Sonic Hedgehog pathway is upregulated in adamantinomatous craniopharyngiomas

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Abstract

Objectives: Pituitary stem cells play a role in the oncogenesis of human adamantinomatous craniopharyngiomas (aCPs). We hypothesized that crosstalk between the Wnt/ β -catenin and Sonic Hedgehog (SHH) pathways, both of which are important in normal pituitary development, would contribute to the pathogenesis of aCPs.

Design: To explore the mRNA and protein expression of components of the SHH signaling pathway in aCPs and their relationship with the identification of *CTNNB1*/ β -catenin mutations and patients outcomes.

Patients and methods: In 18 aCP samples, *CTNNB1* was sequenced, and the mRNA expression levels of SHH pathway members (*SHH*, *PTCH1*, *SMO*, *GLI1*, *GLI2*, *GLI3*, and *SUFU*) and *SMO*, *GLI1*, *GLI3*, *SUFU*, β -catenin, and Ki67 proteins were evaluated by quantitative real-time PCR and immunohistochemistry respectively. Anterior normal pituitaries were used as controls. Associations between molecular findings and clinical data were analyzed.

Results: The aCPs presented higher mRNA expression of *SHH* (+400-fold change (FC); $P < 0.01$), *GLI1* (+102-FC; $P < 0.001$), and *GLI3* (+5.1-FC; $P < 0.01$) than normal anterior pituitaries. Longer disease-free survival was associated with low *SMO* and *SUFU* mRNA expression ($P < 0.01$ and $P = 0.02$ respectively). *CTNNB1*/ β -catenin mutations were found in 47% of the samples. aCPs with identified mutations presented with higher mRNA expression of *SMO* and *GLI1* (+4.3-FC; $P = 0.02$ and +10.2-FC; $P = 0.03$ respectively). *SMO*, *GLI1*, *GLI3*, and *SUFU* staining was found in 85, 67, 93, and 64% of the samples respectively. Strong *GLI1* and *GLI3* staining was detected in palisade cells, which also labeled Ki67, a marker of cell proliferation.

Conclusions: The upregulation of SHH signaling occurs in aCPs. Thus, activation of Wnt/ β -catenin and SHH pathways, both of which are important in pituitary embryogenesis, appears to contribute to the pathogenesis of aCP.

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Introduction

Craniopharyngiomas are epithelial tumors that are classified as papillary craniopharyngiomas or adamantinomatous craniopharyngiomas (aCPs) and account for 1.2–4.0% of primary brain tumors in children (1). aCPs are embryonic tumors that result from the transformation of embryonic squamous cell nests of the involuted craniopharyngeal duct (1, 2) and represent 90% of all craniopharyngiomas, occurring mainly in children (1). aCPs present benign histological features classified by WHO as grade I tumors (3). However, they present

significant morbidity related to their local invasion of CNS structures (4) and to the surgical approach itself (1).

Almost 80% of aCPs show aberrant accumulation of β -catenin in the cytoplasm and nucleus (5, 6). In addition, the prevalence of *CTNNB1* (β -catenin) mutations in aCPs has been shown to vary from 16 to 100%, including in our own series (5, 7, 8, 9, 10). In a previous study, a mouse expressing a degradation-resistant mutant form of β -catenin in early Rathke's pouch progenitor cells demonstrated that cells that accumulated β -catenin formed

clusters resembling human aCPs. These tumors arise from the activation of β -catenin in pituitary progenitors during embryogenesis based on the observation that these clusters expressed the stem cell markers SOX2, SOX9, and p27KIP1 (11, 12). Recently, it was demonstrated that the activation of oncogenic β -catenin in Sox2 positive stem cell in the adult pituitary results in tumors resembling human aCP (13). Of note, mutated Sox2-positive cells were not the cell-of-origin of the tumors, but they gave rise to β -catenin-accumulating clusters with tumor-inducing activities (13).

The Hedgehog (HH) pathway is also involved in pituitary formation during early vertebrate embryogenesis. Its activation is triggered by HH ligand binding to a receptor complex formed by the transmembrane protein patched 1 (PTCH1). In the presence of the ligand, the frizzled class receptor, smoothened (SMO), is released from PTCH1 inhibition, and activates the transcription factors glioma-associated oncogene family zinc finger, GLI1, GLI2, and GLI3 (14). After the appearance of Rathke's pouch, Sonic HH (SHH) expression is excluded from this region but remains in surrounding areas (15). Moreover, the SHH pathway plays an important role in adult stem cell maintenance, and *GLI1* expression is considered as a marker of SHH-responsive cells and as a marker of progenitor/stem cells (16). The expression of the stem cell markers, SOX2 and OCT4, has been observed in human aCPs (17).

Andoniadou *et al.* (11) used the β -catenin mutant transgenic mice model to demonstrate the co-expression of *SHH* mRNA in the cluster of cells expressing β -catenin in tumors resembling aCPs. Furthermore, increased mRNA expression of *SHH* was found in cells that accumulate β -catenin. *In situ* hybridization performed in a small number of human aCP sections revealed *SHH* and *PTCH1* expression in epithelial nodules, whereas *PTCH1* was also found in palisade cells (11). Interestingly, there are reports of aCPs in patients with *PTCH1* mutations (Gorlin syndrome), and over-active SHH signaling occurs in numerous human cancers (18, 19). These data support a role for pituitary stem cells in the oncogenesis of human aCPs. Thus, we hypothesized that crosstalk between Wnt/ β -catenin and SHH pathways, which are important during normal pituitary development, could contribute to the imbalance in intracellular signaling in the molecular pathogenesis of aCPs.

In this study, we have analyzed the expression of several components of the SHH pathway in human aCPs, in which *CTNNB1* mutations were identified or not. Our findings revealed a significant increase in the main components of the SHH pathway and the presence of

an association between the *CTNNB1* gene mutation and SHH signaling activity in aCPs.

Subjects and methods

Patients

This study was approved by the Local Ethics Committee (protocol no. 2009/1188), and informed written consent was obtained from the patients or from their parents. Eighteen patients with aCPs diagnosed at the University Hospital of the Ribeirao Preto Medical School, University of Sao Paulo, were enrolled in the study. The diagnosis considered clinical, hormonal, ophthalmological, and neural system imaging findings, as previously described (8), and it was confirmed by histopathology after surgery. Normal anterior pituitaries ($n=7$), which were obtained from adults who died from natural causes and underwent routine autopsy, were chosen as control tissue considering the probable embryonic common origin of aCPs (17).

Quantitative real-time PCR

The tumoral and normal pituitary tissues were micro-dissected by an experienced pathologist (F Saggioro) and frozen at -70°C until RNA extraction using TRIzol reagent (Invitrogen). RNA was quantified by spectrometry, and its integrity was evaluated by the 260:280-nm absorbance ratio and by agarose gel electrophoresis. The cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit and MultiScribe enzyme (Applied Biosystems).

The relative mRNA expression was analyzed by quantitative real-time PCR using specific TaqMan assays (Applied Biosystems): *SHH* (Hs00179843_m1), *PTCH1* (Hs00181117_m1), *SMO* (Hs01090242_m1), *GLI1* (Hs01110766_m1), *GLI2* (Hs01119974_m1), *GLI3* (Hs00609233_m1), and *SUFU* (Hs00171981_m1). The values were normalized based on the mean expression of the endogenous controls, *GUSB* (4326320E) and *PGK1* (4326318E). The reactions were performed according to the manufacturer's recommendation, and the relative mRNA expression values were determined using the $2^{-\Delta\Delta\text{Ct}}$ method (20).

Immunohistochemical analyses

In addition to routine hematoxylin–eosin staining, immunohistochemical (IHC) was performed according to the availability of formalin-fixed, paraffin-embedded aCPs in 14 samples using the primary antibodies anti-SMO (sc-13943), anti-GLI1 (sc-20687), anti-GLI3 (sc-20688),

anti-SUFU (sc-28847), anti- β -catenin (sc-7963; Santa Cruz Biotechnology, Inc.) and Ki67 (NCL-Ki67-MM1; Novocastra, Newcastle, UK). The staining was developed with 3,3'-diaminobenzidine-tetrahydrochloride (DAB; Vector Laboratories, Inc., Burlingame, CA, USA) and counterstained with Harris' hematoxylin. Staining was evaluated randomly in at least ten representative high-power fields ($\times 400$) for all of the markers. The immunolabeling percentage was evaluated by the ratio of unequivocal nuclei labeled in 100 counted cells or the percentage of labeled area using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). Adult normal testicular tissues were used as positive controls (detailed methodology and IHC standardization figure are shown in [Supplementary Methods](#), see section on [supplementary data](#) given at the end of this article and [Supplementary Figure 1](#)).

Statistical analyses

Continuous variables are expressed as the mean and range or s.d. Differences between variables were evaluated using the Mann–Whitney *U* test. Disease-free survival was defined as the length of time after primary surgery and the return of signs/symptoms of tumor expansion, and the analyses were performed using Kaplan–Meier curves and the log-rank test. The level of significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 5.02 (GraphPad, San Diego, CA, USA).

Results

Clinical presentation

Among all 18 patients, ten were females (55%). The mean age at the time of diagnosis was 16.4 years (6–30); 11 of the patients were children. At the time of diagnosis, 15/18 patients (83%) presented with endocrine abnormalities: ten (55%) multiple pituitary hormone deficiencies; two (11%) isolated growth hormone deficiency; one (5%) diabetes insipidus; one (5%) hypothyroidism; and one (5%) hyperprolactinemia. Hypothalamic dysfunctions, mainly obesity, were present in 13/18 patients (72%). Neurological and/or visual disturbances were found in 15/18 (83%) of these patients. All of the patients underwent surgical treatment, and 28% received radiotherapy as a second therapy. Exon 3 *CTNNB1* mutations were identified in 8/17 cases (47%). The mean time between surgery and tumor progression/recurrence was 55 months, which was observed in 11/18 (61%) patients. The mean

overall follow-up of the patients was 75.3 months (8–248 months). The clinical data for the patients are presented in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. A portion of these clinical data has been previously reported (8).

mRNA expression of SHH pathway genes

The expression of SHH pathway components was detected in aCPs and in control tissues. Compared with normal pituitaries, aCPs presented higher mRNA expression levels of *SHH* (+400-fold change (FC); $P < 0.01$), *GLI1* (+102-FC; $P < 0.001$), and *GLI3* (+5.1-FC; $P < 0.01$). However, *PTCH1*, *SMO*, *GLI2*, and *SUFU* mRNA expression did not differ between aCPs and controls ([Fig. 1A](#)).

The aCPs with identified *CTNNB1* mutations presented higher mRNA expression levels of *SMO* (+4.3-FC; $P = 0.02$) and *GLI1* (+10.2-FC; $P = 0.03$) compared with aCPs where mutations were not identified ([Fig. 1B](#)). In comparison with pediatric and adult aCPs, no differential mRNA expression of the *SHH*, *GLI1*, *GLI3*, *PTCH1*, *SMO*, *GLI2*, or *SUFU* genes was detected (data not shown).

Patients who developed tumor progression after surgery presented higher expression levels of *SMO* mRNA than patients without tumor progression (+5.4-FC; $P < 0.01$). Longer disease-free survival was observed in patients with tumors expressing lower *SMO* ($P = 0.001$) and *SUFU* ($P = 0.02$) mRNA levels than the median of the expression observed in the aCPs ([Fig. 1C](#)). There was no association between disease-free survival and *GLI1* ($P = 0.06$), *GLI2* ($P = 0.06$), *GLI3* ($P = 0.05$), *SHH* ($P = 0.25$), or *PTCH1* ($P = 0.39$) expression.

Immunohistochemical

The IHC analyses revealed the expression of SHH pathway proteins in aCPs. Positive immunostaining was observed for *SMO*, *GLI1*, *GLI3*, and *SUFU* in 85, 67, 93, and 64% of the samples respectively ([Fig. 2](#)). *GLI1*, *GLI3*, *SUFU*, and *SMO*-positive staining was observed in the epithelial palisade cells, the same type of cells in which Ki67 staining was found, as well as in the clusters of β -catenin positive cells.

Discussion

This study has demonstrated that the SHH pathway is activated in human aCPs. The expression of SHH pathway components occurred in β -catenin-accumulating cells that formed clusters in human aCPs. These data suggest that

Figure 1

Relative mRNA expression in controls and in adamantinomatous craniopharyngiomas (aCPs). (A) aCPs ($n=16$) presented higher mRNA expression of *SHH*, *GLI1*, and *GLI3* than normal anterior pituitaries (controls; $n=7$). There was no differential expression of *PTCH1*, *SMO*, *GLI2*, and *SUFU*. (B) aCPs with identified exon 3 *CTNNB1* mutations ($n=8$) presented higher mRNA expression of *SMO* and *GLI1* than aCPs ($n=8$) where mutations were not

identified. A and B were evaluated by Mann–Whitney *U* test and presented as median, interquartile range; percentile 5–95; * $P<0.05$; ** $P<0.01$. (C) Patients ($n=8$) with aCPs expressing *SMO* mRNA levels lower than the median of the expression detected in the all tumors presented longer disease-free survival than those patients ($n=8$) with aCPs expressing *SMO* mRNA levels higher than the median (Kaplan–Meier curves and log-rank test).

concomitant activation of SHH and WNT pathways occur in aCPs. Our data, obtained in a larger number of human tumor samples, confirmed at both mRNA and protein levels that activation of the SHH pathway occurred similarly to what was observed in tumors resembling human aCPs found in a β -catenin mutant transgenic mouse model (11).

A high level of *SHH*, *GLI1*, and *GLI3* mRNA expression in aCPs was confirmed by protein expression analyses, demonstrating strong SHH pathway activation. GLI1 and GLI3 nuclear staining was mainly observed in palisade cells, which are the same cells that express the cell proliferation marker Ki67 and other progenitor/stem cell markers. The increased *SHH* mRNA expression in aCPs suggests that the SHH pathway activation is ligand-dependent. In this condition, via autocrine and paracrine actions, the secreted SHH would promote tumor growth, infiltration, and angiogenesis by the activation of transcription factors in palisade cells (21).

We found that aCPs with identified *CTNNB1* mutations presented higher *SMO* and *GLI1* expression. It is well known that in the presence of ligand, SMO, which is a positive SHH pathway transducer, is released from PTCH1 inhibition and activates GLI transcription factors (21). Patients with aCPs expressing higher levels of *SMO* and *SUFU* display tumor progression and shorter disease-free survival after surgery, which suggests that SMO contributes to the activation of SHH signaling in aCPs, and this phenomenon might be related to tumor progression. Moreover, these findings suggest a concomitant activation of the SHH and Wnt pathways.

We also found SHH pathway activation in aCPs without identified *CTNNB1* mutations. This finding may suggest that SHH activation may be not exclusively driven by Wnt/ β -catenin pathway or that this pathway can also be activated by abnormalities in other genes, as discussed below. Conversely, we must consider the possibility of

Figure 2

Immunohistochemical analyses in adamantinomatous craniopharyngiomas (aCPs). (A, B, D and E) Representative images of GLI1, GLI3, SMO, and SUFU staining (scale 100 µm; inserts show higher magnification of highlighted areas). (C and F) β-catenin and Ki67 staining (scale 50 µm). Cytoplasmic and nuclear GLI1 staining found in the palisade cells and in the cluster of cells.

failure to identify *CTNNB1* mutations by direct sequencing of PCR products if the number of cells carrying the mutations is very low in the samples analyzed. Indeed, this possibility is important when considering the current idea of non-cell autonomous model of aCP tumorigenesis. In this scenario, a small number of β-catenin-mutated stem cells secrete paracrine signals, including SHH, WNT, BMP, and FGF, that drive tumor formation in adjacent tissue. The cells containing the mutated β-catenin may represent a very small percentage of the total cells in the tumor (13, 22).

It is interesting to note that elevated *CTNNB1* mRNA expression and diffuse cytoplasmic and nuclear β-catenin accumulation have been identified in adrenocortical tumors with or without *CTNNB1* mutations, which suggests that other factors might modulate Wnt/β-catenin pathway activation (23). Indeed, using integrated genomic characterization of adrenocortical carcinoma, the *ZNRF3* gene was the most frequently altered gene (21%) and represents a potentially new tumor suppressor gene that is related to the β-catenin pathway (24). Taken together, our findings suggest that not only the Wnt/β-catenin, with a firmly established crucial role in aCP etiology, but also the

GLI3 (mostly cytoplasmic), SMO (nuclear and cytoplasmic), and SUFU (mostly cytoplasmic) proteins presented diffuse staining, both in the palisade cells as well as in the cluster of cells. Protein staining was developed with DAB (brown) and counterstained with Harris' hematoxylin (blue).

activation of the SHH pathway participate in the pathogenesis of aCPs. However, the details of the crosstalk between the Wnt/β-catenin and SHH pathways underlying aCP tumorigenesis require further research.

In conclusion, we demonstrate the upregulation of SHH pathway components in human aCPs. Thus, the activation of Wnt/β-catenin and SHH pathways, both of which are important in pituitary progenitors embryogenesis, appears to contribute to the pathogenesis of aCPs. In addition, longer disease-free survival was associated with a low expression level of SHH pathway components, suggesting that inhibition of the SHH pathway might be a future therapeutic target for recurrent or invasive aCPs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-14-0934>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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