Clinical and biological significance of adamantinomatous craniopharyngioma with *CTNNB1* mutation

Takuma Hara, MD,¹ Hiroyoshi Akutsu, MD, PhD,¹ Shingo Takano, MD, PhD,¹ Hiroyoshi Kino, MD,¹ Eiichi Ishikawa, MD, PhD,¹ Shuho Tanaka, MD, PhD,² Hidetaka Miyamoto, MD,² Noriaki Sakamoto, MD, PhD,³ Keiichiro Hattori, MD,⁴ Mamiko Sakata-Yanagimoto, MD, PhD,⁴ Shigeru Chiba, MD, PhD,⁴ Takashi Hiyama, MD,⁵ Tomohiko Masumoto, MD, PhD,⁵ and Akira Matsumura, MD, PhD¹

Departments of ¹Neurosurgery, ²Otolaryngology, ³Diagnostic Pathology, ⁴Hematology, and ⁵Radiology, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

OBJECTIVE The Wnt/ β -catenin signaling pathway is strongly implicated in the pathogenesis of adamantinomatous craniopharyngioma (adaCP). However, there is no evidence that the *CTNNB1* mutation activates the target gene of Wnt/ β -catenin signaling, and it is unknown whether it affects the tumorigenesis of adaCP. To assess the effect of the *CTNNB1* mutation of adaCP, the authors analyzed the correlation between the mutation and clinical, radiological, pathological, and biological findings.

METHODS Between 2003 and 2015, 42 patients (24 male and 18 female, median age 42 years) with either papillary craniopharyngioma (papCP) or adaCP underwent tumor resection at the authors' institution. *BRAF* V600E and *CTNNB1* in papCP and adaCP samples were sequenced by next-generation sequencing and the Sanger method, and mRNA expression levels of *Axin2* and *BMP4* were evaluated by RT-PCR. *Axin2*, *BMP4*, β -catenin, and *BRAF* expression were evaluated by immunohistochemistry. Other data were collected from clinical reports.

RESULTS The *BRAF* V600E mutation was detected in all 10 cases of papCP (100%). *CTNNB1* exon 3 mutations were detected in 21 of 31 (68%) cases of adaCP, excluding 1 case for which there were no available sequence data. The mRNA expression level of *Axin2* was significantly higher in adaCPs with a *CTNNB1* mutation than in those without ($p < 0.05$). The immunohistochemical findings of *Axin2* and *BMP4* did not correlate with *CTNNB1* mutation positivity. When patients who received adjuvant radiation therapy were excluded, progression-free survival was shorter in the mutation-positive group than in the mutation-negative group (log-rank test, $p = 0.031$). Examination of clinical characteristics and immunohistochemical findings of adaCPs showed that there was no significant correlation between *CTNNB1* mutation positivity and age, sex, tumor volume, gross-total resection, optic tract edema, calcification, or T1 signal intensity of cyst fluid on MRI, β -catenin, and MIB-1 index.

CONCLUSIONS These results raise the possibility that the *CTNNB1* mutation in adaCP may be associated with disease recurrence, and genes related to the Wnt/ β -catenin signaling pathway might represent a therapeutic target.

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KEYWORDS *BRAF*; craniopharyngioma; *CTNNB1*; Wnt/ β -catenin; oncology

CRANIOPHARYNGIOMAS (CPs) are histologically benign, solid or mixed solid-cystic epithelial tumors of the sellar and suprasellar region.⁸ They originate from the epithelium of Rathke's pouch and are divided into adamantinomatous-type CP (adaCP) and papillary-type CP (papCP) according to the World Health Organization classification.¹⁸ These 2 subtypes differ not only in

histopathology but also in epidemiological, radiological, genomic, and transcriptomic characteristics.²⁶ CPs are the third most common pediatric brain tumors and account for 2.3% of all primary intracranial neoplasms.⁵ AdaCP is the most common non-neuroepithelial intracerebral neoplasm in children.^{12,27} Treatment of both types of CPs remains difficult due to anatomical proximity of these le-

ABBREVIATIONS ada = adamantinomatous; CP = craniopharyngioma; FFPE = formalin-fixed paraffin-embedded; GSK3- β = glycogen synthase kinase 3- β ; GTR = gross-total resection; IHC = immunohistochemistry; NGS = next-generation sequencing; pap = papillary; PFS = progression-free survival; RT = radiation therapy; RT-PCR = real-time polymerase chain reaction.

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sions to important functional structures, for example, the optic nerve, hypothalamus, and pituitary stalk. Achieving gross-total resection (GTR) of either type of CP without any complications remains challenging,⁵ and non-GTR results in recurrence requiring reoperation or adjuvant radiation therapy (RT). In Japan, the 5-year overall survival rate is 96.5% for patients with either type of CP, and the 5-year progression-free survival (PFS) rate is 69.7%;⁵ recurrence of tumor in young patients is problematic. The long-term management of both types of CPs is difficult and complex, and there is no clear consensus on the optimal management of residual tumor.

In recent years, the Wnt/ β -catenin signaling pathway has been strongly implicated in the pathogenesis of adaCP. Genetic analyses have shown that 64%–99% of adaCPs have activating mutations in exon 3 of the β -catenin gene *CTNNB1*.^{1,3,4,12,26} *CTNNB1* mutation causes aberrant β -catenin protein accumulation and translocation to the nucleus, where it facilitates transcription of β -catenin target genes and stimulates cellular proliferation and other Wnt-regulated cellular processes.^{2–4,16} However, there is no clear evidence that *CTNNB1* mutation activates the target genes of Wnt signaling (*AXIN2*, *BMP4*, etc.), and it is unknown whether it affects the tumorigenesis of adaCPs.

In previous reports, certain factors, including tumor volume, histopathologic type, lesion position, degree of residual tumor after resection, region of invasion, and aberrant membranous expression of β -catenin, have been shown to correlate with the risk of recurrence.^{17,21} However, the relationship between the biological findings of both types of CPs and PFS remains unclear. Furthermore, the clinical, radiological, pathological, and biological features of adaCP with *CTNNB1* mutation and the features of adaCP without *CTNNB1* mutation are unclear. From the standpoint of treatment, although the *BRAF* V600E mutation has been identified as a molecular and therapeutic target of papCP,^{14,15} the therapeutic target of adaCP remains unknown.

In this study, in order to assess the effect of the *CTNNB1* mutation in adaCP, we examined whether a positive mutation in adaCP is present using targeted next-generation and Sanger sequencing. Using those data, we analyzed the correlation between the mutation and clinical, radiological, pathological, and biological findings (Wnt/ β -catenin signaling pathway).

Methods

Patients

This study was approved by the ethics committee of University of Tsukuba Hospital, and informed consent was obtained from the patients or their parent/guardian. Thirty-two patients with adaCP and 10 patients with papCP as the control group—patients whose cases had been diagnosed at the department of pathology in our university—were included in this study. The operations were performed by senior surgeons (S. Tanaka and H.A.) at our hospital from 2003 to 2015. All patients underwent endoscopic endonasal or transcranial surgery depending on tumor localization. However, we performed endoscopic transventricular cyst fenestration or intended partial/subtotal tumor resec-

tion followed by adjuvant RT in some adults, especially elderly patients in whom aggressive resection might result in poor performance status or severely impaired quality of life. If postoperative adjuvant RT was needed, fractionated radiotherapy or CyberKnife treatment was initiated within 3 months after surgery. In treating pediatric patients, we tried to avoid RT in order to prevent late radiation toxicity. The following were inclusion criteria for this study: 1) availability of hematoxylin and eosin–stained sections for histological review of the case; and 2) availability of formalin-fixed paraffin-embedded (FFPE) tissue for molecular characterization (extraction of DNA and RNA). A total of 62 surgeries for CP were performed in 54 patients during the study period. Twelve patients who did not meet the inclusion criteria were excluded, because we could not obtain sufficient tissue sample for extracting DNA and RNA in some cases of adaCP, especially in cases in which endoscopic transventricular cyst fenestration was performed, followed by RT. As a result, 42 patients were included in this study. Histologically, 10 (24%) cases were papCPs and 32 (76%) were adaCPs. PFS was analyzed in adaCP patients with at least 12 months of follow-up. PFS time was defined as the interval between surgery and confirmation of recurrence by radiological findings.

Histology and Immunohistochemistry

All tissue specimens were fixed in 10% formaldehyde and paraffin embedded according to routine protocols. Serial 4- μ m-thick sections were then prepared, and immunostaining for Ki67 (Immunotech Laboratories Inc.) was performed. Ki67 positivity of nuclei was determined by counting at least 1000 tumor cells in a homogeneously stained area. Immunostaining was performed with a commercially available polyclonal *Axin2* antibody (ab32197, dilution 1:500; Abcam plc) at 1 mg/ml; a polyclonal *BMP4* antibody (ab39973, dilution 1:100; Abcam) at 1 mg/ml; a *BRAF* V600E antibody (E19290, dilution 1:50; Spring Bioscience); and an LSAB2 kit (DAKO) with Tris-EDTA antigen retrieval buffer (pH 9.0). Immunostaining was also conducted with a monoclonal β -catenin antibody (M3539, dilution 1:200; DAKO) at 58.7 μ g/ml and EnVision + Dual Link (DAKO).

All histological findings were independently evaluated by 2 different observers (N.S. and H.K.). The observers were blinded to patients' data. When discrepancies occurred, the 2 observers discussed the case to reach a consensus. Cases with > 50% of cells stained were defined as strongly positive (++; *Axin2*, *BMP4*, β -catenin), and cases with < 50% of cells stained were defined as weakly positive (> 10%, +) or negative (< 10%, –). “Catenin positive” means positive catenin nuclear staining. In this study, strong and weak positivity on immunohistochemistry (IHC) were regarded as positive findings. Negative controls (X090302, DAKO) at the same protein concentration to primary antibody were used for evaluation of IHC (*Axin2*, *BMP4*).

DNA and cDNA Preparation

For DNA extraction, snap-frozen (–80°C) and FFPE tissue samples were used. The frozen sections of all tissue

samples were microscopically analyzed to confirm tumor content. We collected DNA from FFPE and frozen tissue as much as possible. DNA in frozen and FFPE tissue was extracted using DNeasy Blood & Tissue Kit (Qiagen) and QIAamp DNA Mini Kit (Qiagen), respectively.

For RNA extraction, the FFPE sample was scraped under microscope guidance from three 10- μ m-thick sections, using RNeasy FFPE kit (Qiagen). RNA of adaCP was considered to be of sufficient purity at A260/A230 and A260/A280 ratios of RNA > 1.7 measured by Nano Drop 2000 (Thermo Fisher Scientific). The median RNA integrity number was 2.4 (1.8–2.5) measured by Agilent 2100 bioanalyzer (Agilent Technologies) and 31 of 32 RNA integrity numbers fell within the range of 2.3–2.5. Subsequently, for reverse transcription, SuperScript IV Master Mix with ezDNase Enzyme (Thermo Fisher Scientific) was used according to the manufacturer's instructions.

Mutation Analysis

Genomic DNA was used for each amplification reaction using primers specific for *BRAF* and for *CTNNB1*. We prepared 2 different primers: one for next-generation sequencing (NGS) and one for Sanger sequencing. The polymerase chain reaction (PCR) was performed according to the manufacturer's instructions for KOD-plus-neo (TOYOBO). The NGS primer sequence of *BRAF* forward was 5'-TTTCCTTTACTTACTACACCTCAGA-3' and the reverse was 5'-CCATCCACAAAATGGATCCAGAC-3'. The primer sequence of *CTNNB1* forward was 5'-ATGG CCATGGAACCAGACAG-3' and the reverse was 5'-TG GGAGGTATCCACATCCTCT-3'. NGS targeted sequencing was performed for all cases. The libraries were made by using the Ion Plus fragment library kit (Life Technologies) according to the protocol described in "Prepare Amplicon Libraries Requiring Fragmentation Using the Ion Xpress Plus Fragment Library Kit" (available at thermofisher.com). PCR amplicons were ligated to barcoded adapters and P1 adapters and then amplified. The libraries were then subjected to deep sequencing on Ion Torrent PGM according to the standard protocol using Ion 318 chips (Thermo Fisher Scientific). The data were analyzed by Valiant caller 4.2 (Thermo Fisher Scientific). Single-nucleotide variants or insertions/deletions with a frequency $\geq 1\%$ were adopted as mutations.¹⁹ All candidate mutations were validated by genomic PCR followed by Sanger sequencing. The Sanger primer sequence of *BRAF* forward was 5'-TGCTTGCTCTGATAGGAAAATG-3' and the reverse was 5'-CCACAAAATGGATCCAGACA-3'. The primer sequence of *CTNNB1* forward was 5'-GGCC ATGGAACCAGACAGAA-3' and the reverse was 5'-GC ATTCTGACTTTCAGTAAGGCAAT-3'.

mRNA Expression Level by RT-PCR

Axin2 is a well-recognized inhibitor and target gene of β -catenin.¹⁴ *BMP4* expression is said to be enhanced in tumors with oncogenic β -catenin elevation.¹⁵ *Axin2* and *BMP4* are well-known indicators of Wnt signaling activated by nuclear β -catenin accumulation in cells. Relative mRNA expression was analyzed by real-time poly-

merase chain reaction (RT-PCR) using specific TaqMan assays (Applied Biosystems): *Axin2* (Hs01063170_m1) and *BMP4* (Hs00370078_m1). The values were normalized with respect to the mean expression of the endogenous control: *PGK1* (Hs99999906_m1). The reactions were performed according to the manufacturer's recommendation, and relative mRNA expression values were determined using the $2^{-\Delta\Delta C_t}$ method.

Evaluation of Radiological Findings

All radiological findings of adaCPs and papCPs were independently evaluated by 2 neuroradiologists (T.M. and T.H.), who were blinded to patient data. When discrepancies occurred, the 2 neuroradiologists discussed the case to reach a consensus. We evaluated tumor volume by MRI (estimated by $[AP \times LR \times SI]/2$, where AP is the anteroposterior measurement, LR is the left-right measurement, and SI is the superior-inferior measurement). Edema of the optic tract,²³ calcification, and high intensity of cyst fluid on T1-weighted MRI are the representative radiological findings of adaCPs.^{8,16} All of these findings were evaluated on preoperative MRI and CT to investigate whether there was a correlation between the mutation and any radiological finding.

Statistical Analyses

All statistical analysis was performed using SPSS 22.0 software (IBM Corp.). The chi-square test or Fisher's exact test was used to evaluate relationships between 2 variables. Differences between variables were evaluated using the Mann-Whitney U-test. PFS analysis was performed using the Kaplan-Meier method, and statistical significance was determined by the log-rank test. $p < 0.05$ was considered statistically significant.

Results

Clinical and Immunohistochemical Findings

Of the 42 patients, 24 were male (54%). The patients' median age at the time of surgery was 42 years (range 2–73 years); 11 (26%) of the patients were less than 15 years old. Five patients (12.0%) underwent repeated operations. (The tumor specimens used for analysis, however, were all from the initial operation.) GTR was accomplished in 17 cases (40.5%), subtotal in 19 (45.2%), and partial in 6 (14.3%). The duration of follow-up ranged from 4 to 170 months (median 46 months). Nineteen patients received adjuvant RT (fractionated radiotherapy was used in 16 patients and CyberKnife treatment in 3 patients). During the follow-up period, tumor recurrence was detected on MRI in 9 (21.4%) patients.

As for radiological findings, the tumor volume ranged from 0.5 to 104.3 cm³ (median 9.5 cm³). Edema of the optic tract was observed in 9 (90%) cases of papCP and in 13 (40.6%) cases of adaCP. Calcification within the tumor was not observed in papCPs, but it was seen in 30 (93.8%) cases of adaCP. High intensity of cyst fluid on T1-weighted MRI was observed in 1 (10%) case of papCP and in 22 (68.8%) cases of adaCP.

In regard to immunostaining, none of the cases of papCP showed positive immunohistochemical intranucle-

TABLE 1. Clinical, radiological, and pathological data of the 42 patients included in the study

Case No.	Path Findings	Age (yrs), Sex	Reop Case*	GTR	Adj RT	FU (mos)	Tumor Vol in cm ³ †	Optic Tract Edema	T1WI Intensity of Cyst Fluid	Calcification	IHC β -Catenin	IHC VE1
Pap-1	Pap	32, F	–	–	+	88	18.5	+	Hypo	–	–	++
Pap-2	Pap	56, M	–	–	+	4	21.8	+	Hyper	–	–	++
Pap-3	Pap	54, M	–	–	+	6	12.5	+	Hypo	–	–	++
Pap-4	Pap	57, M	–	–	–	83	14.7	+	Hypo	–	–	++
Pap-5	Pap	64, F	–	–	+	59	5.3	+	Not cystic	–	–	++
Pap-6	Pap	40, F	–	–	+	16	9.8	+	Not cystic	–	–	++
Pap-7	Pap	20, F	–	+	–	14	1.5	+	Hypo	–	–	++
Pap-8	Pap	43, F	–	+	–	36	8.7	+	Iso	–	–	++
Pap-9	Pap	60, M	–	–	+	8	3.9	+	Hypo	–	–	++
Pap-10	Pap	64, F	–	–	+	29	1.3	–	Iso	–	–	++
Ada-1	Ada	71, F	–	–	+	22	2.9	–	Iso	–	+	–
Ada-2	Ada	4, F	–	+	–	20	7.0	+	Hypo	+	++	–
Ada-3	Ada	25, F	–	+	–	33	18.9	–	Hyper	+	++	–
Ada-4	Ada	54, M	–	+	–	16	12.4	+	Hyper	+	++	–
Ada-5	Ada	7, M	–	–	–	48	5.1	+	Hypo	+	+	–
Ada-6	Ada	20, M	–	–	+	170	28.1	–	Hypo	+	+	–
Ada-7	Ada	49, F	–	–	–	4	15.4	–	Hyper	+	+	–
Ada-8	Ada	59, M	–	–	–	56	23.2	–	Hyper	+	++	–
Ada-9	Ada	2, M	–	+	–	21	104.3	–	Iso	+	++	–
Ada-10	Ada	5, F	–	+	–	34	3.1	–	Hyper	+	–	–
Ada-11	Ada	3, M	–	–	–	29	15.5	–	Hyper	+	++	+
Ada-12	Ada	62, F	+	–	+	52	12.5	+	Hyper	+	+	++
Ada-13	Ada	56, M	–	–	+	110	5.8	+	Hypo	+	+	–
Ada-14	Ada	35, M	–	–	+	95	12.9	+	Hyper	+	+	–
Ada-15	Ada	10, M	–	–	+	66	5.3	+	Hyper	+	+	+
Ada-16	Ada	52, F	+	–	+	65	19.4	+	Hyper	+	–	–
Ada-17	Ada	12, F	–	+	–	64	10.2	–	Hyper	+	–	++
Ada-18	Ada	70, F	–	+	–	33	2.5	+	Iso	+	+	++
Ada-19	Ada	59, M	+	–	+	69	26.9	–	Hyper	+	++	++
Ada-20	Ada	10, M	–	+	–	47	16.7	+	Hyper	+	++	–
Ada-21	Ada	54, F	–	+	–	57	2.0	+	Hypo	+	–	–
Ada-22	Ada	22, M	+	+	–	18	0.5	–	Hyper	+	+	–
Ada-23	Ada	64, M	–	–	–	30	6.9	+	Hyper	+	+	–
Ada-24	Ada	59, M	+	+	–	24	1.3	–	Hyper	+	+	–
Ada-25	Ada	7, M	–	+	–	17	1.8	–	Hyper	+	–	++
Ada-26	Ada	16, M	–	+	–	45	3.0	–	Hypo	+	++	–
Ada-27	Ada	73, F	–	+	–	71	9.2	–	Hyper	+	–	–
Ada-28	Ada	25, M	–	–	–	62	1.8	–	Hyper	+	–	–
Ada-29	Ada	15, M	–	–	+	90	4.0	–	Hyper	+	+	++
Ada-30	Ada	44, M	–	–	+	93	39.0	+	Iso	+	+	++
Ada-31	Ada	10, M	–	–	+	90	67.7	–	Hyper	+	+	–
Ada-32	Ada	6, F	–	–	+	109	19.8	–	Hyper	–	+	–

Ada = adamantinomatous; adj = adjunctive; FU = follow-up; hyper = hyperintense; hypo = hypointense; IHC = immunohistochemistry; iso = isointense; pap = papillary; path = pathological; RT = radiation therapy; T1WI = T1-weighted imaging.

* Indicates a case in which repeated operation was required.

† $(AP \times LR \times SI)/2$, where AP is the anteroposterior measurement, LR is the left-right measurement, and SI is the superior-inferior measurement.

ar staining of β -catenin while all cases had strong positive staining of VE1. Among the cases of adaCP, 6 (19%) showed strong positive immunohistochemical staining of

VE1 and 7 (22%) were negative for β -catenin. These data are presented in Table 1 and in panels A–D of the Supplementary Figure (available online).

TABLE 2. The mutation analysis data of patients with craniopharyngioma

Case No.	<i>BRAF</i> V600E Mutation (NGS)	Allele Frequency	<i>BRAF</i> V600E Mutation (Sanger)	<i>CTNNB1</i> Mutation (NGS)	Allele Frequency	<i>CTNNB1</i> Mutation (Sanger)	Final Eval of <i>CTNNB1</i> Mutation
Pap-1	+	46.7%	+			–	
Pap-2	+	37.8%	+			–	
Pap-3	+	34.8%	+			–	
Pap-4	+	34.3%	+			–	
Pap-5	+	33.1%	+			–	
Pap-6	+	32.5%	+			–	
Pap-7	+	32.1%	+			–	
Pap-8	+	29.4%	+			–	
Pap-9	+	19.1%	+			–	
Pap-10	+	10.2%	+			–	
Ada-1			–	T41I (122C>T)	46.3%	T41I (122C>T)	+
Ada-2			–	T41I (122C>T)	41.2%	T41I (122C>T)	+
Ada-3			–	T41I (122C>T)	41.1%	T41I (122C>T)	+
Ada-4			–	T41I (122C>T)	38.0%	T41I (122C>T)	+
Ada-5			–	D32G (95A>G)	35.5%	D32G (95A>G)	+
Ada-6			–	T41A (121A>G)	35.5%	T41A (121A>G)	+
Ada-7			–	T41I (122C>T)	29.9%	T41I (122C>T)	+
Ada-8			–	T41I (122C>T)	28.9%	T41I (122C>T)	+
Ada-9			–	S33F (98C>T)	26.5%	S33F (98C>T)	+
Ada-10			–	G34E (101G>A)	23.3%	G34E (101G>A)	+
Ada-11			–	D32V (95A>T)	20.2%	D32V (95A>T)	+
Ada-12			–	G34R (100G>A)	17.2%	G34R (100G>A)	+
Ada-13			–	D32Y (94G>T)	15.5%	D32Y (94G>T)	+
Ada-14			–	S33C (98C>G)	13.4%	S33C (98C>G)	+
Ada-15			–	S33F (98C>T)	13.1%	S33F (98C>T)	+
Ada-16			–	S33P (97T>C)	12.2%	S33P (97T>C)	+
Ada-17			–	S33C (98C>G)	10.9%	S33C (98C>G)	+
Ada-18			–	S37C (110C>G)	8.4%	S37C (110C>G)	+
Ada-19			–	D32N (94G>A)	6.2%	D32N (94G>A)	+
Ada-20			–	D32N (94G>A)	5.2%	D32N (94G>A)	+
Ada-21			–	S37F (110C>T)	4.8%	S37F (110C>T)	+
Ada-22			–	S33F (98C>T)	9.4%	–	–
Ada-23			–	S33F (98C>T)	6.5%	–	–
Ada-24			–	G34R (100G>C)	3%	–	–
Ada-25			–	D32N (94G>A)	2.8%	–	–
Ada-26			–	–	0%	–	–
Ada-27			–	–	0%	–	–
Ada-28			–	–	0%	–	–
Ada-29			–	–	0%	–	–
Ada-30			–	–	0%	–	–
Ada-31			–	–	0%	–	–
Ada-32			–	NA	NA	NA	NA

Eval = evaluation; NA = not available.

***BRAF* and *CTNNB1* Mutations in CP**

Next-generation sequencing (NGS) and validation by Sanger sequencing were used to detect *BRAF* V600E and *CTNNB1* exon 3 mutations in DNA extracted from tumor tissue samples. These mutation analysis data are

summarized in Table 2. The *BRAF* V600E mutation was detected in all 10 (100%) cases of papCP, and *CTNNB1* exon 3 mutations were detected in 21 of 31 (68%) cases of adaCP; the sequence data of 1 case (ada-32) could not be analyzed. Among the detected *CTNNB1* mutations, the

TABLE 3. Summary of characteristics of adamantinomatous CPs according to *CTNNB1* genetic status

Characteristic	CTNNB1 Mutation Status		p Value
	Positive	Negative	
No. of cases	21	10	
Clinical			
Allele frequency (%), mean	22.5 ± 13.0	2.2 ± 3.2	<0.001
Age			
Median	35	23.5	0.755
Range	2–71	7–73	
Sex			
Male	11	9	0.055
Female	10	1	
Tumor vol in cm ³ , mean	16.7 ± 21.1	13.5 ± 20.2	0.072
GTR	9 (43%)	5 (50%)	1.000
Adj RT	8 (38%)	3 (30%)	0.712
Recurrence	7 (33%)	1 (10%)	0.141
Radiological			
Optic tract edema	11 (52%)	2 (20%)	0.129
T1WI high intensity of cyst fluid	13 (62%)	8 (80%)	0.429
Calcification	20 (95%)	9 (90%)	1.000
IHC			
β-catenin ++ & +	17 (81%)	7 (70%)	0.652
Axin2 ++ & +	19 (90%)	7 (70%)	0.296
BMP4 ++ & +	16 (76%)	8 (80%)	1.000
MIB-1 (%)*	7.0 ± 4.1	7.1 ± 3.9	0.950
RT-PCR			
<i>Axin2</i> (relative mRNA expression level), mean	90.7 ± 58.3	49.0 ± 42.6	0.043
<i>BMP4</i> (relative mRNA expression level), mean	2.1 ± 1.6	1.5 ± 1.2	0.186

Values are numbers of cases (%) unless otherwise indicated. Means are presented with SDs.

most frequent point mutation was T41I (122C > T), which was seen in 6 of 31 (19%) cases. Four cases were excluded from the *CTNNB1* mutation-positive group after validation. The relationship between mutations and allele frequency was examined. Four of 8 (50%) cases with NGS allele frequency of less than 10% were confirmed to be mutation positive by Sanger sequencing. All cases (17 of 17 [100%]) that had an allele frequency of more than 10% were confirmed to be mutation positive. Allele frequency of *CTNNB1* mutation was significantly higher in mutation-positive cases (22.5% ± 13.0%) than in mutation-negative cases (2.2% ± 3.2%) ($p < 0.001$).

Comparison of Cases of adaCP With and Without *CTNNB1* Mutation

Clinical and Immunohistochemical Differences

Correlations between *CTNNB1* mutation positivity and clinical characteristics and immunohistochemical findings of adaCPs are summarized in Tables 1 and 3. No significant correlation was seen between *CTNNB1* mutation positivity and clinical and radiological factors (age, sex,

tumor volume, GTR, optic tract edema, calcification, and high intensity of cyst fluid on T1-weighted MRI). The immunohistochemical findings of positive catenin staining and MIB-1 index did not correlate with *CTNNB1* mutation positivity (Table 3). For analysis of PFS as a prognostic factor, we performed Kaplan-Meier survival analysis to assess the relationship between *CTNNB1* mutation positivity and PFS in all of the cases except for the one in which the sequence data could not be analyzed (ada-32) and one with short follow-up (ada-7). The PFS was not significantly shorter in the mutation-positive group ($n = 20$; 1-year PFS, 95%; 2-year PFS, 68.9%; 5-year PFS, 61.3%) than in the mutation-negative group ($n = 10$; 1-year PFS, 100%; 2-year PFS, 100%; 5-year PFS, 83.3%) (log-rank test, $p = 0.141$) (Fig. 1A). We performed Kaplan-Meier survival analysis to assess the relationship between adjuvant RT and PFS (log-rank test, $p = 0.004$) (Fig. 1B).

Finally, we performed Kaplan-Meier survival analysis to assess the relationship between *CTNNB1* mutation positivity and prognosis, excluding the 11 patients who had undergone adjuvant RT. The PFS was significantly shorter in the mutation-positive group ($n = 12$; 1-year PFS, 91.7%; 2-year PFS, 45.7%; 5-year PFS, 22.9%) than in the mutation-negative group ($n = 7$; 1-year PFS, 100%; 2-year PFS, 100%; 5-year PFS, 66.7%) (log-rank test, $p = 0.031$) (Fig. 1C). There was no significant association between *CTNNB1* mutation and other clinical characteristics, including age, sex, and extent of resection.

Differences of Wnt Signal Expression

In order to examine the biological effect of the *CTNNB1* mutation on Wnt signaling, mRNA expression of *Axin2* and *BMP4* was evaluated. AdaCPs with or without a *CTNNB1* mutation seemed to have higher mRNA expression levels of *Axin2* and *BMP4* compared with papCPs. The mRNA expression level of *Axin2* of adaCPs with *CTNNB1* mutations was significantly higher than that of adaCPs without mutations ($p < 0.05$, Fig. 2 left). There was no association between adaCPs with *CTNNB1* mutations and *BMP4* mRNA expression ($p = 0.186$, Fig. 2 right). The statistical data are shown in Fig. 2. The immunohistochemical findings of *Axin2* and *BMP4* had no significant correlation with *CTNNB1* mutation positivity (Table 3) (Supplementary Figure panels E and F).

Discussion

In this study, we examined *BRAF* and *CTNNB1* mutations in 42 CPs using 2 different sequence analyses and investigated the association between *CTNNB1* mutation and the clinical, pathological, radiological, and biological characteristics of CPs, as well as its relationship to the prognosis of these patients. We found that there was a significant correlation between the presence of *CTNNB1* mutation and the mRNA expression level of *Axin2* in adaCPs. We also found that patients with adaCPs harboring a *CTNNB1* mutation who did not undergo adjuvant RT had shorter PFS. These results suggest that presence of a *CTNNB1* mutation affects Wnt/β-catenin signaling pathway activation and is a risk factor for poor PFS in patients with adaCP.

FIG. 1. A: Correlation of *CTNNB1* mutation positivity with PFS in all adamantinomatous craniopharyngioma (adaCP) cases. **B:** Correlation of PFS with surgery or surgery plus adjuvant RT in patients with adaCP. **C:** Correlation of *CTNNB1* mutation positivity with PFS in patients with adaCP, excluding patients treated with adjuvant RT. PFS was assessed using the Kaplan-Meier method, and statistical significance was determined by the log-rank test.

Many studies have suggested the important role played by β -catenin in tumorigenesis, progression, and invasion of adaCP.^{2,11,13,24} Various analyses revealed that genetic mutation in exon 3 of the β -catenin gene *CTNNB1* is present in 64%–99% of patients with adaCPs.^{1,3,4,12} However, the existence of adaCP without *CTNNB1* mutation had not previously been confirmed and the clinical significance of *CTNNB1* mutation in adaCP patients is still unclear. In the present study, we validated the sequencing results using 2 different methods. Compared with an NGS mutation allele frequency of more than 10%, judgment of Sanger sequencing was more difficult if the NGS mutation allele frequency was less than 10% because of the lower peak. Finally, we confirmed the existence of adaCP without *CTNNB1* mutation and the frequency of the mutation was approximately 70%.

The *CTNNB1* mutation affects the functional domains of β -catenin that interact with its regulators, including glycogen synthase kinase 3- β (GSK3- β). These sites encode a

degradation targeting motif, the mutation of which confers resistance to the β -catenin destruction complex.^{3,26} As a result, the β -catenin protein accumulates and translocates to the nucleus, where it facilitates transcription of β -catenin target genes and Wnt-regulated cellular processes.^{2–4,16} Furthermore, it was shown that EGFR and SHH signaling pathways are also upregulated in adaCP and are associated with tumor cell migration.^{9,10} Although many reports have highlighted these β -catenin mechanisms in adaCP, there are only a few studies that have investigated clinical and radiological differences between *CTNNB1* mutation-positive and -negative adaCPs.⁹ We found that the *CTNNB1* mutation of adaCP affects 2 things: disease behavior as seen biologically and clinically, such as mRNA expression level of *Axin2*, and PFS. Analysis of the relationship of *CTNNB1* mutation and PFS showed that adjuvant RT had the most significant effect on PFS in our study. We explain the reason why the difference of PFS was observed only after excluding the group that received RT as follows.

FIG. 2. Relative mRNA expression in papillary and adamantinomatous CPs (papCPs and adaCPs, respectively) with *CTNNB1* mutations and without mutations. **Left:** The adaCPs with *CTNNB1* mutations had higher mRNA expression levels of *Axin2* compared with adaCPs without mutations ($p = 0.043$). **Right:** There was no association between disease-free survival and *BMP4* mRNA expression ($p = 0.186$). These data were evaluated by the Mann-Whitney U-test and presented as median (horizontal line), interquartile range (box), 5th–95th percentile (whiskers); * $p < 0.05$.

CTNNB1 mutation was associated with a higher rate of tumor recurrence in patients who did not receive RT in our study. However, RT strongly prevented recurrence whether the tumor was *CTNNB1* mutation positive or not. Accordingly, the difference of PFS was not observed between the entire groups of patients with and without *CTNNB1* mutation because of the decreased rate of recurrence due to RT in the group with *CTNNB1* mutation.

Axin2 is a recognized inhibitor and target gene of β -catenin.¹⁴ *BMP4* expression is enhanced in tumors with oncogenic β -catenin elevation.¹⁵ The *Axin2* and the *BMP4* proteins are assumed to be markers of Wnt/ β -catenin signaling pathway activation.¹¹ However, it was uncertain whether mRNA expression levels of the 2 genes are elevated. We chose these 2 genes and their corresponding proteins as an indicator of Wnt/ β -catenin signaling pathway. First, we confirmed that these 2 genes were upregulated in adaCPs compared with papCPs, as described previously.¹¹ Next, we found that among adaCPs, *Axin2* mRNA expression but not *BMP4* mRNA expression was significantly higher in the *CTNNB1* mutation–positive group compared with the *CTNNB1* mutation–negative group.

Elevation of the mRNA level of *Axin2* indicates an activated negative feedback loop that opposes Wnt/ β -catenin signaling in adaCP. Hölsken et al. reported that *BMP4* mRNA expression of adaCPs was higher than that of papCPs, but the difference of *BMP4* mRNA expression between papCPs and adaCPs was significantly lower than that of *Axin2*.¹¹ In our study, we confirmed that *BMP4* mRNA expression of adaCPs was significantly higher than that of papCPs. However, there was no difference in *BMP4* mRNA expression between adaCPs with a *CTNNB1* mutation and those without. We assumed that a small sample number is the reason why the expected result—that *BMP4* mRNA expression of adaCPs with a *CTNNB1* mutation is significantly higher than that of those without—was not obtained. The discrepancy between the statistical analysis of protein and mRNA expression in *Axin2* depends on the method of evaluation used for these analyses and the sensitivity and specificity of the experiment. In evaluating

the IHC results, digitization and comparing the degree of the staining was very difficult and not sensitive. It was presumably difficult to discover a slight difference in protein expression between adaCPs with *CTNNB1* mutation and those without the mutation by IHC only. As for detection of *Axin2* expression, mRNA level by RT-PCR had a superior sensitivity compared with examination of protein level by IHC. To reanalyze and evaluate IHC sensitivity, we applied another monoclonal *Axin2* antibody (ab109307, dilution 1:150; Abcam) to detect *Axin2* protein in tissue. In the reanalysis of *Axin2* protein semiquantitative expression, there was no significant difference between adaCPs with *CTNNB1* mutation and those without *CTNNB1* mutation (data not shown). Finally, we supposed that the difference of specificity and sensitivity in each assay was the cause of the discrepancy.

Many clinical, pathological, radiological, and biological factors predictive of CP recurrence have been suggested,²⁵ such as male sex, occurrence in childhood,²⁰ incomplete removal,²⁸ no RT,²² larger tumor size,⁷ presence of whorl-like arrays,²⁸ high expression of p53,²⁸ and higher levels of *SMO* mRNA expression.⁹ Moreover, Li et al. reported that aberrant β -catenin expression significantly correlated with a poor survival rate in patients with CP and *CTNNB1* mutation.¹⁷ However, their study included both adaCPs and papCPs; therefore, the biological marker related to PFS in adaCPs remains unclear. In our study, the PFS was shorter in the *CTNNB1* mutation–positive group than in the mutation–negative group of adaCPs that had not been subjected to radiotherapy. We consider that *CTNNB1* mutation, which correlates with aberrant β -catenin expression, is somehow related to prognosis and recurrence of adaCPs.

We showed that *CTNNB1* mutation leads to activation of the Wnt/ β -catenin signaling by comparing the mRNA expression level of *Axin2* of *CTNNB1* mutation–positive and –negative samples. Many previous reports described that Wnt/ β -catenin signaling activation plays an important role in the pathogenesis of adaCPs and tumor invasion.^{9,11,17} These studies suggest that Wnt/ β -catenin and SHH signaling activation induced subsequent gene activation, includ-

ing activation of *Axin2*, *BMP4*, *SMO*, and *GLII*. However, the detailed mechanism after nuclear accumulation of β -catenin has not been elucidated due to the interaction of these molecules, and we need to further investigate the function and mechanism of these molecules.

Axin2 plays an important role in the regulation of the stability of β -catenin in the Wnt/ β -catenin signaling pathway and mutations of *Axin2* have been associated with colorectal cancer.¹⁴ This gene is recognized as a negative regulator of the Wnt/ β -catenin signaling pathway.¹⁴ It is unclear whether *Axin2* has the potential to become a direct target of molecularly targeted drugs. There is a report of inhibition of brain tumor growth by downregulation of the Wnt/ β -catenin signaling pathway by microRNA.²⁹ Further investigations targeting the Wnt/ β -catenin signaling pathway, including such possible targets as *Axin2*, are needed to improve the prognosis of patients with adaCPs.

Our study has limitations. The number of patients was small and the treatment strategy was heterogeneous. The first choice of surgical approach changed from transcranial surgery to endoscopic endonasal surgery during the study period. Moreover, we recently have tended to choose endoscopic endonasal surgery for the treatment of recurrent tumors, considering the reported excellent results of endoscopic endonasal surgery for recurrent CPs.⁶ Furthermore, we could not completely exclude the possibility of selection bias for RT. Regarding technical aspects of the study, in patients with adaCPs who underwent partial resection, the quality and quantity of tissue sample were not sufficient. On the other hand, we tended to collect more optimal tissue samples in a recent study period. Because this was a retrospective single-center study, institution-specific factors may limit generalizability; a prospective multicenter study is needed.

Conclusions

This study demonstrated biological and clinical differences between adaCPs with a *CTNNB1* mutation and those without. The mRNA expression level of *Axin2* was higher in the *CTNNB1* mutation-positive group than in the mutation-negative group. Although adjuvant RT had the most significant effect on PFS, in patients with adaCP, except for those who received adjuvant RT, PFS was shorter in the *CTNNB1* mutation-positive group than in the mutation-negative group. These results raise the possibility that the *CTNNB1* mutation may be associated with disease recurrence and genes related to the Wnt/ β -catenin signaling pathway. The *CTNNB1* mutation is associated with adaCP and can be a target for treatment of these tumors.

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Disclosures

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Author Contributions

Conception and design: Hara. Acquisition of data: Takano, Hara, Akutsu, Kino, Tanaka, Miyamoto. Analysis and interpretation of data: Takano, Hara, Sakamoto, Hiyama, Masumoto. Drafting the article: Hara. Critically revising the article: Takano, Hara, Akutsu. Reviewed submitted version of manuscript: Takano, Hara, Akutsu. Approved the final version of the manuscript on behalf of all authors: Takano. Statistical analysis: Hara. Administrative/technical/material support: Hattori, Sakata-Yanagimoto, Chiba. Study supervision: Ishikawa, Matsumura.

Supplemental Information

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Correspondence

Shingo Takano: University of Tsukuba, Ibaraki, Japan. shingo4@md.tsukuba.ac.jp.