [34]	Brazil		6	6	Cells around ghost cells	Cytoplasm + Nucleus (Strong) Cytoplasm Cytoplasm + membrane	Ghost cells -Stromal cells plexiform and acanthomatou-sameloblastomas	-
		CCOT Ameloblastom (Follicular, Plexiform, Acanthomatous)	17	14 (follicular ameloblastomas)	-Epithelial lining cells			
Present study, 2022	India	Calcifying odontogenic Cyst Dentinogenic Ghost cell tumour	16	16	Cells around ghost cells and focally aggregated basaloid cells (strong)	Cytoplasm + Nucleus (16)	Ghost cells	-
			4	4	Cells around ghost cells and focally aggregated basaloid cells (strong)	Cytoplasm + Nucleus (04)	Ghost cells	-

[Table/Fig-9]: Brief review of literature on β-catenin and ghost cell lesions studies reported [25-27,29,34].

Limitation(s)

Larger sample size of CCOT and DGCT needs to be analysed. Comparison with malignant CCOT needs to be studied. Role of APC and axin in the pathogenesis of odontogenic ghost cell lesions needs to be identified.

CONCLUSION(S)

In conclusion, Wnt/β-catenin/TCF signal transduction is one of the key components in pathogenesis of odontogenic ghost cell lesions. Present study reinforces the role of β-catenin in histogenesis of CCOT and DGCT. Though there is no association between β-catenin mutation and expression, increased β-catenin levels are pathognomonic of benign odontogenic ghost cell lesions. Since

there was no significant difference observed in the level of abnormal β-catenin expression between CCOT and DGCT, CCOT can be considered as a neoplasm comparable to DGCT. And the term CCOT is preferable. However, definite molecular changes resulting in development of CCOT's remains unknown.

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