J. Maxillofac. Oral Surg. (Oct–Dec 2023) 22(4):1123–1129
https://doi.org/10.1007/s12663-023-02014-2

ORIGINAL ARTICLE

Evaluation of Expression of WNT1 and PTCH Genes in Peripheral Blood of Patients with Odontogenic Cysts and Tumours of the Jaws by Quantitative RT-PCR: A Pilot Study

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Received: 8 March 2022 / Accepted: 1 September 2023 / Published online: 23 September 2023
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Abstract

Introduction Odontogenic lesions of the maxillofacial region constitute a complex group of lesions with diverse histopathologic types and clinical behaviour. Early diagnosis is important to minimize the need for radical surgery and to improve quality of life of the patients. Tumour markers play an essential role in the molecular level understanding of Odontogenic lesions and also used for early diagnosis and target therapies which improves the quality of life of the patients. Patched, a tumour suppressor gene encodes the transmembrane protein PTCH and is a receptor for the morphogen Sonic Hedgehog. It is evident that PTCH gene mutations occur in odontogenic keratocysts and the Hedgehog signalling pathway has an important role during tooth formation. WNT 1 is a key signal molecule that controls cell growth and proliferation. WNT pathway abnormalities are reported to induce tumour occurrence. Hence, my study was to determine the presence of WNT1 and PTCH in peripheral blood of patients with Odontogenic lesions using quantitative RT-PCR.

Materials and Methods In this cross-sectional study, two groups were included: Group 1—blood samples from 8 individuals with odontogenic cysts and tumours, and Group 2—blood samples of 8 individuals without Odontogenic lesions. 2 ml of blood sample was collected from radial veins into PAX gene tubes containing RNA stabilizing agent and stored at a temperature of 2 to 4 degrees and transported to Enable Biolabs India Pvt Ltd., Chennai. PAX gene tubes were subjected to centrifugation at 8000 rpm to separate plasma fraction. Reverse transcription of mRNA was performed using miScript II RT Kit (Cat#218161, Qiagen, Germany) to synthesize cDNA. GAPDH house-keeping gene used as control.

Results The study group had 3 males and 5 females ($n=8$) with a mean age group of 32.6 years and the control group had 2 males and 6 females ($n=8$) with mean age of 35.2 years. Group I (study group) showed 37.5% positive expression of WNT1 gene with a p value of 0.055 ($p>0.05$) and 50% positive expression of PTCH with a p value of 0.021 ($p<0.05$) (Figs. 3 and 4) which was statistically significant when compared with control group. Group II (control group) showed 100% negative expression for WNT1 and PTCH genes.

Conclusion WNT1 and PTCH genes were expressed in peripheral blood of patients with odontogenic lesions. WNT1 and PTCH genes may be potential predictors in individuals who would develop odontogenic lesions. Further studies on expression of WNT1 and PTCH genes with larger number of samples might give a future scope for target therapy in odontogenic lesions.

Keywords Odontogenic lesions · Cyst and tumours · WNT1 · PTCH · Tumour markers · RT-PCR

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Introduction

Odontogenic lesions are the most commonly occurring pathologies in the maxillofacial region. Odontogenic cysts and tumours constitute a complex group of lesions with diverse histopathology types and clinical behaviour. Some of these lesions are true neoplasms and may rarely exhibit malignant characteristics with metastatic potential. These maxillo-mandibular lesions that occur across all patient demographics such as age, sex, race and socio-economic status have altered remnants of dental developmental tissues. Odontogenic lesions can raise diagnostic challenges, due to its overlapping histopathology. Appropriate diagnosis is crucial in the prognosis and management of these lesions. Tumour markers play an important role in confirmatory diagnosis of histopathologically confounding lesions.

Patched, the human homologue of the *Drosophila* segment polarity gene, a tumour suppressor mapped to chromosome 9 [1], that encodes the transmembrane protein PTCH, which is a receptor for the morphogen Sonic Hedgehog which is activated in two different pathways—(i) canonical signalling: by ligand-dependent interaction or receptor-induced signalling and (ii) non-canonical signalling, when there's a mechanism of activation downstream of smoothened [2].

Sonic Hedgehog signalling controls the fate of the cell. PTCH expression is intended to down-regulate and stop the signalling triggered by the arrival of the SHH secreted protein at the cell. PTCH gene auto-regulates to ensure limited programmed proliferation. And this auto-regulation fails if the gene is altered by deletion or mutation. It results in uncontrolled cell proliferation and continuous and abnormal synthesis of the non-functional PTCH protein.

The first direct association of tooth development and WNT signalling pathway was reported in 1990 [3]. WNT signalling pathway is widely involved in various process of biological development like embryogenesis, tissue haemostasis and wound healing by regulating cell proliferation, differentiation, polarization and apoptosis [4].

Three distinct pathways which are activated by WNT proteins include the canonical pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca2 + pathway [5]. WNT1 is a canonical Beta-catenin-dependent gene involved in the development of tooth. Mutation or abnormal expression of WNT genes results in aberrant activation of WNT signalling pathway leading to tumour development and progression [6]. Hence, this study was done to determine the presence of WNT1 and PTCH in peripheral blood of patients with Odontogenic cysts and tumours using quantitative RT-PCR. The main objective was to assess the expression of WNT1 and PTCH in individuals with and without odontogenic cysts and tumours of the jaws and to compare the expression among the two groups.

Methodology

The present study has been carried out in the department of Oral and Maxillofacial surgery, from July 2019 to February 2020. Two groups were included in this study. Group 1 included blood samples from 8 individuals with odontogenic cysts and tumours Group 2 included blood samples of 8 individuals without Odontogenic lesions. Malignant Odontogenic lesions were excluded from this study. 2 ml of blood was collected from radial veins in PAX gene tubes which contained RNA stabilizing agent. The PAX gene Blood RNA Tube containing a proprietary reagent was used to stabilize the intracellular RNA immediately after collection. The tube was kept upright in room temperature for minimum of 2 h and a maximum of 72 h and stored at 2–4 °C in a refrigerator and transported to Enable Biolabs India Pvt Ltd., Urapakkam, Chennai.

Blood sample collected in PAX gene tubes were subjected to centrifugation at 8000 rpm to separate plasma fraction. Following centrifugation, 250 µl of plasma was transferred carefully into a fresh 1.5 ml sterile, DNase/RNase free microfuge tube (cat#500,011, Tarson, India) without disturbing the white blood cell fraction at the interphase. The sample was subjected to RNA extraction using a microRNA extraction kit, as per manufacturer's instruction (cat#740,981.50, Machery-Nagel, Germany). MicroRNA kit was used instead of normal total RNA extraction kit because the RNA in plasma is often found to be fragmented (in-house observation). The yield of RNA from plasma fraction was found to be consistently higher with use of microRNA kit than regular total RNA extraction kit.

Briefly, 75 µl of MLP buffer was added to 250 µl of plasma supplemented with 20 µl of protease (Mat#1,016,330, Qiagen, Germany), mixed and incubated at room temperature for 3 min, which resulted in lysis of plasma proteins. Following lysis, 25 µl of MPP buffer was added and incubated at room temperature for 1 min, during which plasma proteins were denatured. Following denaturation, the lysate was centrifuged at 12,000 rpm for 3 min at room temperature to pellet the denatured proteins. Following centrifugation, 300 µl of the lysate was transferred to a fresh 1.5 ml sterile, DNase/RNase free microfuge tube (cat#500,011, Tarson, India) and 300 µl of isopropanol was added to precipitate RNA. The lysate along with precipitated RNA was transferred to an RNA capture column and centrifuged at 12,000 rpm for 30 s to enable capture of the RNA in the silica gel present in the capture column. Subsequently, the RNA in the silica gel was treated with RNase free DNase (rDNase, cat#740,963, Machery-Nagel, Germany) to remove contaminating genomic DNA. Following this step, the RNA in the silica gel was washed twice with 500 µl of wash buffer and total RNA was eluted in 20 µl of DNase/RNase free water.

Reverse transcription of mRNA was performed using miScript II RT Kit (Cat#218,161, Qiagen, Germany) to

synthesize complementary DNA (cDNA). This kit was used as it is capable of transcribing both messenger RNA and small RNA molecules with high efficiency. Briefly, 1 µg of RNA eluate was mixed with 4 µl of Hiflex buffer, 2 µl of nucleic-acid mix (containing random hexamers for reverse transcription), 1 µl of RNase inhibitor (cat#N2511, rRNasin Ribonuclease Inhibitor, Promega, USA) and 2 µl of reverse transcriptase enzyme in a total volume of 20 µl. The mix was incubated at 37 °C for 60 min to allow reverse transcription reaction. The reaction was terminated by incubating the mix at 95 °C for 5 min.

In order to identify the quantitative presence of WNT1 and PTCH mRNA molecules in the plasma samples, cDNA samples were subjected to real time polymerase chain reaction (PCR) amplification with 10 µM of gene specific primers at a final concentration of 2 µM. The primers were designed in such a manner to selectively amplify only WNT1 or PTCH cDNA but not any other RNA molecules during real time PCR reactions.

The following gene specific primer sequences were used

Gene	Primer sequence	Size of amplicon (bp)
WNT1	CTGACCTGATGCAGACGCAAG AGGAGCCACCTGTAGCTCTCATGTA	139
PTCH	CTGCTGGTATGCTCGGGACTG TAAATCGCTCGGAGTTTCTGG	175

To detect presence of WNT1 and PTCH signals in the samples, quantitative PCR amplification with gene specific primers was performed in the presence of Type-IT HRM Kit (Cat#206,542, Qiagen, Germany) in a 20 µl reaction. The following common amplification condition was used for quantitative determination of both WNT1 and PTCH mRNA expression in the samples: after an initial denaturation at 95 °C for 4 min to activate the hotstart taq polymerase, the samples were subjected to 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 10 s with capture of fluorescence after extension. To confirm for the specificity of amplification of WNT1 and PTCH signals, the samples were subjected to melt curve analysis, which involved a ramp step that ranged between 60 and 95 °C with an initial hold for 90 s followed by a rise of 1 °C at each step with a 5 s hold to enable the melting process. Analysis of melt curve showed distinct peak intensities for both WNT1 and PTCH between 85 and 90 °C (Fig. 1).

Interpreting Amplification Curves: Type-IT HRM is a green fluorescent dye that binds with minor groove of amplified DNA, which causes it to emit fluorescence in green wavelength. When a particular sample contains WNT1 or PTCH cDNA, the primers would anneal within the genome of that specific cDNA molecule and amplify during the PCR amplification process. Whenever amplification happens, the Type-IT HRM would bind with the amplified cDNA molecule and emit fluorescence in green range.

The emitted fluorescence is captured by sensors present within the Rotorgene Q real time system and displayed as amplification curve on the monitor by in-built software of the system. Based on the amount of cDNA molecules present in a given sample, the cut off (CT) value of amplification curve would vary. For example, when the amount of WNT1 cDNA in a sample is very high, the fluorescence would be detected early, and hence, the software would produce an amplification curve with a CT between 20 and 25 cycles. Similarly, if the amount of WNT cDNA in a sample is low, the fluorescence would be detected later in the amplification, and hence, the software would produce amplification curve with a CT later than 25 cycles (Fig. 2).

Statistical Analysis were performed using SPSS 16.5 software. Chi-square tests were used for comparisons. p value < 0.05 was considered as statistically significant.

Results

A total of 16 blood samples were collected from the participants of both study and control groups by using standard methods. The study group had 3 males and 5 females with a mean age group of 32.6 years and the control group had 2 males and 6 females with mean age of 35.2 years (Tables 1 and 2). Group I (study group) showed 37.5% positive expression of WNT1 gene with a p value of 0.055 ($p > 0.05$) and 50% positive expression of PTCH with a p value of 0.021($p < 0.05$) (Fig. 3 and 4) (Tables 3 and 4) which was statistically significant when compared with control group. Group II (control group) showed 100% negative expression for WNT1 and PTCH genes.

Discussion

WNT1, the first gene of WNT family, was identified by Varmus and Nusse in 1982 [7]. It was originally named integration site 1 (Int-1) as it encodes a cysteine rich protein. It was renamed in 1987 as WNT1 because of the homology between Wingless (*Drosophila Melanogaster)* and Int-1 [8]. WNT1 (wingless protein family) is a key signal molecule that controls cell growth and proliferation.

It transfers regulatory information between cells and serves an important role in stem cell differentiation and neural development. WNT1 gene induces phosphorylation of β-catenin in the nucleus which activates the classic WNT pathways, inducing cell proliferation which helps in tooth development and inhibiting apoptosis to generate anomalous growth, resulting in tumour occurrence.

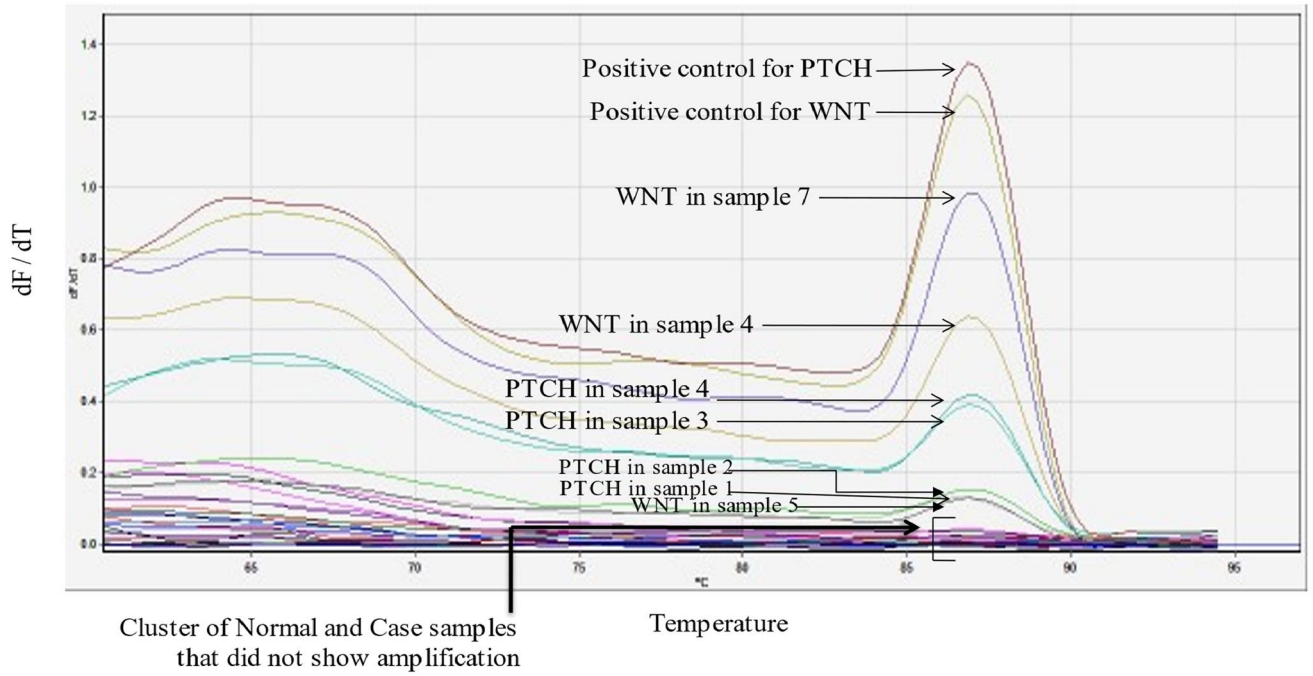


Fig. 1 Melt curve analysis of WNT1 and PTCH to confirm the specificity of amplification

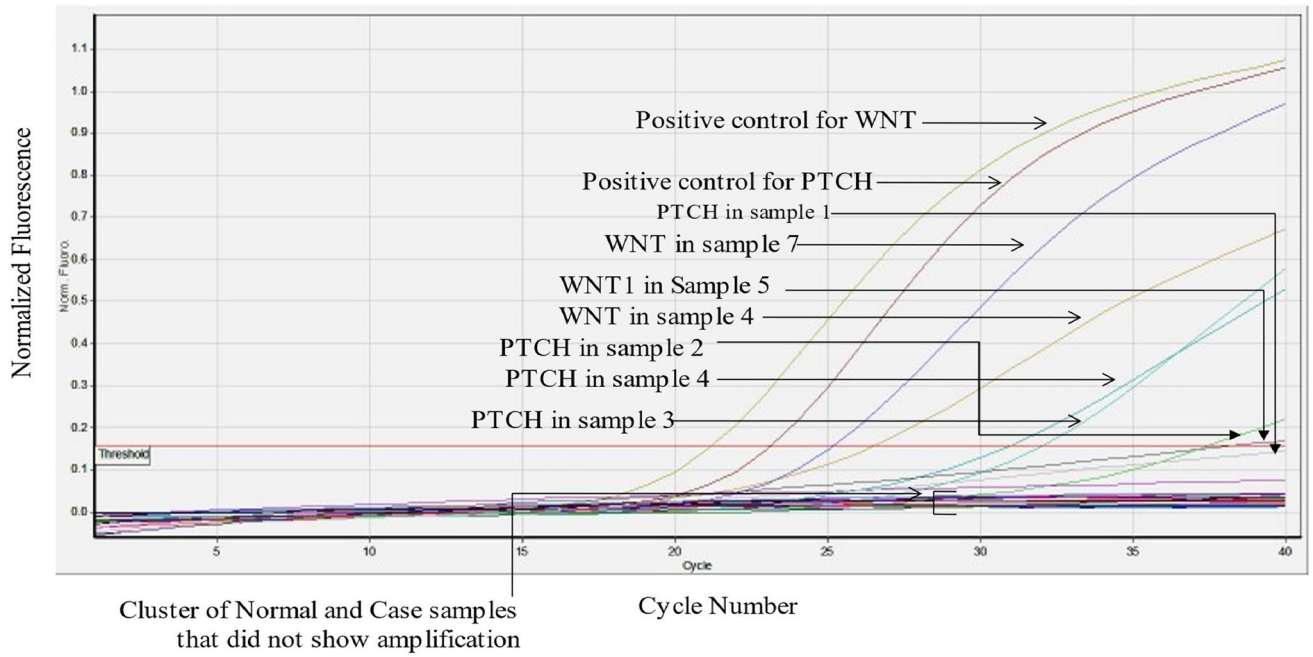


Fig. 2 Amplification curves of WNT and PTCH genes

Kee seng chuah et al. [9] in 2009 demonstrated increased expression of WNT1 in recurrent unicystic ameloblastomas using Immunohistochemistry of tissue specimens. Siar et al. [10] in 2011 demonstrated increased expression of WNT1 in different types of ameloblastoma (unicystic, solid/multicystic, desmoplastic and recurrent ameloblastomas) from immunohistochemical analysis and showed that WNT1 would be a key signalling molecule in the tumourigenesis of ameloblastoma.

Table 1 Expression of WNT1 and PTCH genes in Patients with Odontogenic Cysts and Tumours

S.No	Age/Sex	Type of odontogenic lesion	Radiographic pattern	Site	WNT1 expression	PTCH expression
1	29/F	Odontogenic Keratocyst Para-keratinized	Multilocular	Mandible	–	+
2	16/F	Dentigerous cyst	Unilocular	Maxilla	–	+
3	55/F	Ameloblastoma Plexiform	Unilocular	Mandible	–	+
4	50/M	Dentigerous cyst	Unilocular	Mandible	+	+
5	59/F	Ameloblastoma Unicystic	Unilocular	Mandible	+	–
6	35/F	Odontogenic Keratocyst	Unilocular	Mandible	–	–
7	55/F	Ameloblastoma Unicystic	Unilocular	Mandible	+	–
8	33/M	Inflammatory Odontogenic Keratocyst	Multilocular	Mandible	–	–

Table 2 Expression of WNT1 and PTCH genes in individuals without odontogenic cysts and tumours

S.No	Age/Sex	WNT1	PTCH
1	23/M	–	–
2	25/F	–	–
3	16/F	–	–
4	33/M	–	–
5	24/F	–	–
6	55/M	–	–
7	50/F	–	–
8	35/F	–	–

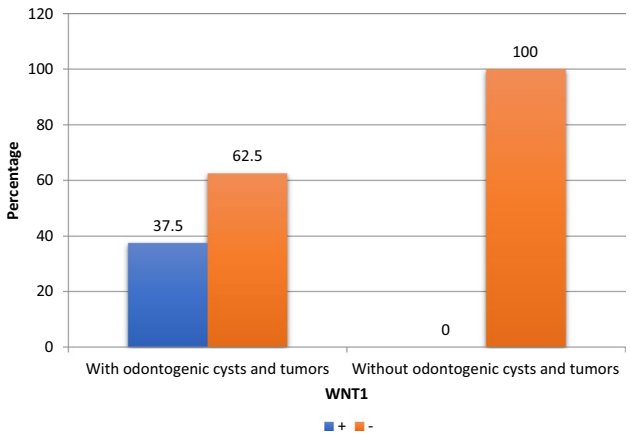


Fig. 3 Percentage of positive and negative expression of WNT1 gene

Other than ameloblastomas, abnormal expression of WNT1 was noted in calcifying cystic odontogenic tumour [11]. Wang et al. [12] in 2018 compared normal mucosa with ameloblastoma and found increased expression of WNT1 protein in ameloblastoma. In our group of odontogenic lesions, we identified positive expression of WNT1 in dentigerous cyst and unicystic ameloblastoma from peripheral blood samples using RT-PCR.

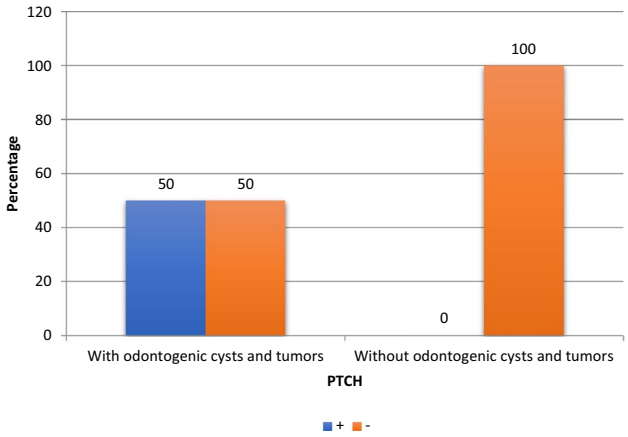


Fig. 4 Percentage of Positive and negative expression of PTCH gene

The human homolog of the Drosophila segment polarity gene (PTCH) encodes a 1447 amino acid transmembrane glycoprotein (Hahn et al. 13, Johnson et al.[14]). PTCH is involved in hedgehog (HH) signalling, where it is thought to act as a receptor for Hedgehog (Stone et al., 1996) [15]. The binding of PTCH and Sonic Hedgehog results in inhibition of another transmembrane protein Smoothened (SMO) thereby the downstream factor GLI-1 is activated. It enters the nucleus and transactivate the genes which results in cell proliferation [16].

Barreto et al. [17] found mutations of PTCH in peripheral blood of sporadic odontogenic keratocysts from Polymerised Chain Reaction. In our study on odontogenic lesions, we detected PTCH gene expression in peripheral blood using quantitative RT-PCR. In comparison, PTCH expressions among dentigerous cyst, Odontogenic keratocyst and radicular cysts by Pavelic et al. [18] showed similarity based on immunohistochemistry. Li et al. [19] in his study on Naevoid Basal cell carcinoma syndrome (NBCCS) associated odontogenic cysts, mutation of PTCH gene was found both in tissues and peripheral blood. Till now, no studies were done

Table 3 Comparison of WNT1 expression as determined by RT-PCR

WNT1	Group				Total		Chi square	*p*
	With odontogenic cysts and tumours		Without odontogenic cysts and tumours		*N*	%		
	N	%	*N*	%				
+	3	37.5			3	18.75	3.69	0.055
−	5	62.5	8	100.0	13	81.25		
Total	8	100.0	8	100.0	16	100.00		

Table 4 Comparison of PTCH expression as determined by RT-PCR

PTCH	Group				Total		Chi square	*p*
	With odontogenic cysts and tumours		Without odontogenic cysts and tumours		*N*	%		
	N	%	*N*	%				
+	4	50.0	–	–	4	25.00	5.33	0.021*
−	4	50.0	8	100.0	12	75.00		
Total	8	100.0	8	100.0	16	100.00		

on the expression of both WNT1 and PTCH gene in peripheral blood. With very few literatures evidence on individual expression of WNT1 and PTCH genes in peripheral blood, our study showed strong positivity of WNT1 and PTCH expression although in limited number of samples showing statistically significant results with PTCH expression.

Limitations of the Study

The distribution of odontogenic lesions was diverse with limited number of samples.

Conclusion

WNT1 and PTCH genes were expressed in peripheral blood of patients with odontogenic lesions. WNT1 and PTCH genes may be potential predictors in individuals who would develop odontogenic lesions. Further studies on expression of WNT1 and PTCH genes with larger number of samples might give a future scope for target therapy in odontogenic lesions.

Acknowledgements Nil

Funding Nil.

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