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ORIGINAL ARTICLE

Immunohistochemical evaluation of Sonic Hedgehog signaling pathway proteins (Shh, Ptch1, Ptch2, Smo, Gli1, Gli2, and Gli3) in sporadic and syndromic odontogenic keratocysts

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Abstract

Aims The aim of this study was to compare the clinical and demographic features of 62 patients presenting sporadic odontogenic keratocysts (OKCs) or OKCs associated with nevoid basal cell carcinoma syndrome (NBCCS). In conjunction with this, we also evaluated the immunohistochemical expression of Shh, Ptch1, Ptch2, Smo, Gli1, Gli2 and Gli3 proteins in 86 OKCs. By doing this, we add to the understanding of the biology of this type of lesion, providing tools that will help facilitate the early diagnosis of NBCCS in those patients where the first manifestation is that of OKCs.

Methods This is a retrospective study; patients were classified into two groups: group 1 which consisted of those who were not affected by NBCCS (49 patients and 57 OKCs) and group 2 which consisted of those who were diagnosed with NBCCS (13 patients and 29 OKCs). The clinical and demographic features were studied and the immunohistochemical expression of Sonic Hedgehog proteins (Shh, Ptch1, Ptch2, Smo, Gli1, Gli2, and Gli3) was analyzed in all samples.

Results There was an increase in the expression of three proteins in the syndromic OKC, when compared to that of sporadic cysts. Shh and Gli1 showed higher cytoplasmic expression, while Smo revealed stronger nuclear and cytoplasmic expressions.

Conclusion and clinical relevance Our findings suggest that the expression patterns of important Shh pathway proteins can represent valuable markers for early diagnosis of NBCCS-associated OKCs, as the major criterion for the diagnosis of NBCCS is currently based on the late appearance of basal cellular carcinomas. Thus, standardizing a new diagnostic tool for diagnosis of NBCCS could be of great importance in the identification of therapeutic targets. We therefore suggest, as based on our findings, that OKCs showing high expression of Shh, Smo, and Gli1 are potentially associated with NBCCS.

Keywords Odontogenic keratocyst · Nevoid basal cell carcinoma syndrome · Oral diagnosis · Odontogenic cysts · Sonic Hedgehog

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Introduction

An odontogenic keratocyst (OKC) is derived from odontogenic epithelium. Radiographic features present as solitary or multiple lesions, with the latter most often being associated with nevoid basal cell carcinoma syndrome (NBCCS) [1]. The World Health Organization (WHO) changed the classification of OKCs from that of a cyst to a tumor, in 2005 [2]. The evidence for reclassification was based on "aggressive growth," recurrence after treatment, the rare occurrence of a "solid" variant of OKC, and, most importantly, mutations in the PTCH gene. PTCH gene mutations have been documented in up to 85% of syndromic OKCs and around 30% of non-syndromic OKCs [3].

In 2017, the WHO reclassified OKCs back to a cyst, justifying that the neoplasias continue to grow even when the stimulus is eliminated. In addition, Neoplasms should not regress spontaneously, and yet, OKCs are well documented to completely regress following decompression. Also, the lining of many decompressed cysts appears more like oral mucosa than OKCs histologically. It is important to note that the WHO consensus panel is not necessarily saying that OKCs are not neoplastic, but rather, that they believe there is a lack of current evidence to justify continuing to classify keratocystic odontogenic tumors as a type of tumor [3].

NBCCS is a rare autosomal dominant disease related to the tumor suppressor gene Patched (PTCH), which is located on chromosome 9q22.3 [4]. Approximately 40–85% of patients diagnosed with NBCCS have germ-line mutations in this gene [5, 6]. Clinical manifestations include basal cell carcinomas, OKCs, palmar/plantar pits, and skeletal disorders. Approximately 70% of patients with NBCCS present multiple OKCs and are diagnosed during the first decade of their life [7–9].

The pathogenesis of OKCs is still under intense investigation due to its aggressive nature. The transformation and neoplastic cell proliferation typically involve the deregulation of signaling pathways which participate in normal embryonic development [10], mainly the Sonic Hedgehog (Shh) pathway [11–13]. The expression of certain Shh signaling pathway proteins have been detected in several odontogenic cysts and tumors, suggesting that this pathway plays an important role in epithelial interactions and in cell proliferation during tumor growth [14–16]. Activation of the Shh pathway occurs when an extracellular Shh protein binds to Patched membrane receptors (Ptch1, Ptch2), which then activates the Smo protein. Further biochemical events are activated downstream, which induces proteins from the Gli family (Gli1, 2, and 3) to stimulate cell proliferation [11, 13]. Although the role of the Shh signaling pathway is not well established in the development of OKCs, it has been suggested that the activation of the pathway may be correlated with OKCs' aggressive clinical behavior [17].

The main purpose of this study was to compare the clinical and demographic features of 62 patients with the presence of sporadic OKCs or associated with NBCCS, as well as to evaluate the immunohistochemical expression of Shh, Ptch1, Ptch2, Smo, Gli1, Gli2, and Gli3 proteins in 57 sporadic OKC lesions and 29 OKCs associated with NBCCS syndrome. The findings may contribute to a better understanding of the biology and pathogenesis of this odontogenic lesion, providing tools that will help facilitate the early diagnosis of NBCCS in those patients where the first manifestation is odontogenic keratocysts, even when the patient has no other clinically observable manifestations.

Patients and methods

Study characterization

Based on a previous departmental study, we evaluated data from 62 patients who presented isolated or multiple lesions (86 OKCs) and were subsequently treated at the A.C. Camargo Cancer Center in Sao Paulo, Brazil, between 1970 and 2009. This study was approved by the Institutional Ethic Committee in Research, and registered as protocol no.1322/09.

Clinical features of all patients were obtained from their medical records, including age, gender, presence of recurrence and association with NBCCS. Patients were classified into two groups: Group 1, those who were not affected by NBCCS, was composed of 49 patients and 57 OKCs (44 patients presented a single lesion, while 3 patients had three lesions and 2 patients two). Group 2, those who were diagnosed with NBCCS, was composed of 13 patients and 29 OKCs (3 patients had only one lesion, 6 had two lesions, 2 had three lesions, and 2 had four lesions). The OKCs were classified according to the WHO [3] and the NBCCS diagnosis criteria were based on those listed by Bree and Shah [9].

Recurrence was considered when a new OKC occurred in the same location as that of a previously treated lesion. The follow-up was calculated from the date of surgery up to the date of the last evaluation available. Eighty-six lesions were included in this study, of which 52 were primary OKCs (38 from group 1 and 14 from group 2) and 34 were recurrent (19 from group 1 and 15 from group2).

Immunohistochemistry

Slides of 3 μm were pre-heated for 24 h at 60 °C, deparaffinized in xylene, and hydrated in decreasing alcohol solutions. For each antibody, except the Gli2 antibody, the slides were subjected to antigen retrieval with citrate buffer (pH 6.0). For the Gli2 antibody, antigen retrieval was carried out using Tris-EDTA (pH 9.0). Endogenous peroxidase activity was blocked by incubating the slides in a solution of 3% hydrogen peroxide (Merck, Brazil). The slides were then incubated with the primary antibodies for 18 h at 4 °C (Table 1). All antibodies were diluted in PBS containing 1% bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA) and 0.1% sodium azide. The reactions were detected using the streptavidin-biotin-peroxidase system according to manufacturer specifications (LSAB DakoCytomation, Dako, Carpinteria, CA, USA). Diaminobenzidine was used as the chromogenic substrate (DakoCytomation, Dako, Carpinteria, CA, USA). For the negative control, the primary antibodies were omitted in the reactions. All experiments were duplicated.

Double-blind evaluation of the immunohistochemical results was performed by two researchers (AMH and EK)

Table 1 List of primary antibodies with respective title, source and clones

Antibody	Clone	Dilution	Supplier
Shh	Monoclonal	1:100	Abcam, Cambridge, MA, EUA
Ptch1	Polyclonal	1:400	Abcam, Cambridge, MA, EUA
Ptch2	Polyclonal	1:300	Lifespan, Seattle, Washington, EUA
Smo	Polyclonal	1:100	Abcam, Cambridge, MA, EUA
Gli1	Polyclonal	1:500	Thermo Scientific, Rockford, IL, EUA
Gli2	Polyclonal	1:200	Abcam, Cambridge, MA, EUA
Gli3	Polyclonal	1:50	Sigma, St. Louis, EUA

previously linked to this study. Only the epithelium of the OKCs was considered and it was divided into the basal and suprabasal layers for comparative analysis. Cells from the basal layer were defined as cells in contact with the basement membrane, whereas the suprabasal layer consisted of cells positioned two rows above the basal layer cells. Areas of inflammation were excluded from the evaluation and only areas with typical characteristics were analyzed. Immunoreactivity was evaluated in a similar manner to that of the analysis carried out by Vered et al. (2009) [18], where a score of 1 indicated 0–10% positive cells (low), 2 indicated 11–50% positive cells (intermediate), and 3 indicated more than 50% positive cells (high). Cytoplasmic/nuclear staining was evaluated for Shh, Gli1, Gli2, and Gli3 whereas Ptch1, Ptch2, and Smo were evaluated via cytoplasmic/membrane staining.

Statistical analysis

Clinical data were presented according to their frequency and distribution, and then statistical analysis was performed. The expressions of Shh, Ptch1, Ptch2, Smo, Gli1, Gli2, and Gli3 proteins were correlated with OKCs from groups 1 and 2, using Pearson's chi-squared test. In cases where the frequency was less than 5, Fisher's exact test was applied. For statistical analysis, GraphPad Prism 5 software was used (GraphPad Software Inc, La Jolla, CA, USA) and p values less than 0.05 were considered statistically significant.

Results

The age of the participants ranged from 8 to 74 years, with a mean age of 35.37 years and a median age of 31.5 years. The mean age of group 1 was 37.18 years, ranging from 9 to 74 years (median of 34), whereas the mean age of group 2 was 28.53 years, ranging from 8 to 66 years (median of 26). In both groups, women were more affected by OKCs than men (53.1% of group 1 and 69.3% of group 2). However, analysis suggested that there was no significant statistical difference between gender and association with NBCCS.

Of a total of 86 OKCs, 52 were primary lesions (60.5%) and 34 were recurrent lesions (39.5%), of which 19 belonged to group 1 (33.3%) and 15 to group 2 (51.7%). Although the percentage of recurrence was higher in group 2 than in group 1, there was no statistical difference ($p = 0.10$).

The follow-up was calculated from the date of surgery to the date of the last evaluation available. In Group 1, the time ranged from 0 days to 491 months (~4 years and 11 months), with a mean of 83.8 months (~ 7 years). In Group 2, the follow-up time ranged from 7 days to 295 months (~ 24 years and 7 months), with a mean of 78.44 months (~ 6 years and 6 months).

Immunohistochemical results

Immunohistochemistry analysis of Shh, Ptch1, Ptch2, Smo, Gli1, Gli2, and Gli3 proteins was performed in 86 OKCs. The expression of each marker was correlated with the presence or absence of NBCCS (Table 2). Figure 1 illustrates the expression pattern of each protein in both the sporadic and syndromic groups.

Cytoplasmic expression of Shh protein was higher in the syndromic group

In this study, all cases in groups 1 and 2 showed low nuclear expression within the basal and suprabasal layers. However, cytoplasmic expression of Shh was intermediate in both layers (Fig. 1(A, B)), with higher levels in syndromic-associated cysts (Table 2). However, within group 2, the cytoplasmic expression of Shh was only statistically significant in the basal layer when compared to group 1 ($p = 0.03$).

Ptch protein expression showed no difference in the association with NBCCS

There was high nuclear and cytoplasmic expression (score 3) of Ptch1 protein in both groups. Similar findings were observed for Ptch2, where high nuclear and cytoplasmic expressions were detected in both the basal and suprabasal layers. No statistically significant differences in Ptch1 and Ptch2 expressions were observed between syndromic and non-syndromic groups (Table 2) (Fig. 1(C–F)).

Table 2 Semi-qualitative analysis of Shh proteins in OKCs between non-syndromic (group 1) and syndromic (group 2) cases, being classified as cytoplasmic or nuclear expression. Scores were described as 1 = 0–10%; 2 = 11–50%; 3 ≥ 50% of positive cells. *p* value < 0.05. *the expected frequencies were less than 5 for Ptch1 and Gli2 proteins; therefore, Fisher's exact test was applied and the score values were grouped into 1 and 2 + 3

		Basal										Suprabasal									
Protein	Score	Nucleus					Cytoplasm					Nucleus					Cytoplasm				
		Group 1		Group 2			Group 1		Group 2			Group 1		Group 2			Group 1		Group 2		
		(*n*)	(%)	(*n*)	(%)	*p*	(*n*)	(%)	(*n*)	(%)	*p*	(*n*)	(%)	(*n*)	(%)	*p*	(*n*)	(%)	(*n*)	(%)	*p*
SHH	1	57	100	29	100	NA	20	35.0	4	13.8	0.03	57	100	29	100	NA	22	38.6	7	26.9	0.10
	2	0	0	0	0		37	64.9	25	86.2		0	0	0	0		35	61.4	22	75.8	
	3	0	0	0	0		0	0	0	0		0	0	0	0		0	0	0	0	
PTCH1	1	13	22.8	7	24.1	0.92	18	31.6	7	26.9	0.38	13	22.8	7	26.9	0.45	21	36.8	7	26.9	0.23*
	2	3	5.2	1	3.4		3	5.2	0	0		3	5.2	0	0		0	0	0	0	
	3	41	71.9	21	72.4		36	63.1	22	75.8		41	71.9	22	75.8		36	63.1	22	75.8	
PTCH2	1	19	33.3	6	20.7	0.45	19	33.3	6	20.7	0.34	5	8.7	2	6.9	0.45	7	12.3	2	6.9	0.51
	2	6	10.5	3	10.3		6	10.5	2	6.9		9	15.8	2	6.9		7	12.3	2	6.9	
	3	32	56.1	20	68.96		32	56.1	21	72.4		43	75.4	25	86.2		43	75.4	25	86.2	
SMO	1	48	84.2	17	58.6	0.01	45	78.9	17	58.6	0.03	47	82.4	16	55.2	0.02	44	77.1	16	55.2	0.09
	2	6	10.5	5	17.2		9	15.8	5	17.2		6	10.5	8	27.5		9	15.8	8	27.6	
	3	3	5.2	7	24.1		3	5.2	7	24.1		4	7.0	5	17.2		4	7.0	5	17.2	
GLI1	1	30	52.6	10	34.4	0.25	27	47.4	10	34.4	0.38	23	40.3	7	24.1	0.10	25	43.8	6	20.7	0.03
	2	13	22.8	8	27.6		16	28.0	8	27.6		25	43.8	12	41.4		23	40.3	12	41.4	
	3	14	24.5	11	37.9		14	24.5	11	37.9		9	15.8	10	34.4		9	15.8	11	37.9	
GLI2	1	24	42.1	10	34.4	0.69	22	38.6	10	34.4	0.38	2	3.5	2	6.8	0.63	0	0	1	3.4	0.15*
	2	3	5.2	1	3.4		3	5.2	0	0		4	7.0	1	3.4		0	0	0	0	
	3	30	52.6	18	62.1		32	56.1	19	65.5		51	89.4	26	89.7		57	100	28	96.5	
GLI3	1	52	91.2	23	79.3	0.25	50	87.7	22	75.8	0.16	49	85.9	22	75.8	0.36	48	84.2	22	75.8	0.20
	2	4	7.0	4	13.8		6	10.5	4	13.8		7	12.3	5	17.2		0	14.0	4	13.8	
	3	1	1.7	2	6.9		1	1.7	3	10.3		1	1.7	2	6.9		1	1.7	3	10.3	

NA not applicable

Smo nuclear and cytoplasmic expression patterns were elevated in the syndromic group

There was a significant difference in the expression patterns of the Smo protein between sporadic and syndromic OKCs (Fig. 1(G, H)). Both nuclear and cytoplasmic expressions were higher in syndromic OKCs, especially in the basal layer (Table 2), with a statistical significance of $p = 0.03$. However, in most cases the expression of Smo was low (score 1) for both layers in groups 1 and 2.

Gli1 protein showed higher cytoplasmic expression in syndromic OKCs

Nuclear and cytoplasmic expressions of the Gli protein family (Gli1, 2, and 3) in syndromic group 2 were higher when compared to those in sporadic OKCs, where both intracellular expressions were more evident for Gli1 (Fig. 1(I, J)) than for Gli2 and Gli3 (Fig. 1(K–N)). Although slightly higher in group 2, Gli3 protein had low expression within both groups. The higher cytoplasmic expression of Gli1 in the suprabasal layer of syndromic lesions compared to that in the suprabasal layer in sporadic lesions was the only statistically significant difference ($p = 0.03$) (Table 2).

Discussion

OKCs have aroused the interest of many researchers [2, 3, 19], because these odontogenic lesions present specific histopathological characteristics, exhibit aggressive behavior, and may be associated with chromosomal abnormalities and NBCCS. This retrospective study revealed the expression patterns of several Shh signaling pathway protein markers in 86 OKCs samples, comparing syndromic and sporadic OKCs.

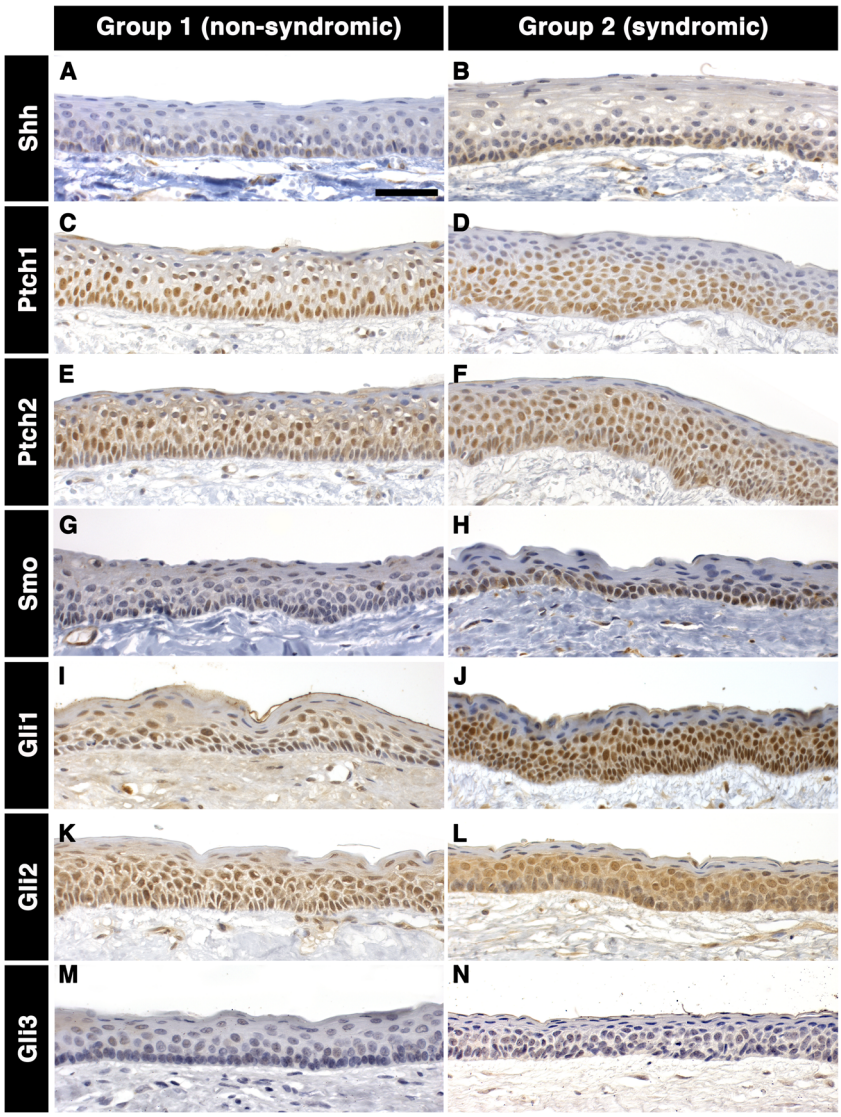

Fig. 1 Expression of Shh signaling pathway proteins in sporadic and syndromic OKCs. (A, B) Epithelial expression of protein Shh. (C, D) Epithelial expression of protein Ptch1. (E, F) Epithelial expression of protein Ptch2. (G, H) Epithelial expression of protein Smo. (I, J) Epithelial expression of protein Gli1. (K, L) Epithelial expression of protein Gli2. (M, N) Epithelial expression of protein Gli3
.

The Shh pathway has been shown to play an important role in different processes of cancer progression, but the exact mechanisms of action have not been elucidated yet [12–14]. In this study, Shh protein expression was predominantly observed in the cytoplasm of syndromic cysts, which was also the case when compared to sporadic lesions, with the greatest significance being in the basal layer. This may indicate greater activation of the Shh signaling pathway leading to increased cell proliferation. The expression pattern of this protein in syndromic and sporadic OKCs is similar to that seen in breast tumors, which also showed more prominent cytoplasmic Shh expression when compared to normal tissues [20]. Shh, Ptch, Smo, and Gli1 expression in sporadic and syndromic OKCs were all positive in the epithelial layers [21].

By regulating the activation of Gli family transcription factors and therefore stimulating cell proliferation, Smo plays an important role in the Shh signaling pathway. Increased expression of Smo may therefore be responsible for the development of OKCs and NBCCS [22]. Applying this information to our results, cytoplasmic Smo expression in syndromic OKCs was higher than that in sporadic lesions, which could indicate more aggressive behavior in this group.

Overexpression of either *Gli1* or *Gli2* leads to tumor development in transgenic mice, suggesting that these transcription factors contribute to tumorigenesis associated with alterations in upstream components of the Shh pathway [22]. Gli1 overexpression in the nucleus is associated with increased cell proliferation and differentiation, which is reflected in our work because this protein is highly expressed in syndromic cysts. However, the nuclear expression of Gli1 alone cannot be associated to neoplastic activation of the Shh pathway, because, in the absence of Shh pathway activation, Gli1 will relocate to the nucleus instead, suppressing cell proliferation in target cells [23]. Therefore, we suggest that Gli1 can be used as a diagnostic marker in syndromic OKCs for therapeutic purposes.

The recurrence rate of OKCs in the literature ranges from 5 to 62.5%. Recurrence has been correlated with high epithelial mitotic activity and the presence of satellite cysts and epithelial remnants from the cystic capsule [6, 15, 24, 25]. In this study, 39.5% of the 86 OKCs were recurrent (34 samples), of which 33.3% were sporadic cysts and 51.7% were associated with NBCCS.

Previous reports have shown more evident positivity for Smo in recurrent sporadic OKCs, suggesting that Smo may be a candidate marker for recurrence [6, 26]. Takahiro et al. [6] found cells positive for Smo, Shh, and Ptch mainly in the intermediate layer of the OKCs, whereas our results showed concentrated expression of these proteins in the basal and suprabasal epithelial layers. There are no reports showing the expression of Shh pathway proteins in recurrent OKCs of syndromic patients.

Studying the Shh signaling pathway is essential for understanding the biological development of different types of cancer. However, the mechanisms of activation for this pathway remain unclear and require further investigation [17, 27, 28]. This study highlighted the expression patterns of important Shh pathway regulators that can represent valuable markers for early diagnosis of NBCCS-associated OKCs. This is of extreme clinical relevance because, currently, the major criterion for NBCCS diagnosis is based on the late appearance of basal cellular carcinomas, which rarely appear before the age of 20 [29]. Thus, standardizing a new diagnostic tool for early diagnosis of NBCCS could be of great importance in the earlier identification of therapeutic targets. We therefore suggest, as based on our findings, that OKCs showing high expression of Shh, Smo, and Gli1 are potentially associated with NBCCS.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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