Immunohistochemistry or Molecular Analysis: Which method is better for subtyping craniopharyngioma?

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Abstract

Craniopharyngioma (CP) is mainly classified into two pathological subtypes: adamantinomatous (ACP) and papillary (PCP). *CTNNB1* (β-catenin) mutations are detected in ACPs, and the *BRAF* V600E mutation is detected in PCPs. However, genetic analysis is not always possible in general medical practice. In this study, we investigated whether immunohistochemistry could replace genetic analysis as an aid in subtype diagnosis. Here, 38 CP patients who had undergone their first tumor resection were included. Among the 38 cases, 22 were morphologically diagnosed as ACP, 10 cases were diagnosed as PCP, and six cases were diagnosed as undetermined CP that were morphologically difficult to classify as either ACP or PCP. Results of immunohistochemistry and genetic analysis and clinical features were compared. Based on the immunohistochemistry, 26 (22 ACPs and four undetermined CPs) showed nuclear β-catenin expression, 11 (nine PCPs and two undetermined CPs) exhibited positive BRAF V600E immunostaining and one PCP showed membranous β-catenin expression and negative for BRAF V600E immunostaining. Among the 26 nuclear β-catenin expression cases, 11 had *CTNNB1* mutations; however, 15 cases had mutations of neither *CTNNB1* nor *BRAF* V600E. All 11 BRAF V600E immunopositive cases had *BRAF* V600E mutations. When comparing clinical features between, pediatric patients and those with tumor calcification and less solid components on MRI more commonly had nuclear β-catenin expression tumors than BRAF V600E immunopositive tumors, reflecting the differences in clinical

features between ACP and PCP. Accordingly, immunohistochemistry can replace genetic analysis as an aid to determine the subtype diagnosis of CP in general medical practice.

Keywords: craniopharyngioma, CTNNB1, β-catenin, BRAF V600E

Introduction

Craniopharyngioma (CP) is mainly classified into two pathological subtypes: adamantinomatous craniopharyngioma (ACP) and papillary craniopharyngioma (PCP). *CTNNB1* (β-catenin) mutations have previously been detected in ACP [1], and the *BRAF* V600E mutation has been detected in 100% of PCPs by exome sequencing [2]. These reports indicate that these subtypes have different mechanisms of tumorigenesis. In addition, β-catenin and BRAF V600E can be evaluated using immunohistochemistry [3-5].

It is important to accurately differentiate the CP subtype during diagnosis because ACP and PCP differ from each other not only in their clinical characteristics [6-8] but also in their responses to dabrafenib, a BRAF inhibitor, as dabrafenib is effective for PCP but not ACP [9-13].

Although subtype diagnosis of craniopharyngioma is difficult by hematoxylin and eosin (HE) staining alone in some cases, these genetic mutations aid in the subtype diagnosis. However, genetic analysis to detect *CTNNB1* and *BRAF* V600E mutations is not always possible in general medical practice. In this study, we compared the morphological diagnoses determined by HE staining and immunohistochemistry as well as the genetic analysis results and clinical features to demonstrate that an immunohistochemical study can be fully substituted for genetic analysis for the subtype diagnosis of CP.

Methods

In this study, 38 cases of CP who underwent their first tumor resections in Toranomon Hospital between 2013 and 2017 were included. The clinical features of the 38 CP cases were as follows: their ages ranged from 2 – 77 years (median 38 years); 12 cases were children, and 26 cases were adults; and 16 cases were male, and 22 cases were female. Regarding tumor location, tumors were subdiaphragmatic in eight cases and suprasellar in 30 cases. The maximum tumor diameter was 13 – 44 mm (median 29 mm). Among the 38 cases of CP, 22 were morphologically diagnosed as ACP by HE staining, 10 were diagnosed as PCP, and six were diagnosed as undetermined CP, which were morphologically difficult to classify as either ACP or PCP. Ciliated CP, another reported subtype of CP, was excluded in this study [14].

Results of morphological subtype diagnosis by HE staining, immunostaining for β-catenin and BRAF V600E, and *CTNNB1* and *BRAF* V600E mutations were compared to determine whether immunostaining can be substituted for genetic analysis for the subtype diagnosis in CP. Furthermore, the clinical features of patients were classified by immunohistochemistry results and compared to determine whether the diagnosis subtype determined by immunohistochemistry reflected clinical differences.–

Genetic analysis

Genomic DNA was extracted from frozen tumor tissue. Tumor DNA was amplified by PCR using primers that were specific for *CTNNB1* exon 3 or *BRAF* exon 15, including V600, and direct DNA sequencing was performed to detect mutations in *CTNNB1* exon 3 and *BRAF* V600E. The primer sequences for *CTNNB1* were forward, 5'-tactgaattggggctctgct-3' ~~a~~nd reverse, 5'-tgtcagttcagggattgcac-3'. The primer sequences for *BRAF* V600E were forward, 5'-ctgcagcatcttcattccaa-3' and reverse, 5'-tgatttttgtgaatactgggaac-3'. The resultant PCR products were analyzed with the ABI 3500xL sequencing analyzer. Moreover, competitive allele-specific TaqMan PCR (castPCR) using the TaqMan Mutation Detection Assay (BRAF_476_mu, Assay ID: Hs00000111_mu., Thermo Fisher Scientific, Waltham, MA, USA), which is highly sensitive and specific, was applied for cases in which the *BRAF* V600E mutation could not be detected by direct DNA sequencing due to an insufficient number of cells with the mutation.

Pathological diagnosis

Tumor tissue was fixed in 10% buffered neutral formalin and paraffin-embedded for histopathology and immunohistochemistry. Morphological diagnosis was performed by HE staining. ACP was diagnosed when the majority of tumor tissue showed distinctive peripheral palisading of epithelial cells with a stellate reticulum and/or wet keratin. When the epithelial cells had disappeared due to a xanthogranulomatous change, the tumor was also diagnosed as ACP if wet keratin was observed. PCP was diagnosed when the tumor tissue showed papillary proliferation of squamous epithelium with a

fibrovascular core and lacked wet keratin. When the arrangement of the squamous epithelium was difficult to classify as either ACP or PCP and wet keratin was lacking, the tumor was diagnosed as undetermined CP. Commercially available monoclonal antibodies for β-catenin (clone 17C2, Leica Biosystems, Newcastle Upon Tyne, UK) and BRAF V600E (clone VE1, Spring Bioscience, Pleasanton, CA, USA) immunostaining were used. The tissues were considered positive for the *CTNNB1* mutations when immunostaining showed nuclear accumulation of β-catenin (nuclear β-catenin expression) in even a part of the tumor tissue but negative when immunostaining showed a cell membranous localization of β-catenin in all tumor cells (membranous β-catenin expression) [3]. The anti-BRAF V600E antibody showed cytoplasmic staining for only the *BRAF* V600E mutated protein but did not reveal other mutant BRAF V600 epitopes or the wild-type protein. The differences of β-catenin and BRAF V600E immunostaining between ACP and PCP were showed in Fig. 1.

Comparison of clinical features

Based on the immunohistochemistry results, CP was divided into two groups (nuclear β-catenin expression group and BRAF V600E immunopositive group) after excluding CP with membranous β-catenin expression and BRAF V600E immunonegativity. The clinical features of the patients who underwent tumor resection, including percentage of pediatric patient (< 18 or ≥ 18 years), sex, tumor location (subdiaphragmatic or suprasellar), cystic component on pituitary MRI, proportion of solid

component (< 25% or ≥ 25% of tumor volume), calcification on head CT, pituitary function (normal or hypopituitarism), hyperprolactinemia (≥ 31.2 or < 31.2 ng/mL), tumor resection rate (total or subtotal), and recurrence rate, were compared between two groups.

Continuous variables are expressed as means and standard deviations after confirming normal distribution by the Shapiro-Wilk test and were compared using Student's t-test. Categorical variables are expressed as numbers and percentages and were compared using Fisher's exact test. A P value < 0.05 was considered to be statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 21.0.

Results

Comparisons among the immunostaining, genetic analysis and HE staining results

Among ~~the~~ 38 cases of CP, 26 (68%) showed nuclear β-catenin expression and negative BRAF V600E immunostaining, 11 (29%) exhibited positive BRAF V600E immunostaining and membranous β-catenin expression, and one (3%) showed membranous β-catenin expression and negative BRAF V600E immunostaining. No case showed both nuclear β-catenin expression and BRAF V600E immunopositivity. The genetic analysis of 38 cases showed that 11 (29%) had a *CTNNB1* mutation, 11 (29%) had a *BRAF*

V600E mutation, and the remaining 16 (42%) had neither mutation. *CTNNB1* mutations were detected in 11 of 26 cases that exhibited nuclear β-catenin expression (42%), and 15 cases (58%) did not have a *CTNNB1* mutation. *BRAF* V600E mutations were detected in all 11 cases with positive BRAF V600E immunostaining. The case that showed membranous β-catenin expression and BRAF V600E immunonegativity did not show any mutation.

In a comparison of these results with the morphological diagnoses determined by HE staining, all 22 cases of ACP showed nuclear β-catenin expression, nine of which had *CTNNB1* mutations and 13 of which did not have either mutation. Among 10 PCP cases, nine were positive for BRAF V600E immunostaining and had a *BRAF* V600E mutation. A case of PCP was negative for BRAF 600E immunostaining but showed membranous β-catenin expression did not have *CTTNB1* and *BRAF* V600E mutations. Among six cases of undetermined CP, four showed nuclear β-catenin expression in a part of tumor tissue, two of which had *CTNNB1* mutations and two of which did not have either mutation. These six cases were negative for BRAF V600E immunostaining. The other two cases of undetermined CP showed positive BRAF V600 immunostaining and membranous β-catenin expression, and had *BRAF* V600E mutations.

These results indicate that the results of β-catenin and BRAF V600E were unique to CP, that the results of immunostaining and genetic mutations were not reversed, and that β-catenin

immunostaining was more positive than *CTNNB1* mutations. Undetermined CP could also be classified as either subtype by immunostaining (Fig. 2).

The case with no mutations for C*TNNB1* and *BRAF* V600E, membranous β-catenin expression and BRAF V600 immunonegativity

A case that was morphologically diagnosed as PCP by HE staining did not have either a *CTNNB1* or *BRAF V600E* mutation and showed membranous β-catenin expression and negative for BRAF V600E immunostaining. This pediatric case had marked inflammatory cell infiltration with papillary proliferation and keratinization in squamous epithelia of a cystic lesion; therefore, this case had been morphologically diagnosed as PCP by HE staining. The CP was a subdiaphragmatic mono-cystic lesion without a solid component and with calcification on a head CT scan (Fig. 3).

Comparisons of clinical features of subtypes classified by immunostaining

Based on these results, the clinical features of the 26 cases with nuclear β-catenin expression and 11 cases with positive BRAF V600E immunostaining were compared. The case that showed

membranous β-catenin expression and negative for BRAF V600E immunostaining was excluded from this comparison.

Pediatric patients (< 18 years old) were found only in the nuclear β-catenin expression group (11 of 26 cases, 42%), resulting in a significant difference between nuclear β-catenin expression cases and BRAF V600E immunopositive cases ($P = 0.015$). Regarding patient sex, there were 12 males and 14 females in the nuclear β-catenin expression group (male-to-female ratio = 0.86) and four males and seven females in the BRAF V600E immunopositive group (male-to-female ratio = 0.57). There was no significant difference in the male-to-female ratio between the two groups ($P = 0.72$). Regarding tumor location, in the nuclear β-catenin expression group, six cases had the subdiaphragmatic type (23%), and 20 cases had the suprasellar type (77%); in the BRAF V600E immunopositive group, one case had the subdiaphragmatic type (9%), and 10 cases had the suprasellar type (91%). There were no significant differences between the two groups ($P = 0.65$). The maximum tumor diameter was 30.7 ± 7.9 mm in the nuclear β-catenin expression group and 26.4 ± 12.0 mm in the BRAF V600E immunopositive group. There were no significant differences between the two groups ($P = 0.19$). A cystic component on pituitary MRI was found in all 26 cases in the nuclear β-catenin expression group (100%) and in nine cases in the BRAF V600E immunopositive group (82%). There was no significant difference between the two groups ($P = 0.83$). However, the number of cases with a solid component ≥ 25% of the tumor volume was six in the nuclear β-catenin expression group (23%) and eight in the BRAF V600E immunopositive group

(73%), and the number of such cases was significantly higher in the BRAF V600E immunopositive group than in the nuclear β-catenin expression group ($P = 0.008$). Calcification on head CT was found in 24 cases in the nuclear β-catenin expression group (92%) and in one case in the BRAF V600E immunopositive group (9%); the number of such cases was significantly higher in the nuclear β-catenin expression group than in the BRAF V600E immunopositive group ($P < 0.001$). Pituitary function was preserved in 11 cases in the nuclear β-catenin expression group (42%) and three cases in the BRAF V600E immunopositive group (27%); there was no significant difference between the two groups ($P = 0.48$). However, hyperprolactinemia was seen significantly more often in the nuclear β-catenin expression group (21 cases, 81%) than in the BRAF V600E immunopositive group (four cases, 36%) ($P = 0.018$). Total resection was performed in 25 cases in the nuclear β-catenin expression group (96%) and 10 cases in the BRAF V600E immunopositive group (91%); there was no significant difference between the two groups ($P = 0.51$). When the cases with subtotal tumor resection (one in the nuclear β-catenin expression group and one in the BRAF V600E immunopositive group) were excluded, tumor recurrence was observed in two of 23 cases in the nuclear β-catenin expression group (9%) and in none of the cases in the BRAF V600E immunopositive group. The recurrence rate was not significantly different between the two groups ($P = 1.00$). These results are summarized in Table 1, and they reflect the clinical features of ACP and PCP.

Discussion

These results indicate that the detection of *CTNNB1* (β-catenin) and *BRAF* V600E mutations in CP by genetic analysis or immunohistochemistry is exclusive to and specific for the respective CP subtype, as in previous reports [2,5,15,16]. There were no cases with both mutations. The morphological diagnosis determined by HE staining, immunohistochemistry and genetic analysis were well correlated. Among the cases with nuclear β-catenin expression, only 42% had detectable *CTNNB1* mutations by genetic analysis. However, the BRAF V600E immunostaining results matched the gene mutations. In addition, undetermined CPs were clearly diagnosed as either ACP or PCP by immunostaining. Among undetermined CPs, the *CTNNB1* mutation was detected in only half of the cases with nuclear β-catenin expression. These results suggest that immunostaining is useful for subtype diagnosis of CP in addition to HE staining without genetic analysis in general clinical practice.

Nuclear accumulation of β-catenin is not found in the whole tumor but mainly in the whirl-like structures [3]. However, CPs often have heterogeneous tissue due to inflammation or other factors, such that some ACPs do not exhibit nuclear accumulation of β-catenin, even according to the pathological results. Similarly, the part containing whirl-like structures exhibiting nuclear accumulation of β-catenin may not have been included in the tumor tissue used for genetic analysis. This may explain why the detection rate of *CTNNB1* mutations was lower than that of β-catenin immunostaining in this study. In

previous reports, the detection rate of *CTNNB1* mutations by genetic analysis in ACP was not high, but β-catenin immunostaining exhibited better positive results, which may be due not only to the technique but also to inflammatory changes [17].

The case with no mutations for CTNNB1 and BRAF V600E, membranous β-catenin expression and BRAF V600 immunonegativity

In one case, neither *CTNNB1* nor *BRAF* V600E mutation was detected by genetic analysis, and the case was also showed membranous β-catenin expression and negative BRAF V600E immunostaining. Although this case was clinically suggested to be ACP, it was morphologically diagnosed as PCP by HE staining. *BRAF* V600E mutations and their immunopositivity have been reported to be detected in most PCPs [2,15]. All of the other PCPs in our study had *BRAF* V600E mutations and immunopositivity. Since only this case was negative, the subtype may have been ACP. Similarly, squamous metaplasia is also caused by inflammation in Rathke cleft cyst, making it difficult to differentiate a Rathke cleft cyst from PCP [18,19]. It is also possible that this case was an inflammatory Rathke cleft cyst. However, it was difficult to determine the actual subtype of this case. This case suggests that inflammation confounds the morphological subtype diagnosis of CP by HE staining, and in such cases, the diagnosis of subtype is difficult even with immunostaining and genetic analysis. In addition, tumors showing squamous cystic

proliferation may be morphologically similar to PCP, so biomarker including BRAF V600E should be routinely used for differential diagnosis.

Undetermined CP

In our series, six of the 38 cases (16%) could not be morphologically diagnosed as either ACP or PCP by HE staining. However, all six cases showed either nuclear β-catenin expression or positive BRAF V600E immunostaining, although both mutations were not detected in two cases. These results indicate that undetermined CP is difficult to diagnose by HE staining and can be classified as ACP or PCP using immunohistochemistry. Therefore, these findings suggest that ACP and PCP have different oncogenic mechanisms and that immunostaining may be useful for subtype diagnosis even in cases that are difficult to diagnose according to subtype by HE staining.

Mixed-type CP comprising both ACP and PCP histology in a single tumor was reported [20]. Immunostaining may also allow classification as either ACP or PCP in such a case. However, this type of CP remains controversial because ACPs with both *CTNNB1* and *BRAF* V600E mutations have been reported [21].

Conclusions

The results of morphology by HE staining, immunohistochemistry and genetic analysis for the diagnosis of CP subtype correlate with each other. *CTNNB1* and *BRAF* V600E mutations are useful for the diagnosis of CP subtypes because they are exclusive to and specific for their respective subtypes. However, pathological diagnosis with immunostaining is sufficient for the diagnosis of subtype, and genetic analysis is not necessarily required in clinical practice, as immunostaining has a higher positivity rate than genetic analysis. In addition, immunohistochemistry should be routinely used for the diagnosis of CP subtypes because it is also useful for confirming the subtype diagnosis even in the case that can be diagnosed morphologically.

Author's contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Noriaki Fukuhara, Takeo Iwata, Naoko Inoshita, Katsuhiko Yoshimoto, Hirokazu Fukuhara, Keita Tatsushima, Mitsuo Yamaguchi-Okada, Akira Takeshita, Junko Ito, Yasuhiro Takeuchi, Shozo Yamada, and Hiroshi Nishioka. The first draft of the manuscript was written by Noriaki Fukuhara and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Information

This work was supported in part by a grant from the Foundation for Growth Science.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Statement

This study is a retrospective case series and was approved by the institutional review board of Toranomon Hospital (No. 1616) and Tokushima University (No. 714-2).

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Table 1. Comparison of clinical features between nuclear β-catenin expression cases and BRAF V600E immunopositive cases

	Nuclear β-catenin expression 26 cases	BRAF V600E immunopositive 11 cases	*P*
Age (pediatric/adult)	11/15	0/11	0.015
Sex (male/female)	12/14	4/7	0.72
Location (subdiaphragmatic/suprasellar)	6/20	1/10	0.65
Maximum tumor diameter (mean ± SD mm)	30.7 ± 7.7	26.4 ± 12.0	0.19
Cystic component (+/-)	26/0	9/2	0.83
Solid component (< 25%/≥ 25%)	20/6	3/8	0.008
Calcification on CT (+/-)	22/2	1/10	<0.001
Pituitary function (normal/hypo)	11/15	3/8	0.48
Hyperprolactinemia (+/-)	21/5	4/7	0.018
Resection (total/subtotal)	25/1	10/1	0.51
Recurrence (+/-) *	23/2	10/0	1.00

*Cases with subtotal tumor removal were excluded.

Fig. 1. Pathological findings of HE staining and immunostainings in adamantinomatous and papillary craniopharyngioma

A, B, C: amadantinomatous craniopharyngioma. D, E, F: papillary craniopharyngioma. A, D: HE staining. B, E: β-catenin immunostaining. C, F: BRAF V600E immunostaining. These ACP and PCP were relatively resemble morphologically (A, D), however, immunohistochemistry showed distinct differences. In adamantinomatous craniopharyngioma, β-catenin immunostaining showed nuclear expression in some cells, especially in the part of whirl-formation. This finding is not seen in the whole tumor tissue (B). On the other hand, β-catenin immunostaining showed only membranous expression in papillary craniopharyngioma (E). BRAF V600E immunostaining showed cytoplasmic positivity in papillary craniopharyngioma (F), although negative in adamantinomatous craniopharyngioma (C).

Fig. 2. Correlations between immunochistochemistry, genetic analysis and morphological diagnosis

IHC: immunohistochemistry *: membranous β-catenin expression and BRAF V600E immunonegative. †: undetermined.

Immunostaining was able to classify the highest number of cases into ACP or PCP.

Fig. 3. Pathological findings of a case of papillary craniopharyngioma with no genetic mutation, immunostaining showing nuclear β-catenin expression and negative BRAF V600E

A: HE staining, B: β-catenin immunostaining, C: BRAF V600E immunostaining. This case was diagnosed as papillary craniopharyngioma by HE staining; however, immunostaining showed membranous β-catenin expression and negative BRAF V600E. In addition, neither a *CTNNB1* nor *BRAF* V600E mutation was detected by genetic analysis.

Fig 1

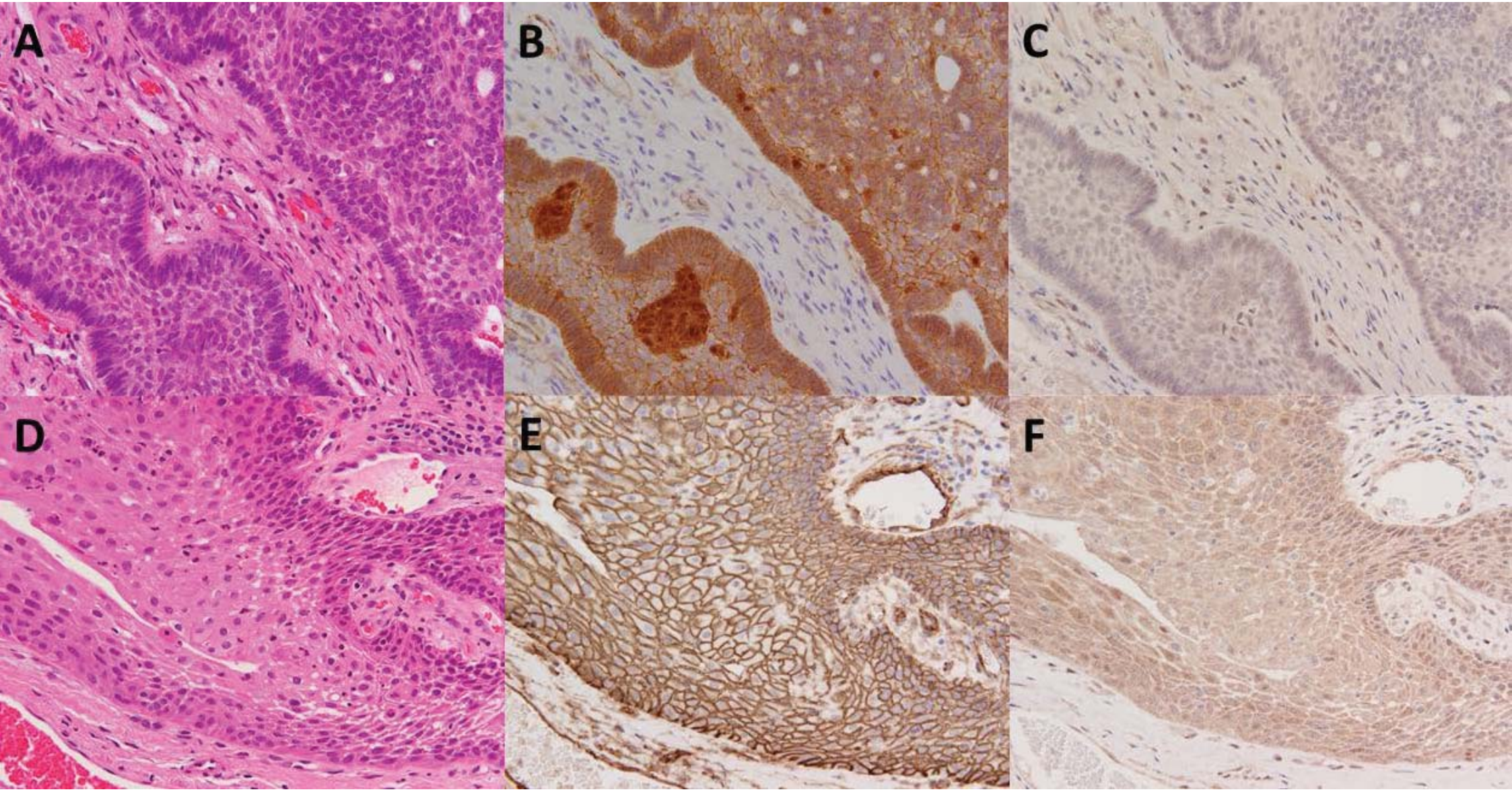

Fig 2

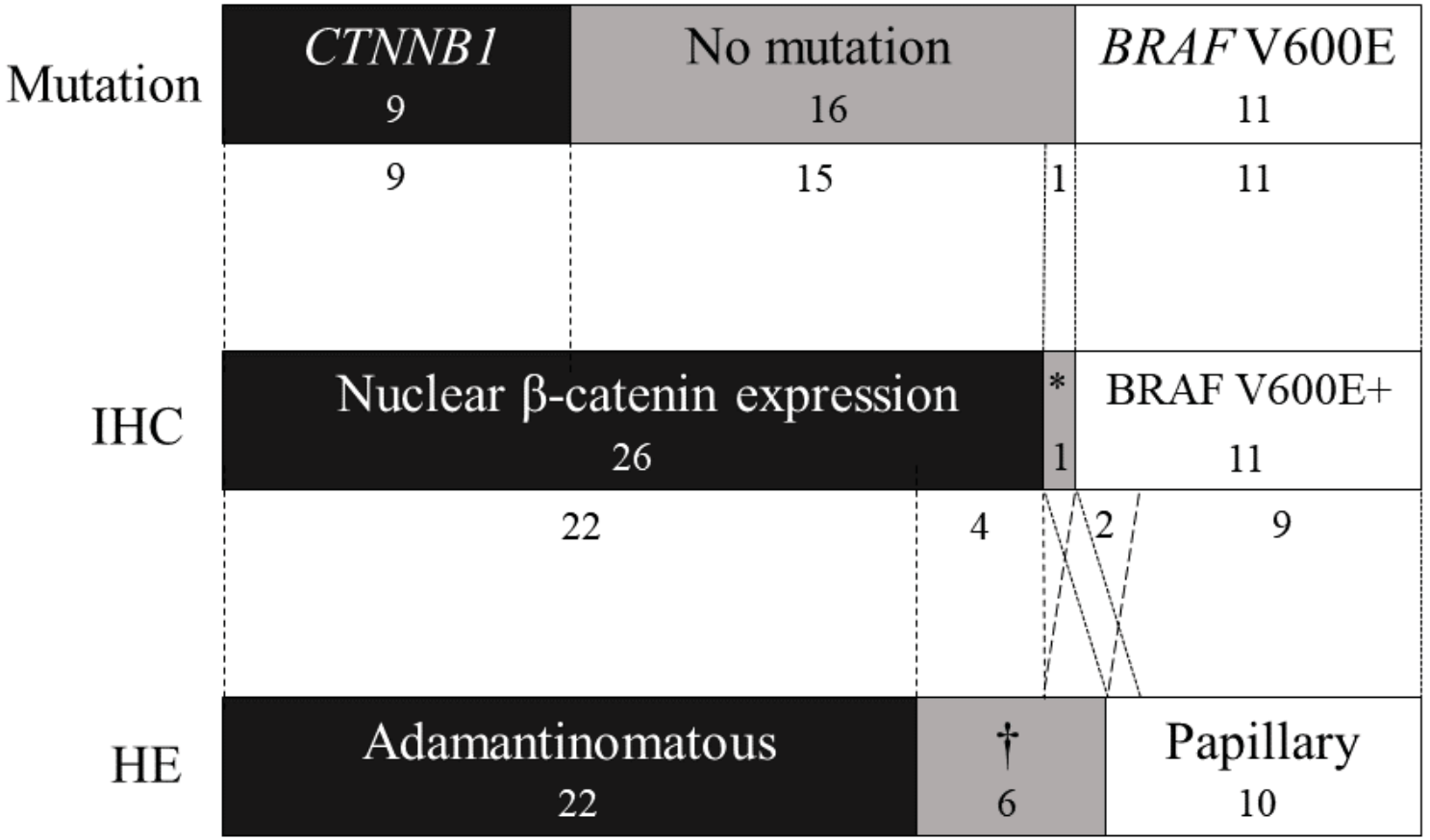
Mutation
IHC
HE
CTNNB1
9
No mutation
16
BRAF V600E
11
9
15
1
11
Nuclear β-catenin expression
26
*
1
BRAF V600E+
11
22
4
2
9
Adamantinomatous
22
†
6
Papillary
10

Fig 3