CTNNB1 Gene Mutations, Pituitary Transcription Factors, and MicroRNA Expression Involvement in the Pathogenesis of Adamantinomatous Craniopharyngiomas

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Abstract Genes involved in formation/development of the adenohypophysis, *CTNNB1* gene, and microRNAs might be implicated in the craniopharyngioma pathogenesis. The objective of this study is to perform the molecular analysis of *HESX1*, *PROP1*, *POU1F1*, and *CTNNB1* genes and evaluate a panel of miRNA expression in craniopharyngioma. We also verified whether the presence of *CTNNB1* mutation is associated with clinical findings and miRNA expression. The study included 16 patients with adamantinomatous craniopharyngioma (nine children and seven adults; eight females and eight males; 6–55 years, median 15.5 years). DNA, RNA, and cDNA were obtained from craniopharyngioma and normal pituitaries. DNA was also extracted from peripheral blood of healthy subjects. All genes were amplified by polymerase chain reaction and direct sequenced. Relative quantification of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. We found no mutations in *HESX1*, *PROP1*, and *POU1F1* genes and four polymorphisms in *PROP1* gene which were in Hardy–Weinberg equilibrium and had similar allelic frequencies in craniopharyngioma and controls. We found seven different mutations in *CTNNB1* in eight of 16 patients. Younger patients presented more frequently *CTNNB1* mutation than adults. We observed hyperexpression of miR-150 (1.7-fold); no different expression of miR-16-1, miR-21, and miR23a; and an underexpression of miR-141, let-7a, miR-16, miR-449, miR-145, miR-143, miR-23b, miR-15a, and miR-24-2 (ranging from −7.5 to −2.5-fold; $p=0.02$) in craniopharyngioma. There was no association between tumor size or the recurrence and the presence of *CTNNB1* mutations. miR-16 and miR-141 were underexpressed in craniopharyngioma presenting *CTNNB1* mutations. miR-23a and miR24-2 were hyperexpressed in patients who underwent only one surgery. Mutations or polymorphisms in pituitary transcription factors are unlikely to contribute to the adamantinomatous craniopharyngioma pathogenesis, differently of *CTNNB1* mutations. Our data suggest the potential involvement of the deregulation of miRNA expression in the craniopharyngioma pathogenesis and outcome and also that the miRNA could modulate the Wnt signaling pathway in craniopharyngioma tumorigenesis.

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Introduction

Craniopharyngiomas (CP) are benign epithelial, slow growing tumors of the sellar region. These tumors are among the most common childhood brain tumors but may be also diagnosed during adult life. Despite their benign histological appearance, they often show an unpredictable growth pattern and are frequently associated with severe adverse effects, significant morbidity, reducing quality of life and long-term survival [1]. CP pathogenesis is poorly understood. A subset of CP is monoclonal in origin [2]. Chromosomal imbalances are rare events in both adamantinomatous and papillary CP [3], although translocation, deletion, and an increase in DNA copies have been demonstrated [4]. In contrast, mutations in the p53 tumor suppressor gene [5] and the *Gsp* or *Gip* oncogenes have not been found [2].

Two hypotheses have been considered regarding the genesis of CP, which could result from metaplasia of adenohypophyseal cells in the pituitary stalk or gland or could arise from neoplastic transformation of embryonic squamous cell rests of the involuted craniopharyngeal duct [6, 7]. In humans, at approximately 3 weeks of gestation, the oral ectoderm invaginates to form the rudimentary pituitary primordium, the Rathke's pouch. By the end of the second month, a definitive Rathke's pouch separates from the oral ectoderm. Then, the anterior side of this pouch undergoes a period of intense proliferation and differentiation to form the anterior lobe of the pituitary gland development [8]. By birth, the anterior pituitary is comprised of five cell types that are defined by the hormone(s) they secrete. Differentiation of these cell types occurs in a temporally and spatially restricted manner. Several classes of homeodomain-containing transcription factors play important roles in both lineage specification and cell-type-specific gene expression [8]. Based on the hypothesis that CP can arise from embryonic remnants of Rathke's pouch, which is the primordium of the anterior and intermediate lobes of the pituitary gland, these transcription factors might be involved in the pathogenesis of CP.

Mutations of the glycogen synthase kinase 3β binding domain of *CTNNB1* gene have been detected in calcifying odontogenic cysts, pilomatricomas, and in adamantinomatous CP [9–12], suggesting a potential role of the associated Wnt/wingless signaling pathway during tumorigenesis of these particular neoplasms. Recently, nuclear β-catenin accumulation was shown as a highly significant finding in adamantinomatous CP [13]. As a key player of the canonical Wnt signaling pathway, β-catenin plays an important role during development cellular proliferation, differentiation, and migration [14].

MicroRNAs (miRNAs) are an abundant class of endogenous small RNA molecules that act as negative regulators of the protein-coding gene expression. miRNAs have been related to control multiple biological processes, including stem cell growth, apoptosis, and cancer [15], by regulating the expression of oncogenes and tumor suppressor genes [16]. Several deregulated miRNAs have been involved not only in pituitary cell proliferation and apoptosis, but also in neoplastic transformation [17]. There are few studies on miRNAs in pituitary tumors demonstrating underexpression of miR-15a and miR-16 in growth hormone (GH)- and PRL-secreting pituitary adenomas and underexpression of miR-145, miR-21, miR-141, let-7a, miR-150, miR-15a, miR-16, and miR-143 in adrenocorticotropic hormone (ACTH)-secreting pituitary tumors [18, 19]. miR-15a and miR-16 were inversely correlated with tumor diameter and directly correlated with secretion of the anti-neoplastic cytokine p43 [20], and patients with ACTH-secreting pituitary tumors presenting lower miR-141 expression showed better chance of remission [19]. Recently, it was proposed that miRNAs potentially activate Wnt signaling pathways, suggesting that miRNAs via Wnt might be involved in the tumor formation [21]. There are lacking studies on miRNA expression in CP.

In the present study, we performed the molecular analysis of three homeodomain-containing transcription factors, *HESX1*, *PROP1*, and *POU1F1*, and the *CTNNB1*gene as well as the evaluation of a panel of miRNA expression in adamantinomatous CP in order to elucidate their contribution to the tumorigenesis. In addition, we also verified whether the presence of *CTNNB1* mutations is associated with age of diagnosis, tumor size, recurrence, or different miRNA expression.

Subjects and Methods

Patients and Clinical Assessment

This study was approved by the Institutional Review Board for Human Research of the University Hospital of School of Medicine of Ribeirão Preto-University of São Paulo and informed consent was obtained from all subjects (process no. 1188/2009). We evaluated sixteen Brazilian patients with CP (eight males and eight females; aged 6 to 55 years, median of 15.5 years, being nine children and seven adults). Clinical criteria for the diagnosis of CP included neurological, endocrinological, and radiological findings as well as histological examinations of tissue specimens in all cases.

All patients underwent biochemical evaluation to confirm the diagnosis of hypothalamic and pituitary abnormalities, as

previously described [22]. Hypothyroidism was diagnosed by basal values of FT_4 and TSH according to the normal age matched values provided by the manufacturer. On the other hand, adrenal, GH, and gonadotrophic deficiencies were confirmed by dynamic tests. To evaluate the adrenal axis, we performed insulin tolerance test (ITT) (normal response: plasma cortisol >19 µg/dl). The GH axis was evaluated by two different dynamic tests, e.g., ITT and L-dopa tests, after priming with estrogen. Glucagon test was used instead of ITT when patients presented seizures (normal response: GH peak >7 ng/ml). Hypogonadism was diagnosed by gonadotropin-releasing hormone test, when patients were in an appropriate chronological and bone ages (normal response: LH >6.8 mUI/ml). To confirm the vasopressin deficiency, serum sodium and plasma and urinary osmolality were determined by flame photometry and freezing depression point, respectively, in basal condition or after the water deprivation test, with or without desmopressin administration. Obesity was diagnosed through the body mass index and classified according to the WHO classification. The hormone levels were assessed by the age, sex, and pubertal stages.

Subjects were evaluated by magnetic resonance image (MRI) performed with a 1.5-T imager (Siemens Vision) with a head coil. All patients were submitted to ophthalmologic evaluation by an expertise.

Molecular Studies

Total DNA and RNA were extracted, using TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA), from 16 craniopharyngioma tumoral tissues obtained during surgery from 2005 to 2008. In addition, 15 normal anterior pituitaries, obtained during autopsies from subjects (mean age, 53 years old; ranging from 36 to 76 years old) who had natural death without previous evidence of any endocrine disease or neurological abnormality, were used as control. Tumoral samples were microdissected by an experienced pathologist in order to separate any non-tumoral tissue. Part of the tumor tissue was processed for routine histopathological examination, and other stored at −70°C.

DNA extraction and purification from fresh frozen CP and normal pituitary tissues were performed as previously described [19]. The coding regions and intron/exon boundaries of POU1F-1, PROP1, HESX1, and the GSK-3β-phosphorylation sites of the CTNNB1 genes were amplified by polymerase chain reaction (PCR) using primers previously published [23–25]. Genomic DNAs from all patients and from 71 healthy Brazilian subjects were sequenced to investigate the presence of mutations in POU1F1, PROP1, HESX1, and CTNNB1 genes. In addition, reverse transcriptase PCR from normal pituitary and tumoral tissues were performed using a first strand cDNA synthesis kit (Taqman RT reagents, Applied Biosystems, Branchburg, New Jersey, USA). PCR amplifications from cDNA product were performed using a specific pair of primers also encompassing the GSK-3β-phosphorylation sites of the CTNNB1 gene (5′GCTGATTTGATGGAGTTGGA/3′GCTACTTGTTCTTGAGTGAA). All PCR products from peripheral blood, tumoral DNA, and cDNA were purified and direct sequenced from both directions on an ABI PRISM 310 autosequencer (Applied Biosystems, Foster City, CA).

The sequences of HESX1, POU1F1, PROP1, and CTNNB1 genes, obtained from each subject, were compared to sequences obtained from Ensembl Gene Report (ENSG00000163666, ENSG00000064835, ENSG00000175325, and ENSG00000168036, respectively).

microRNA Expression Profiling

Identification of potential miRNA target molecules was performed using TargetScan 5.1 (http://www.targetscan.org), miRNAMap 2.0 (http://mirnamap.mbc.nctu.edu/), and miRGene (http://diana.pcbi.upenn.edu/miRGene) databases. The outputs of these databases were merged, and all potential targets of a miRNA predicted. Besides let-7a, miR-15a, miR-16, miR-23a, miR-23b, miR-24-2, miR-150, and miR-449, which direct targeting the Wnt/β-catenin, we also evaluated miR-16-1, miR-21, miR-141, miR-143, and miR-145 expression, using qPCR. Primers and probe mix of the TaqMan® MicroRNA Assay for each miRNA and endogenous controls (RNU44 and RNU49) were also purchased from Applied Biosystems (Foster City, CA, USA). The cycle threshold (Ct) data were performed using default threshold settings. Data were presented as the mean CT for duplicates of each sample of each miRNA normalized by the endogenous controls Ct-median expression and calibrated by ΔCt-median value obtained from all normal pituitary tissues. Relative quantification of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Fold change of the expression of miRNAs observed in CP in relation to normal pituitaries was determined by the median of $2^{-\Delta\Delta Ct}$ values of CP related to median of $2^{-\Delta\Delta Ct}$ values of normal pituitaries.

Statistical Analysis

The expression of miRNAs in CP and normal pituitary are presented as mean, standard error deviation, median, and range. Statistics were carried out using Mann–Whitney test for continuous variables. To analyze whether the presence of CTNNB1 mutations is associated with age of the diagnosis, tumor size (≤3 or >3 cm), recurrence, or different miRNA expression, we used Fisher's exact test. Data were analyzed by GraphPad Prism5 software. Differences were considered significant at $p < 0.05$.

Results

Clinical Assessment

Table 1 shows the clinical and endocrine findings of patients with craniopharyngioma at the time of the diagnosis. Endocrine disorders were found in 12 patients (12/16). Eight patients presented more than one hormone deficiency. Hypothalamic dysfunctions, such as hyperphagia and/or obesity, were observed in eight patients and weight loss in two; somnolence was observed in five patients. Neurological manifestations were observed in 12 patients; the main complaints were headache and central nervous system hypertension. Thirteen out of 16 CF patients had either unilateral or bilateral visual disturbances. The most frequent visual defect was temporal hemianopsia; however, amaurosis was observed in two patients and papilloedema in another two. In two children, the visual field defect was not possible to be determined.

Fifteen patients were assessed by MRI and one by CT (Table 2). The tumor size varied from 1.5 to 6.5 cm ($X\pm$SD: 4.4 ± 1.4 cm; median, 4.4 cm). All tumors presented invasion of surrounding structures, nine showed parasellar and seven suprasellar extensions. Calcification was observed in 87.5% of the patients; tumors were solid–cystic in 62.5%, solid in 31.3% and one was cystic (6.2%). Hydrocephaly was observed in three patients and hemorrhage in 12.5%. Surgical

management was undertaken in all cases, and the decision to perform a complete or a partial resection was made by the neurosurgeon during the operation according to the anatomical findings. All patients were operated by the same surgeon (H.R.M.). The surgical approach was chosen based on the image findings. The transsphenoidal route was performed in 25% of surgical cases, while the remaining patients (75%) were treated by the transcranial approach (Table 2). Gross total removal was defined as the absence of residual tumor in post-operative imaging. Partial removal was based on the detection of residual tumor post-operatively and/or on the neurosurgeon's report confirming incomplete excision. Post-operative imaging confirmed total resection in 56.2% and partial in 43.8% (Table 2). Among seven patients with residual tumor, four (57.1%) underwent at least a second surgery. However, recurrence was also detected in two patients (12.5%) who had total resection. The referral for radiotherapy (conventional fractionated external beam irradiation) was indicated in 18.8%. The follow-up period ($X\pm$SD; 2.9 ± 4.0 years) ranged from 0.5–17 years, median of 1.9 years.

There was no recovery of pre-existing pituitary hormone deficiencies after any surgical intervention; all the 16 patients required post-operative hormonal replacement therapy. By the time of the last evaluation, 15 patients were receiving desmopressin and cortisol; ten of them were receiving also levothyroxine; eight patients were diagnosed

Table 1 Clinical manifestation of patients with craniopharyngioma at the diagnosis

Patients	Sex	Age at the diagnosis (years)	Endocrine involvement	Hypothalamic dysfunctions	Neurological manifestations	Visual disturbances
1	M	6	Hyperprolactinemia	Somnolence	Headache	Diplopia
2	M	6	GHD	OB	Headache, nausea/vomit	Visual deterioration
3	F	7	GHD	Weight loss	Headache, nausea/vomit	TQ RE
4	F	15	GHD, HG, HC	Somnolence	Headache, nausea/vomit	Papilloedema, optic atrophy LE, BH
5	F	8	No	Hyperphagia, OB, somnolence	Headache, hemiparesis, ptosis LE	AM
6	M	9	GHD, HC, hyperprolactinemia	Somnolence	Headache, nausea/vomit	AM LE/TH RE
7	F	10	DI	OB	Headache	Papilloedema, TH LE
8	M	14	No	OB	Headache	BH
9	M	16	GHD, DI, HT, HG, HC	–	–	–
10	M	20	No	OB	–	BH
11	F	21	DI, HT, HG, HC, hyperprolactinemia	–	Headache, nausea/vomit	BH
12	F	22	GHD, HT, HG, HC	OB	Headache	BH
13	M	26	GHD, DI, HT, HG, HC	OB	–	–
14	F	27	DI, HG, HC	Somnolence	Nausea/vomit	–
15	M	30	HT, HG, HC	Hyperphagia, OB, somnolence	Headache	Diplopia
16	F	55	No	Weight loss	–	TQ LE

M male, *F* female, *DI* diabetes insipidus, *GHD* growth hormone deficiency, *HT* hypothyroidism, *HG* hypogonadism, *HC* hypocortisolism, *OB* obesity, *LE* left eye, *RE* right eye, *BH* bitemporal hemianopsia, *AM* amaurosis, *TH* temporal hemianopsia, *TQ* temporal quadranopsia

Table 2 Individual characteristics of imaging, extension of the disease, modality of therapy, follow-up, and mutational analysis of *CTNNB1* in patients with adamantinomatous craniopharyngioma

Patient	CT or MRI	Tumor size (cm)	Extension of disease	Treatment	Tumor characteristics	CNS involvement	Follow-up (years, months)	Nucleotide change	Effect of mutation
1	MRI	6.0	PS	TC/PR	Calcification, solid–cystic	Invasion 3rd ventricle, hydrocephaly	1 month	TCT → TTT (c.98C>T)	p.Ser33Phe
2	MRI	3.1	PS	TC/PR	Calcification, solid–cystic	Hypothalamic and OQ compression, invasion 3rd ventricle	2 years	ACC → GCC (c.121A>G)	p.Thr41Ala
3	MRI	4.7	PS	TC/PR	Calcification, cystic	None	1 year and 7 months	ACC → ATC (c.121C>T)	p.Thr41Ile
4	MRI	4.4	SS	TSS/TR	Calcification, solid	ON/OQ compression, Hydrocephaly	1 year	No	
5	MRI	6.2	PS	TC/PR	Calcification, solid	None	1 year	TCT → TGT (c.98C>G)	p.Ser33Cys
6	MRI	6.5	SS	TC+RTX/TR	Solid–cystic	3rd cranial nerve palsy	1 year and 3 months	GAC →AAC (c.94G>C)	Asp32Phe
7	MRI	5.0	PS	TC+RTX/PR	Calcification, solid–cystic	Invasion 3rd ventricle	1 year and 4 months	No	
8	MRI	2.5	PS	TC/TR	Solid	OQ compression, invasion 3rd ventricle	4 years	TCT → TGT (c.110C>G)	p.Ser37Cys
9	MRI	1.5	PS	TSS/TR	Calcification, solid–cystic	Hypothalamic and OQ compression, hemorrhage	9 months	TCT → TGT (c.110C>G)	p.Ser37Cys
10	MRI	6.5	SS	TC/PR	Calcification, solid	OQ compression	3 years	No	
11	MRI	4.0	SS	TC/TR	Calcification, solid–cystic	Hypothalamic and OQ compression	7 years	No	
12	MRI	4.2	PS	TSS/TR	Calcification, solid–cystic	Hypothalamic and OQ compression, hemorrhage	1 year	No	
13	MRI	2.5	SS	TSS/TR	Calcification, solid–cystic	Hydrocephaly	7 months	No	
14	MRI	4.8	PS	TC/TR	Calcification, solid–cystic	Hypothalamic and OQ compression	1 year and 10 months	GGA → GAA (c.101G>A)	p.Gly34Glu
15	CT	3.2	SS	TC+RTX/PR	Calcification, solid	None	16 years	No	
16	MRI	2.5	SS	TC/PR	Calcification, solid–cystic	None	2 years and 6 months	No	

CT computerized tomography, *SS* suprasellar extension, *PS* parasellar extension, *CNS* central nervous system, *OQ* optic chiasm, *ON* optic nerve, *TC* transcranial surgery, *TSS* transsphenoidal surgery, *RTX* radiotherapy, *TR* total surgical removal, *PR* partial surgical removal

as having hypogonadism. GH replacement was given to six patients (four children and two young adults). Obesity was observed in nine patients and hyperfagia in three patients. Post-operative visual function was maintained in two patients, improved in six, and deteriorated in two patients. All patients showed histological characteristics of an adamantinomatous CP.

Genetic Analysis

In tumoral DNA as well as in cDNA obtained from CP tissues, we found seven different mutations in the *CTNNB1* gene (Table 2) in eight patients with CP. Six of them have been previously described: the g.304G>A, corresponding to c.94G>A at codon 32 (p.N32F); the g.308C>G, corresponding to c.98C>G at codon 33 (p.S33C); the g.308C>T, corresponding to c.98C>T at codon 33 (p.S33F); the g.320C>G, corresponding to c.110C>G at codon 37 (p.S37C) in two patient; the g.331A>G, corresponding to c.121A>G at codon 41 (p.T41A); and the g.332C>T, corresponding to c.122C>T at codon 41 (p.T41I). We also found one new mutation: the g.311G>A, corresponding to c.101G>A at codon 34 (p.G34E). We found no *CTNNB1* gene mutation in genomic DNA obtained from CP patients or in the subset of general Brazilian controls. We also did not find any *CTNNB1* gene mutation in the DNA or cDNA obtained from 15 normal pituitaries. We observed that patients younger than 18 years old presented more frequently *CTNNB1* mutation (seven of nine patients) than adults (one of seven) ($p=0.01$). On the other hand, there was no association between tumor size or the recurrence of the disease and the presence of *CTNNB1* mutation.

No mutations were identified in *HESX1*, *POU1F1*, and *PROP1* genes. However, in tumoral DNA from CP and in genomic DNA from controls, we found four single nucleotide polymorphisms (SNPs) in *PROP1* gene (Table 3). Allelic frequencies of each polymorphism were assessed in DNA from CP tissue and compared to genomic DNA from a subset of the normal Brazilian population. All polymorphisms were in the Hardy–Weinberg equilibrium, and allelic frequencies were similar in CP and genomic DNA from control subjects.

Table 3 *PROP1* SNPs in craniopharyngiomas

SNP	Genotype	Patients (%)	Controls (%)	p
p.A9A	CC	13	28	ns
(rs1135320)	CT	60	41	
	TT	27	31	
IVS1+3G>A	GG	27	31	ns
(rs4072924)	AG	53	38	
	AA	20	31	
p.A142T	GG	64	60	ns
(rs1800197)	GA	36	36	
	AA	–	4	
p.S20N	GG	100	100	ns
(rs7445271)				

ns not significant

microRNA Expression Profiling

Table 4 shows the relative expression of a panel of miRNA in CP and normal pituitaries. Expression of nine microRNAs (miR-141, let7a, miR-16, miR-449, miR-145, miR-143, miR-23b, miR-15a, and miR-24-2) was significantly lower in CP than in normal pituitaries. miR-150 was found to be overexpressed in CP while no differential expression was found in miR-16-1, miR-21, and miR-23a. Considering the age of the patients, there was no difference in the expression pattern of the examined microRNAs between children and adults. Considering the presence or absence of *CTNNB1* mutations, miR-16 was underexpressed in CP presenting *CTNNB1* mutation ($p=0.03$), and there was a tendency ($p=0.06$) to lower expression of miR-141 in tumoral tissues (Fig. 1a, b). We observed that six out of eight patients (75%) with CP without mutation presented both miR-16 and miR-141 upregulated. In addition, in the

other two patients, at least one of these miRNAs was upregulated. miR-23a and miR24-2 were more expressed ($p=0.04$) in patients with CP who underwent only one surgery than CP patients with two or more surgeries (Fig. 1c, d).

Discussion

In the present study, we reviewed the clinical manifestations of patients with adamantinomatous CP, by the time of the diagnosis, and performed the molecular analysis of *HESX1*, *PROP1*, *POU1F1*, and *CTNNB1* genes in order to elucidate their contribution to the tumorigenesis. We found that mutations or polymorphisms in pituitary transcription factors or their co-regulators are unlikely to contribute to the pathogenesis of CP. On the other hand, we reinforced that somatic *CTNNB1* gene mutations are related to the pathogenesis of adamantinomatous CP, mainly in children. We also demonstrated, for the first time, abnormal expression of a panel of microRNAs, including hyperexpression of miR-150 and underexpression of let-7a, miR-16, miR-15a, miR-23b, miR-24-2, miR-141, miR-143, miR-145, and miR-449 in adamantinomatous CP. Our data suggest that these small molecules can inhibit the Wnt signaling via interaction with different compounds of the Wnt pathway.

Endocrine as well as neurological disorders were found in the majority of patients. Three or more hormone deficiencies occurred in 62.5%, which is in agreement to the reported frequencies (54–100%) in previous studies [1, 26, 27]. Headache, central nervous system hypertension, and visual disturbances were observed in most patients probably due to the proximity to and the subsequent

Table 4 miRNA expression in craniopharyngiomas compared to normal pituitary tissues

miRNA	Mean ΔCt normal	Mean ΔCt tumor	Normal mean $2^{-\Delta\Delta Ct}$.median range	Tumor mean $2^{-\Delta\Delta Ct}$.median range	Fold tumor/normal
miR-150	−1.7±1.9	−4.5±2.2	1.3±1.2; 1.0 (0.02–4.6)	12.6 ±17.3; 8.1 (0.3–66.5)	1.7 ($p=0.01$)
miR-16-1	3.4±1.5	4.2±1.9	1.5±1.3; 1.0 (0.1–4.8)	1.5±1.4; 0.7 (0.04–4.4)	ns
miR-21	−6.4±1.4	−6.9±1.7	1.4 ±1.4; 1.0 (0.2–4.7)	2.8 ±4.9; 1.7 (0.2–20.7)	ns
miR-23a	−1.3±0.6	−1.6±1.6	1.0±0.4; 1.0 (0.5–1.8)	1.9±2.1; 1.5 (0.2–8.1)	ns
miR-141	−4.5±1.1	−1.3±2.8	1.4±0.8; 1.0 (0.2–2.7)	0.3±0.43; 0.1 (0–1.5)	−7.5 ($p<0.0001$)
let7a	−8.2±1.8	−4.8±1.9	1.7±1.7; 1 (0.5–6.7)	0.2±0.3; 0.1 (0.01–0.9)	−7.4 ($p<0.001$)
miR-16	−9.7±1.0	−6.7±1.9	1.2 ±0.9; 1.0 (0.2–4.2)	0.2 ±0.2; 0.1 (0–0.9)	−7.2 ($p<0.0001$)
miR-449	4.3±0.6	5.9±2.6	1.0 ±0.4; 1.0 (0.4–1.8)	1.8 ±4.3; 0.2 (0.03–16.3)	−6.6 ($p=0.007$)
miR-145	−1.1±1.1	1.6±2.7	1.2±1.3; 1.0 (0.2–5.5)	0.5±0.8; 0.19 (0–3.3)	−5.4 ($p=0.002$)
miR-143	−1.3±0.8	0.4±2.0	1.3±0.7; 1.0 (0.4–2.9)	1.0±1.8; 0.26 (0.05–7.4)	−3.8 ($p=0.01$)
miR-23b	−5.7±0.6	−4.6±1.5	1.0±0.4; 1.0 (0.4–1.6)	0.8±0.9; 0.31 (0.1–3.2)	−3.2 ($p=0.02$)
miR-15a	−1.4±0.8	0.1±1.5	1.0±0.5; 1.0 (0.3–2.1)	0.6±0.6; 0.39 (0.04–2.1)	−2.5 ($p=0.009$
miR24-2	2.6±0.6	4.2±2.6	1.2±0.5; 1.0 (0.4–2.2)	0.8±1.1; 0.4 (0–4.3)	−2.5 ($p=0.01$)

ns not significant

Fig. 1 miRNA relative expression in craniopharyngiomas. **a**, **b** miR-16 and miR-141 expression in craniopharyngiomas with or without *CTNNB1* mutation. **c**, **d** miR-23a and miR-24-2 expression in craniopharyngiomas from patients who underwent one or more than one surgery
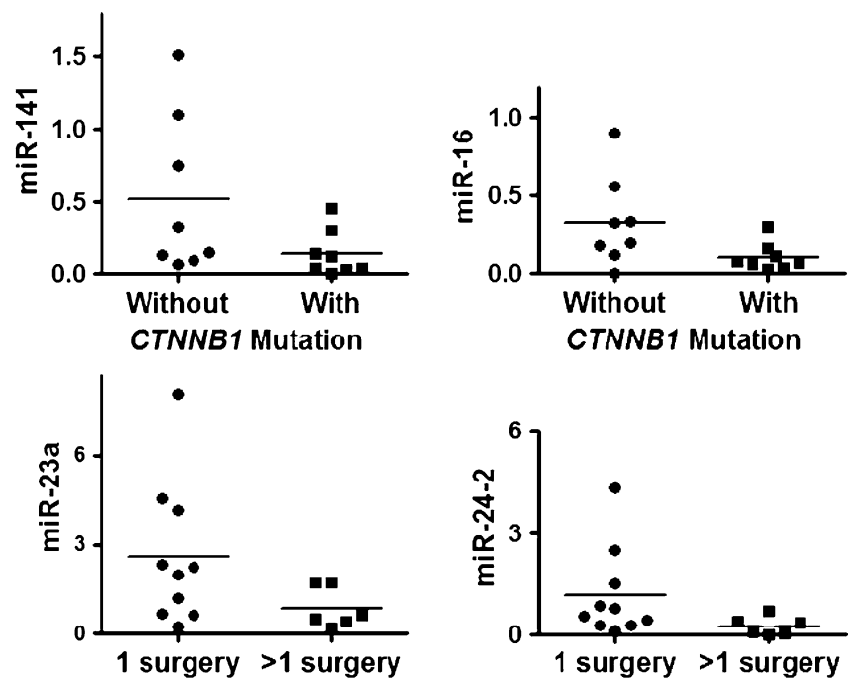
pressure effects of tumor on vital structures of the brain [1, 26, 27]. The invasion and compression of surrounding structures are due to the large tumor size observed in CP. Some reports demonstrated the average in macroscopic specimens of 3.5 cm at the time of initial surgery [1, 28]; in our series, the median tumor size was 4.4 cm, suggesting a delay in the diagnosis. Hyperphagia and obesity are the most distressing manifestations of hypothalamic injury and were observed in 50% of the patients before surgery [1, 27, 29]. Lesion in the pituitary–hypothalamic region has the potential to affect the autonomous nervous system output from the brain influencing gut hormone secretion, so critical for controlling energy homeostasis and food intake. After treatment, there was a slight increment (62.5%) of the number of patients presenting obesity/hyperphagia, which could be exacerbated by unmodulated hunger, reduced physical activity, and increased daytime sleepiness. Indeed, important somnolence was observed in five patients of our series. Recently, it has been demonstrated a blunted postmeal-related changes of PYY and ghrelin in obese CP compared to obese controls [30]. Hyperinsulinemia and decreased insulin sensitivity seem to be important contributing factors for observed gut hormonal changes in CP patient [30].

Surgical management, mainly transcranial approach, was undertaken in the majority of our patients, similarly to others [1, 31]. In our series, total resection (TR) was achieved in 56.3% of the patients, and a recurrence rate was of 22.2% while partial resection showed a recurrence rate of 57.1%; both rates of recurrence are in accordance with recurrence rates in the literature (TR=0–62% and PR=25–100%) at 10-year follow-up [1, 26, 31]. In the present study, 37.5% of the patients underwent a second or even a third surgery. Radiotherapy, which has a limited value in CP [1], was performed in about 20%. Hypopituitarism and diabetes insipidus occurred both in 93.8% of the patients in post-operative period. In fact, there was no recovery of pre-existing pituitary hormone deficiencies after surgical intervention. All patients showed histological characteristics of an adamantinomatous CP, which is the most common type [9] and has a broader age distribution with a predilection for childhood and early adolescence [1, 28, 32].

Pituitary transcription factors involved in formation and/or development of the adenohypophysis might be implicated in CP pathogenesis. Therefore, in the present study, we performed the molecular analysis of three homeodomain-containing transcription factors, *HESX1*, *PROP1*, and *POU1F1*, involved in anterior lobe of the pituitary gland development, and the *CTNNB1* gene, a co-regulator for transcription activity of *PROP1* [33]. Among all these genes, PROP1 may be a commitment factor, since it is initially expressed in the dorsal pouch and developing anterior pituitary where the somatolactotrophs and definitive thyrotrophs will eventually appear [33, 34]. Further, PROP1 is required for expression of *POU1F1*, which itself is necessary for differentiation of the same lineages [35, 36]. For instance, PROP1 is known to extinguish gene expression of HESX1 [37]. No mutations were identified in *HESX1*, *PROP1*, and *POU1F1* genes in CP. SNPs in *PROP1* gene are also unlikely to contribute to CP formation. Of interest, sellar masses have been described in association with inactivating *PROP-1* mutations that

generate nonfunctional proteins, which fail to induce normal development of pituitary cells [38, 39], leading to hyperplasia of the adenohypophysis, cystic pituitary masses mimicking CP, or Rathke's cleft cysts [38, 39]. These findings would suggest a direct involvement of these transcription factors in the pathogenesis of CP. However, our data did not indicate that mutation or SNPs in these pituitary transcription factors, involved in Rathke's pouch and anterior pituitary development, might contribute to the adamantinomatous CP pathogenesis.

During pituitary development, Wnt/β-catenin pathway provides a key signal for determining cell lineages, with direct interactions between β-catenin and the tissue-specific homeodomain factor Prop1, rather than lymphoid enhancer-binding factor 1/T cell-specific transcription factor, serving as the mechanism for transcriptional activation of the POU1F1 gene. A Prop1/β-catenin complex simultaneously represses expression of the Hesx1 paired-like homeodomain factor via recruitment of a series of corepressors, including HDACs 1/2, Reptin, and Groucho/TLEs [40]. These results establish a transcriptional switching mechanism for β-catenin control of cell-fate determination [33]. We found seven different mutations in CTNNB1 gene, including the novel p. Gly34Glu mutation, in 50% of the 16 adamantinomatous CP examined. These mutations were all somatic in origin as long as they were not found in genomic DNA from affected patients. Interestingly, these mutations are significantly more frequent in younger patients. The prevalence of CTNNB1 mutations in adamantinomatous CP observed in different series varies from 16% to 100% [9, 12, 41]. The majority of CTNNB1 gene mutations affected serine or threonine phosphorylation sites encoded by exon 3, which encodes the degradation targeting box of beta-catenin. Accumulation of nuclear β-catenin protein results in enhanced transcriptional activity, as demonstrated in vitro for CTNNB1 variants carrying similar mutations in exon 3 [42]. Furthermore, strong β-catenin expression has been demonstrated in immunohistochemical studies of the adamantinomatous subtype [9, 11, 13] indicating reactivation of the Wnt signaling pathway, which is implicated in the development of several neoplasms including CP [41]. Indeed, recently, study using the primary human adamantinomatous CP cell cultures and small interfering RNA (siRNA) directed against the CTNNB1 gene showed that tumor cell migration was significantly reduced suggesting that activated Wnt signaling serves as a promoter of the epithelial migration machinery [43].

Based on bioinformatics analysis, we chose a panel of miRNAs which target the Wnt pathway in order to better understanding the role of these post-transcriptional regulators on Wnt signaling cascade. We demonstrated, for the first time, hyperexpression of miR-150 and underexpression of let-7a, miR-16, miR-15a, miR-23b, miR-24-2, miR-141,

miR-143, miR-145, and miR-449 in CP. In silico analysis demonstrated that miR-150, miR-23b, miR-24-2, miR-141, and miR-449 are regulators of CTNNB1 gene. All these miRNAs, but miR-150, might act as tumor suppressors. In normal conditions, miRNAs could bind with partial homology to CTNNB1 mRNA leading to mRNA translation inhibition, while binding with high homology would cause mRNA degradation. Thus, we postulated a putative connection between downregulation of tumor suppressive miRNAs and consequently the accumulation of β-catenin in the cytoplasm and nucleus, which would affect transcription of large number of Wnt targets during Wnt signaling in CP. Recently, it has been demonstrated in adrenal tumors that miR-15, miR-16, and miR-24-2 modulate Wnt4 (Wingless tipo 4) and mir-449 modulates Wisp2 (WNT1-inducible signaling pathway protein 2, important players of Wnt signaling pathway [21]. On the other hand, we observed an overexpression of mir-150 in CP, which in silico modulates adenomatous polyposis coli (APC); thus, mir-150 could be acting as an oncoMir. APC proteins have a role in β-catenin degradation, and mutations of the APC gene activate the Wnt pathway and lead to tumor formation [42]. Given the number of Wnt genes and their widely ranging functions, it is not surprising that mis-regulation of such an important pathway is involved in the genesis of many cancers, including craniopharyngioma.

To discover a putative association between the presence of CTNNB1 mutation and the severity of the disease, we studied the correlation of different variables such as age at the diagnosis, tumor size, and recurrence and the micro-RNA expression levels. We observed no association between the presence of CTNNB1 mutation and tumor size or recurrence of the disease, suggesting that the CTNNB1 mutation seems to be one among other mechanisms that might be involved in the formation of CP. Regarding miRNA expression, we observed no difference in the expression of each miRNA between children and adults. On the other hand, miR23a and miR24-2 were more expressed in patients with CP who underwent only one surgery than CP patients with two or more surgeries. More interestingly, miR-16 and miR-141 were underexpressed in CP patients presenting CTNNB1 mutation. Indeed, the majority of CTNNB1 non mutated CP (75%) presented both miR-141 and miR-16 upregulated. These miRNA elevations could compensate for the lack of CTNNB1 mutation and serve to activate the Wnt pathway. Therefore, we suggest that at least two different defects could be involved in the CP pathogenesis and outcome: the presence of CTNNB1 mutations and/or the deregulation of the Wnt pathway by differential miRNA expression. Further studies are needed to predict miRNA target genes for either downregulated or upregulated miRNAs in CP.

In conclusion, our findings indicate that the majority of patients with craniopharyngioma present endocrine disorders and hypothalamic injury, large tumors with invasion and compression of surrounding structures. All therapeutic standard of care did not allow tumor resection or growth control leading patients to undergo repetitive surgeries and to present high prevalence of hypopituitarism and diabetes insipidus. In addition, mutations or SNPs in pituitary transcription factors involved in Rathke's pouch and anterior pituitary development are unlikely to contribute to the adamantinomatous CP pathogenesis. Conversely, *CTNNB1* gene mutation was observed in a high prevalence, confirming that this alteration is a hallmark of the adamantinomatous CP, mainly in children. Finally, we investigated, for the first time, the microRNA signature and its clinical significance in CP; these data suggest the Wnt signaling pathway, via microRNA regulation, might be a mediator of CP tumorigenesis.

Disclosure statement The authors declare that they have no conflict of interest.

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