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Wnt/β-catenin signal alteration and its diagnostic utility in basal cell adenoma and histologically similar tumors of the salivary gland

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ABSTRACT

Differential diagnosis among basal cell adenoma (BCA), basal cell adenocarcinoma (BCAC), adenoid cystic carcinoma (ACC) and pleomorphic adenoma (PA) of the salivary gland can be challenging due to their similar histological appearance. Although frequent nuclear β-catenin expression and *CTNNB1* mutations have been reported in BCA, further details of the Wnt/β-catenin signal alterations are unclear. The aim of this study was to assess the diagnostic utility of Wnt/β-catenin signal alteration in BCA and morphological mimics. We performed immunohistochemical staining for β-catenin and mutation analysis for Wnt/β-catenin-related genes (*CTNNB1*, *APC*, *AXIN1* and *AXIN2*) in BCA (n = 34), BCAC (n = 3), ACC (n = 67) and PA (n = 31). We also analyzed ACC-specific *MYB* and *MYBL1* gene rearrangements by fluorescence in situ hybridization (FISH). Nuclear β-catenin expression (≥3%) was present in 32/34 cases (94.1%) of BCA, and the nuclear β-catenin labeling index was significantly higher than in other tumor types (p = < 0.0001). In BCA, we found mutations in *CTNNB1*, *APC* and *AXIN1* genes (41.1%, 2.9% and 8.8%, respectively). In BCAC, nuclear β-catenin expression with *CTNNB1* mutation was present in 1/3 cases (33.3%). As for ACC, nuclear β-catenin expression was observed in 3/67 cases (4.4%), but all 3 cases harbored either *MYB* or *MYBL1* gene rearrangement. The results suggest that nuclear β-catenin immunoreactivity with appropriate criteria may be helpful to distinguish BCA from histologically similar tumors. However, a minor subset of ACCs with nuclear β-catenin expression require careful diagnosis. In addition, Wnt/β-catenin signal alteration may play a role in the pathogenesis of BCA and BCAC.

1. Introduction

Basal cell adenoma (BCA) is a benign tumor accounting for 1–3% of salivary gland epithelial tumors [1,2], and is histologically characterized by basaloid cell proliferation in an anastomosing jigsaw puzzle-like solid or trabecular pattern with peripheral palisading [3]. Other kinds of salivary gland tumors with basal cell differentiation can share morphologic features with BCA. Such tumors include basal cell adenocarcinoma (BCAC), adenoid cystic carcinoma (ACC), and pleomorphic adenoma (PA). BCAC is the malignant counterpart to BCA, and has a relatively high recurrence rate but favorable prognosis [4–6]. BCA and ACC show similar histological appearance in terms of their focal or extensive cribriform structures [7,8]. ACC is generally indolent, but the long-term prognosis is poor due to persistent local recurrence and distant metastases. Thus, differential diagnosis of these tumors is clinically very important, but sometimes challenging. Precise diagnosis can be particularly difficult for cases in which only biopsy or other small samples are available for analysis.

Characteristic molecular abnormalities have been identified in salivary gland tumors. β-catenin is one of the canonical Wnt signaling pathway components regulating the expression of Wnt target genes. β-catenin is usually maintained at a low level in the cytoplasm due to phosphorylation by a multiprotein complex containing APC, Axin, GSK3β and PP2A. Mutations of *CTNNB1*, *APC* or *AXIN* result in nuclear β-catenin accumulation linked with abnormal activity of Wnt/β-catenin signaling pathway [9], and play a role in the pathogenesis of various diseases [10–12]. Nuclear β-catenin expression is observed in most cases of BCA, and activating mutations of *CTNNB1* have been identified

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in about half of BCAs [13–15]. However, the molecular mechanisms causing nuclear accumulation of β-catenin, other than *CTNNB1* mutation, remain to be clarified. Although nuclear β-catenin expression has been reported in some cases of basal cell adenocarcinoma (BCAC), the pathogenesis of BCAC has been unclear [14].

As for ACC, *MYB-NFIB* fusion gene has been identified in approximately 60% to 70% of cases [16–19]. In a recent study, the presence of *MYBL1* gene rearrangement was reported in about 25% of ACCs without *MYB* gene rearrangement [20]. In a previous report about Wnt/β-catenin signal alteration in ACC, mutations in *CTNNB1*, *APC* or *AXIN1* were found in some populations of ACCs [21]. However, the relationship between gene rearrangement in *MYB* or *MYBL1* and Wnt/β-catenin signal alteration has not been clarified in ACC.

The aim of this study was to further elucidate the Wnt/β-catenin signal alterations in BCA and the diagnostic utility of these alterations in the differentiation between BCA and morphological mimics.

2. Materials and methods

2.1. Case selection

We examined 135 cases of salivary gland tumors, including BCA (n = 34), BCAC (n = 3), ACC (n = 67) and PA (n = 31). These cases were selected from the institutional database of Kyushu University Hospital and its affiliated hospitals, dating from 1983 to 2014. BCA, BCAC and PA occurred in parotid gland (n = 33, n = 3 and n = 22, respectively) and submandibular gland (n = 1, n = 0 and n = 9, respectively). Forty-seven cases of ACC occurred in parotid gland (n = 9), submandibular gland (n = 13), sublingual gland (n = 3) and nasal/paranasal cavity (n = 22). The remaining twenty cases occurred in other sites of head and neck (Table 1). This study was approved by the Institutional Review Boards of Kyushu University (no. 26–185, 29–200) and the National Kyushu Cancer Center (no. 2015-5).

2.2. Immunohistochemical staining for β-catenin

Immunohistochemical staining was performed using 4-μm-thick, formalin-fixed, paraffin-embedded (FFPE) tissue sections and the primary antibodies for β-catenin (clone 14, mouse monoclonal; dilution × 200; BD Biosciences, San Jose, CA). A biotin-free, horseradish peroxidase enzyme-labeled polymer method (EnVision+ system; Dako, Carpinteria, CA) was used. All tissue sections were counterstained with hematoxylin.

We evaluated at least 500 cells in the hot spot, then assessed the labeling index of nuclear immunoreactivity of β-catenin in neoplastic cells. We drew a ROC curve to calculate a cut-off value of the nuclear β-catenin labeling index for BCA compared with other tumors (BCAC, ACC and PA), and found that 3% was the most appropriate value (see Results).

Table 1
Clinical data of BCA, BCAC, ACC and PA.

	BCA	BCAC	ACC	PA
	n = 34	n = 3	n = 67	n = 31
Age (years)[a]	60 (18–78)	61 (30–77)	61 (22–81)	54 (13–84)
Sex				
Male	12 (35.3%)	1 (33.3%)	28 (41.8%)	8 (25.8%)
Female	22 (64.7%)	2 (66.7%)	39 (58.2%)	23 (74.2%)
Site				
Parotid gland	33 (97.1%)	3 (100%)	9 (13.4%)	22 (71.0%)
Submandibular gland	1 (2.9%)		13 (19.4%)	9 (29.0%)
Sublingual gland			3 (4.5%)	
Nasal/Paranasal cavity			22 (32.8%)	
Other head and neck regions[b]			20 (29.9%)	

Abbreviation: BCA, basal cell adenoma; BCAC, basal cell adenocarcinoma; ACC, adenoid cystic carcinoma; PA, pleomorphic adenoma; M, male; F, female; N.A., not available data.
[a] The data are shown by median with the range in the parenthesis.
[b] Other head and neck regions include tongue (n = 7), palate (n = 1), orbit (n = 3), lacrimal duct (n = 2), external ear (n = 4), nasopharynx (n = 2) and trachea (n = 1).

2.3. Mutational analysis for CTNNB1, APC and AXIN

We extracted genomic DNA from FFPE tissue sections by using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To detect mutations in *CTNNB1* (exon3), *APC* (exon15), *AXIN1* (exon5, 7, 10) and *AXIN2* (exon1, 5, 7) [10], we performed a polymerase chain reaction (PCR) followed by Sanger sequencing, as previously described [22]. The primer sequences are summarized in **Supplementary** Table 1. The PCR products were subjected to 2% agarose gel electrophoresis, and the sequence was confirmed by the direct sequencing method using an ABI Prism 310 sequence analyzer (Applied Biosystems, Foster City, CA, USA).

2.4. MYB and MYBL1 fluorescence in situ hybridization (FISH) and evaluation

Fluorescence in situ hybridization (FISH) was done on FFPE tissue sections of ACC to detect *MYB* or *MYBL1* gene rearrangements. We used commercially available break-apart probes for the *MYB* gene (*MYB* Split Dual Color FISH Probe; GSP Laboratory, Kobe, Japan) and *MYBL1* gene (*MYBL1* Split Dual Color FISH Probe; GSP Laboratory, Kobe, Japan) in accordance with the manufacturer's instructions. In the *MYB* FISH probe design, the 5′ *MYB* signal was labeled with spectrum green, and the 3′ *MYB* signal was labeled with spectrum red. In the *MYBL1* FISH probe design, the 5′ *MYBL1* signal was labeled with spectrum red, and the 3′ *MYBL1* signal was labeled with spectrum green. The hybridized slides were reviewed on a fluorescent microscope (Olympus BX53; Olympus, Tokyo, Japan) under a × 100 objective lens with oil immersion using a DAPI/Green/Red triple band-pass filter set. The results were scored by evaluating 100 non-overlapping tumor cells with hybridization signals. Tumor cells lacking hybridization signals were excluded from evaluation. A split signal was defined by 5′ and 3′ signals observed at a distance larger than the signal size, and signals separated by a distance smaller than the signal size were regarded as fused signals. When the ratio of tumor cells having split signals or isolated 5′ signals was more than 20%, the samples was considered to be positive for gene rearrangement.

2.5. Statistical analysis

All calculations were done using JMP Statistical Discovery Software (version 11.0.0; SAS, Cary, NC). We used the Wilcoxon rank sum test to evaluate the comparisons between basal cell tumors and mimics. The results were considered significant when the p-value was < 0.05.

3. Results

3.1. Histological findings and immunohistochemical results for β-catenin

BCA showed diverse histological appearances, including predominantly tubulotrabecular (n = 28) (Fig. 1**A**), cribriform (n = 1) (Fig. 1**B**), solid (n = 4) and membranous (n = 1) patterns. Among these 34 cases of BCA, 10 cases (29.4%) had at least focally cribriform or membranous structure similar to ACC. In most BCAs (n = 32), strong nuclear β-catenin immunoreactivity was present in abluminal cells, but not in luminal cells (Fig. 1**C**). One case of membranous-type BCA with focal tubulotrabecular component showed nuclear β-catenin expression. Two cases without nuclear β-catenin expression were tubulotrabecular-type, but not membranous-type.

BCAC resembled BCA cytologically but exhibited infiltrative growth

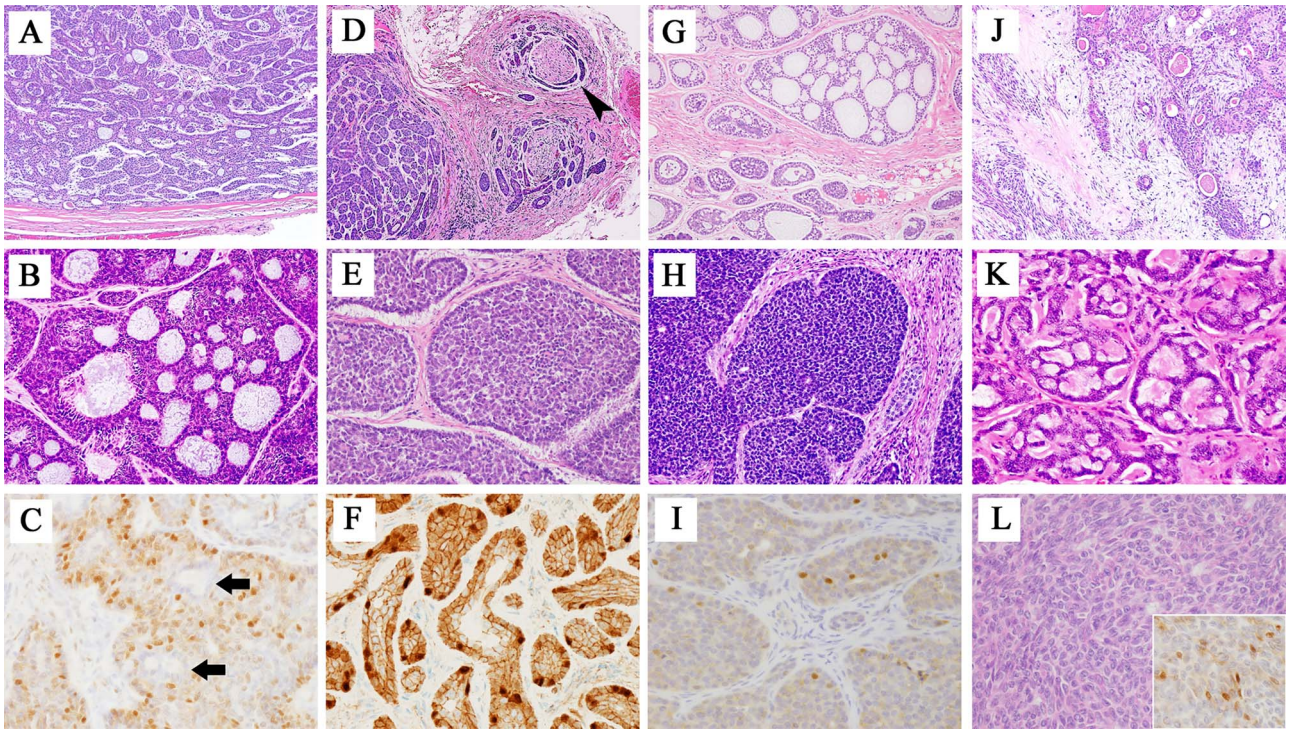

Fig. 1. Histological and immunohistochemical findings in BCA (A-C), BCAC (D-F), ACC (G-I) and PA (J-L) (A, B, D, E, G, H, J, K, L, hematoxylin-eosin stain; C, F, I, L, inset, β-catenin). BCA is surrounded by a fibrous capsule, and is composed of neoplastic basaloid cells with anastomosing jigsaw puzzle-like islands or trabecular arrangement (A). BCA exhibits cribriform structures resembling ACC (B). Nuclear β-catenin expression is diffusely and strongly positive in the neoplastic basaloid/abluminal cells (score 2), whereas luminal cells show no nuclear β-catenin expression (arrows) (C). BCAC exhibits infiltrative growth with occasional perineural invasion (arrowheads) (D). BCAC can exhibit a solid component resembling ACC (E). β-catenin expression is identified predominantly as a membranous pattern, and nuclear β-catenin expression is sparsely observed (score 1) (F). In ACC, infiltrative growth is usually obvious (G). The morphologic patterns of ACC are tubular, cribriform (G) and solid (H). Nuclear β-catenin expression is sparsely observed (score 1) (I). PA shows anastomosing tubules and chondro-myxoid stroma with the melting phenomenon of myoepithelial cells (J). PA can exhibit cribriform structures (K) and hyper-cellular components (L). Nuclear β-catenin expression is focally positive in a case of PA with a hyper-cellular component (score 1) (L, inset). Original magnification × 100 (A, D, G and J), × 200 (B, E, H and K) and × 400 (C, F, I, L and L, inset).

with a predominantly tubulotrabecular (n = 1) (Fig. 1**D**) or solid (n = 2) (Fig. 1**E**) pattern. In one case of BCAC, focal but strong staining for β-catenin expression was observed in the nuclei of abluminal cells (Fig. 1**F**).

ACC also exhibited infiltrative growth with a predominantly tubular (n = 26), cribriform (n = 34) (Fig. 1**G**) or solid (n = 7) (Fig. 1**H**) pattern. In a few cases of ACC, nuclear β-catenin expression was focal with weak-to-strong staining intensity in abluminal cells (Fig. 1**I**).

PA showed variable histological appearances such as predominantly chondro-myxoid matrix-rich (n = 17) (Fig. 1**J**) and hyper-cellular (n = 14) (Fig. 1**L**) patterns; 11 of 31 cases of PA had a cribriform structure focally resembling that of ACC (n = 2) (Fig. 1**K**) or a peripheral palisading structure resembling that of BCA (n = 9). In one case of PA, focal nuclear β-catenin expression was observed in myoepithelial cells in the hyper-cellular component (Fig. 1**L, inset**).

To establish an optimal cut-off value, we assessed the labeling index of nuclear β-catenin-expressing cells in neoplastic cells. The distribution of the labeling index is illustrated in Figs. 2 and 3. The nuclear β-catenin labeling index ranged from 0% to 57% (median: 22.5%; mean: 22.9%) in BCA, from 0% to 8% (median: 0%, mean: 2.6%) in BCAC, from 0% to 10% (median: 0%, mean: 0.4%) in ACC and from 0% to 13% (median: 0%, mean: 0.3%) in PA. The nuclear β-catenin labeling index was significantly higher in BCA than BCAC, ACC or PA ($p = 0.0179$, $p = < 0.0001$ and $p = < 0.0001$, respectively) (Fig. 3). Of note, ten cases of BCA showed a cribriform or membranous structure where the nuclear β-catenin labeling index ranged from 13% to 45% (median: 31.0%, mean: 29.9%); the labeling index was significantly higher than that of ACC ($p = < 0.0001$).

We then compared the ROC curve between BCA and non-BCA (BCAC, ACC and PA). The results showed that the area under the curve (AUC) was 0.963, and that a cut-off value ≥3% was most appropriate (Youden index = 0.891) (Table 2). If the cut-off value was ≥3%, the sensitivity and specificity for BCA were 94% and 95%. Moreover, if the cut-off was ≥15%, the specificity for BCA was 100%, but the sensitivity declined to 76%. Therefore, we used the following scoring system by labeling index: score 0 (negative): 0% to < 3%; score 1: 3% to < 15%; score 2: ≥15%. Samples with a score of 1 or 2 were considered positive for nuclear β-catenin expression. Positivity for nuclear β-catenin expression was observed in 32/34 cases (94.1%) of BCA, 1/3 cases (33.3%) of BCAC, 3/67 cases (4.4%) of ACC and 1/31 cases (3.2%) of PA (Table 3).

3.2. Mutation analysis for Wnt/β-catenin signaling pathway

Table 4 summarizes the results of mutation analysis. *CTNNB1* (exon 3) missense mutations were present in 14/34 cases (41.1%) of BCA; almost all mutations caused residue I35 to be replaced with a T (I35T), and one mutation was T41P. Moreover, we detected *APC* (exon 15) missense mutations in 1/34 cases (2.9%) of BCA; these mutations were E1295 K and E1353 K. *AXIN1* (exon 5) missense mutations were present in 3/34 cases (8.8%) of BCA; these mutations were G430E, P439S, G508D and G514R. We found no missense mutations in *AXIN2* in BCAs. Collectively, 18/34 cases (52.9%) of BCA had missense mutations in either *CTNNB1*, *APC* or *AXIN1*. Among the 32 cases of BCA with nuclear β-catenin expression, 17 cases (50%) had mutations in *CTNNB1* or related genes. *AXIN1* missense mutations were detected in 1 of 2 cases of BCA without nuclear β-catenin expression (Table 5).

CTNNB1 mutation (exon 3) was present in 1/3 cases (33.3%) of

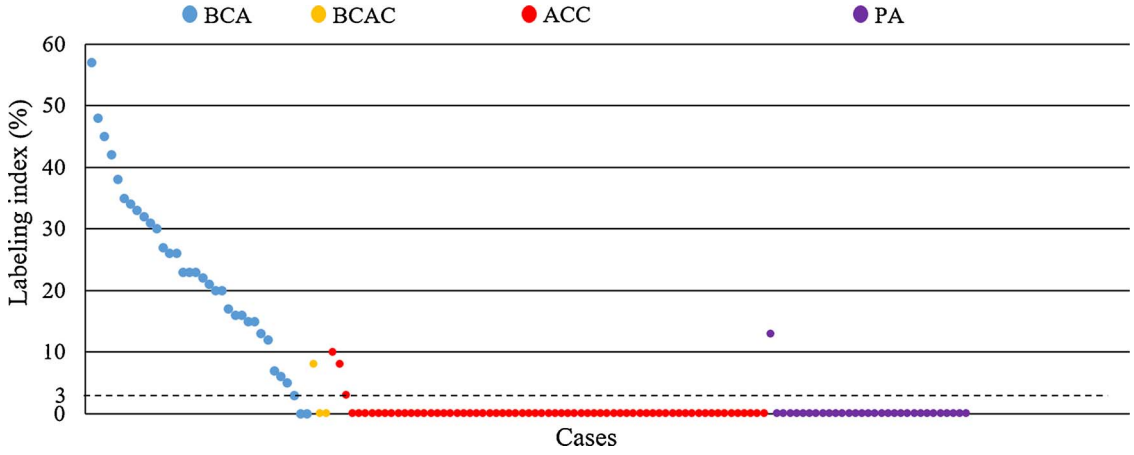

Fig. 2. Distribution of the nuclear β-catenin labeling index for BCA, BCAC, ACC and PA. The cut-off value (3%) is shown by a dotted line.

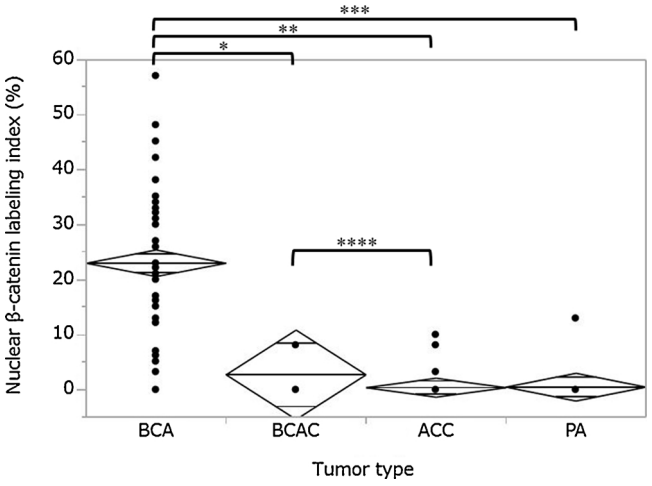

Fig. 3. Distribution of the nuclear β-catenin labeling index in BCA, BCAC, ACC and PA. The nuclear β-catenin labeling index of BCA is significantly higher than those in BCAC, ACC and PA (* p = 0.0179, ** p = < 0.0001, *** p = < 0.0001, respectively). The nuclear β-catenin labeling index of BCAC is significantly higher than that in ACC (**** p = 0.0399).

Table 2
ROC analysis to determine the nuclear β-catenin positivity.

Cut-off	BCA (n = 34)	Non-BCA[a] (n = 101)	Sensitivity	Specificity	Youden index
3%	32	5	94%	95%	0.891
5%	31	4	91%	96%	0.872
10%	28	2	82%	98%	0.803
15%	26	0	76%	100%	0.764
20%	21	0	61%	100%	0.617
30%	11	0	32%	100%	0.323

Abbreviation: ROC, receiver operating characteristic; BCA, basal cell adenoma.

[a] Non-BCA includes basal cell adenocarcinoma, adenoid cystic carcinoma and pleomorphic adenoma.

BCAC; this mutation was I35T. Nuclear β-catenin expression was detected in this case by IHC.

Both *AXIN1* (exon7) and *AXIN2* (exon 1) missense mutations were present in a single case of ACC (1/16 cases, 6.2%); *AXIN1* mutation was H662Y, and *AXIN2* mutations were G39R and G214E (Table 4). Nuclear β-catenin expression was detected in this case by IHC.

In a case of PA with nuclear β-catenin expression by IHC, no mutations were detected in Wnt/β-catenin signal-related genes.

3.3. MYB and MYBL1 gene rearrangements and Wnt/β-catenin signaling pathway alteration in ACCs

MYB FISH and *MYBL1* FISH were successful in 58 cases of ACC. *MYB* and *MYBL1* gene rearrangements were identified in 41/58 cases (70.6%) and 2/58 cases (3.4%) of ACCs, respectively (Fig. 4). Fifteen of 58 cases (25.8%) were negative for both *MYB* and *MYBL1* gene rearrangements. *MYB* and/or *MYBL1* gene rearrangement could not be assessed in the remaining 9 cases due to weak FISH signals. Table 6 summarizes the molecular features in 3 cases of ACC with nuclear β-catenin expression. All 3 cases had either *MYB* or *MYBL1* gene rearrangement, and one case (Case 1) had *AXIN1* and *AXIN2* mutations.

4. Discussion

Differential diagnosis among BCA and other salivary gland tumors with basal cell differentiation (BCAC, ACC and PA) is sometimes challenging due to their similar histological appearance. In several recent studies, nuclear β-catenin immunoreactivity was observed exclusively in BCA and BCAC (Table 7) [13–15]. For example, Kawahara et al. defined ≥1% as positive for nuclear β-catenin immunoreactivity, and found nuclear β-catenin expression in 21/22 cases (95.4%) of BCA and 1/3 cases (33.3%) of BCAC [13]. Similarly, Jo et al. showed that nuclear β-catenin expression was observed in 18/22 cases (81.8%) of BCA and in 2/3 cases (66.6%) of BCAC [14]. Jung et al. used 5% as the cut-off value, and reported that nuclear β-catenin expression was present in 17/20 cases (85%) of BCA, and in all cases of BCAC (7/7 cases) [15]. Although different clones of antibodies were used in these previous reports and the current study, the prevalence of nuclear β-catenin immunoreactivity was essentially similar among the study cohorts. Meanwhile, nuclear β-catenin immunoreactivity was observed in 0/37 cases (0%) of ACC and 0/63 cases (0%) of PA (Table 7) [13,14]. In contrast, Daa et al. detected nuclear β-catenin expression in 2/20 cases (10%) of ACC [21]. One possible explanation for this discrepancy is that there have been no standard criteria for nuclear β-catenin expression. In the current study, the vast majority of BCA and one third of BCACs were positive for nuclear β-catenin expression, whereas a minor subset of ACCs and PAs showed variable levels of nuclear β-catenin immunoreactivity. To establish useful criteria of nuclear β-catenin positivity for use in pathological diagnosis, we assessed a labeling index of nuclear β-catenin immunoreactivity and found that a labeling index of ≥3% was a reasonable cut-off value with 94% sensitivity and 95% specificity (Table 2). With this cut-off value, nuclear β-catenin expression was positive in 32/34 cases (94.1%) of BCA and 5/101 cases (4.9%) of non-BCA tumors. Furthermore, when the cut-off for nuclear β-catenin positivity was designated as ≥15% (score 2), the specificity

Table 3
Nuclear expression of β-catenin in BCA, BCAC, ACC and PA.

	Nuclear β-catenin expression				Labeling index[a]
	Score 0	Score 1	Score 2	Score 1 + 2 (Positive, total)	Mean +SD (%)
BCA (n = 34)	2 (5.8%)	6 (17.6%)	26 (76.4%)	32 (94.1%)	22.9 +1.2 (0–57)
BCAC (n = 3)	2 (66.6%)	1 (33.3%)	0	1 (33.3%)	2.6 +1.2 (0–8)
ACC (n = 67)	64 (95.5%)	3 (4.4%)	0	3 (4.4%)	0.4 +1.2 (0–10)
PA(n = 31)	30 (96.7%)	1 (3.2%)	0	1 (3.2%)	0.3 +0.8 (0–13)

[a] The data are shown by mean +SD with the range in the parenthesis.

Table 4
Mutations of Wnt/β-catenin-related genes in BCA, BCAC, ACC and PA.

	CTNNB1	*APC*	*AXIN1*	*AXIN2*
BCA (n = 34)	14 (41.1%)	1 (2.9%)	3 (8.8%)	0
BCAC (n = 3)	1 (33.3%)	0	0	0
ACC (n = 16)[a]	0	0	1 (6.2%)	1 (6.2%)
PA (n = 1)[b]	0	0	0	0

Table 5
Prevalence of nuclear β-catenin expression and Wnt/β-catenin −related gene mutations in BCAs.

		Mutations in *CTNNB1*, *APC*, *AXN1* and *AXIN2*	
		+ (n = 18)	- (n = 16)
Nuclear β-catenin expression	+ (n = 32)	17 (50.0%)	15 (44.1%)
	- (n = 2)	1 (2.9%)	1 (2.9%)

Percentages in parentheses are relative to total cases (n = 34) of BCAs.

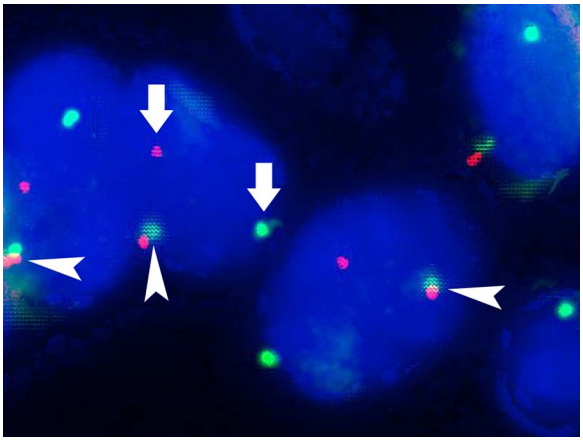

Fig. 4. Split FISH for *MYB* rearrangement in ACC (Original magnification × 1000). Tumor cells show a split (separated) signal pattern of one green and one red (arrow). The intact *MYB* is shown by a fused (yellow) signal or closely juxtaposed red and green signals (arrowhead).

for BCA was 100%, but the sensitivity fell to 76%. Therefore, when the β-catenin labeling index is 3–14% (score 1), careful diagnosis is needed to avoid confusion between BCA and other tumors with basal cell differentiation. In our study, 10/34 cases (29.4%) of BCA had at least focally cribriform or membranous structure similar to ACC, and the β-catenin labeling index was score 2 (≥15%) in the vast majority of these BCAs. On the other hand, in all cases of ACC, the β-catenin labeling index was lower than 15%. Therefore, when the β-catenin labeling index exceeds 15% in a morphologically challenging case with a cribriform or membranous structure, the case is more likely to be BCA.

According to the previous studies [13–15], *CTNNB1* mutations have been detected in about half of the cases of BCA (Fig. 5), but the reason for the discrepancy between the frequency of nuclear β-catenin immunoreactivity and the prevalence of *CTNNB1* mutation has been unclear. In our series, *CTNNB1* missense mutations were identified in about 40% of BCAs; this result was consistent with the previous reports [13–15]. Moreover, interestingly, 4/34 cases (11.7%) of BCA showed *APC* and *AXIN1* missense mutations. Collectively, *CTNNB1*, *APC* and *AXIN1* missense mutations were detected in 18/34 cases (52.9%) of BCA. To the best of our knowledge, the present study is the first report showing the prevalence of Wnt/β-catenin signaling pathway alterations including *CTNNB1*, *APC*, *AXIN1* and *AXIN2* in BCA. Our result suggests that *APC* and *AXIN1* alterations, as well as *CTNNB1* mutation, may be responsible for the accumulation of β-catenin in BCA. However, these mutations could not account for the nuclear β-catenin accumulation in about 40% of BCA cases, and thus alternative molecular mechanisms will be needed to explain the nuclear β-catenin accumulation in these tumors. As one of the possible mechanisms, the methylation of Wnt-suppressing genes such as *SFER*, *WIF1* and *DKK1* could be a cause of nuclear β-catenin expression; this alteration has been reported in colorectal cancer [23–25].

Table 6
Molecular features of ACCs with aberrant nuclear β-catenin expression.

Case	Site	Histological subtype	Nuclear β-catenin labeling index	*MYB*, *MYBL1* FISH	Mutations in Wnt/β-catenin-related genes
Case 1	Maxilla	Cribriform	10%	+, *MYB*	+ (*AXIN1* and *AXIN2*)
Case 2	Maxilla	Cribriform	8%	+, *MYB*	–
Case 3	Nasal cavity	Solid	3%	+, *MYBL1*	–

Abbreviation: FISH, fluorescence in situ hybridization.

In the current study, we found that nuclear β-catenin expression was also present in a certain percentage of the cases of BCAC, ACC and PA, in addition to gene mutations in *CTNNB1*, *AXIN1* or *AXIN2*. In BCAC, the molecular alterations that underlie the development and progression of the tumor are poorly characterized. Some of the previous studies reported that none of BCACs had *CTNNB1* mutation, despite the presence of nuclear β-catenin expression in 33–100% of cases (Fig. 5) [13–15]. In our series, 1/3 cases (33.3%) of BCAC showed focal nuclear β-catenin expression in addition to *CTNNB1* mutation. This genetic commonality suggests that at least some cases of BCAC might develop via malignant transformation from BCA. Recently, Jo et al. reported that one case of BCAC with a focal membranous component had *PIK3CA* mutation, biallelic inactivation of *NFKBIA* (coding IκBα) and focal *CYLD* deletion, but not *CTNNB1* mutation, despite the finding of focal nuclear β-catenin expression [14]. Although Choi et al. reported the loss of heterozygosity (LOH) of *CYLD* in 17/21 (80.9%) cases of sporadic membranous-type BCAs (dermal analogue tumor of salivary gland) [26], *CYLD* loss was not observed in conventional BCAs in the study by Jo et al. [14]. In our series, one case of membranous-type BCA with focal tubulotrabecular component showed nuclear β-catenin

Table 7
Nuclear β-catenin immunoreactivity in BCA, BCAC, ACC and PA in the previous reports and current study.

Authors	Clone	Dilution	Cut-off	BCA	BCAC	ACC	PA
Kawahara A, et al[#] [13]	β-catenin-1 Mouse monoclonal Dako Cytomatin	1: 200	1%	21/22 (95.4%)	1/3 (33.3%)	0/17 (0%)	0/43 (0%)
Jo, et al[#][14]	14 Mouse monoclonal BD Biosciences	1: 1000	1%	18/22 (81.8%)	2/3 (66.6%)	0/20 (0%)	0/20 (0%)
Jung, et al[#][15]	CAT-5H10 Mouse monoclonal Zymed Laboratories	1: 2000	5%	17/20 (85%)*	7/7 (100%)	N.D.	N.D.
Daa T, et al[#][21]	17C2 Mouse monoclonal Novocastra Laboratories Ltd	1: 100	Not shown	N.D.	N.D.	2/20 (10%)	N.D.
Our study	14 Mouse monoclonal BD Biosciences	1: 200	3%	32/34 (94.1%)	1/3 (33.3%)	3/67 (4.4%)	1/31 (3.2%)

Abbreviation: N.D., not done.
* Nuclear β-catenin expression was present in all conventional BCAs (10/10) and 70% (7/10) of tumors classified as "BCA with capsular invasion" (but not infiltrative growth).

expression. These findings suggest a diversity of molecular abnormality of membranous-type BCA. *NFKBIA* and *CYLD* are negative regulators of NF-κB signaling pathway, and dysfunctional alterations in these genes result in transcriptional up-regulation of target genes, leading to cell proliferation and survival [27,28]. Further analysis with a large number of BCAC cases is needed to elucidate the molecular tumorigenic mechanism and genetic relationship with BCA.

As for ACC, most previous reports have revealed that there is no nuclear β-catenin expression. According to the whole genome and exome sequencing data in ACCs, Wnt/β-catenin signal alterations were detected in only 3 of 88 cases (3.4%) in total; those alterations included homozygous loss, nonsynonymous mutation or truncation in *CTNNB1* gene [29,30]. In our series, 3 cases (4.4%) of ACC showed nuclear β-catenin expression, and one of them had *AXIN1* and *AXIN 2* mutations. Our results thus support the idea that only a minor subset of ACCs will exhibit nuclear β-catenin expression, in rare cases with Wnt/β-catenin-related gene alterations.

Here, the fact that nuclear β-catenin expression was present in ACC may evoke a diagnostic dilemma. In our study, all 3 cases of β-catenin-positive ACC had either *MYB* or *MYBL1* gene rearrangement. Therefore, our data suggest that the combination of nuclear β-catenin immunochemistry and *MYB* and *MYBL1* FISH may be helpful for differential diagnosis between BCA and ACC.

In PA, only 1 of 31 cases (3.2%) revealed nuclear β-catenin expression in neoplastic myoepithelial cells (S-100+, α-SMA+, calponin +) of the hyper-cellular component, but this case had no mutation in Wnt/β-catenin-related genes. Like, several publications have reported that nuclear β-catenin expression was not observed in PA (Table 7) [13,14,31,32]. It is well known that rearrangement of *PLAG1* or *HMGA2* genes is frequently present in PA [33,34]. Queimado et al. detected *HMGA2-WIF1* fusion gene in a subset of PAs, and these PA cases showed lower expression of WIF1 (Wnt inhibitor factor 1) protein than normal salivary gland tissue [34]. WIF1 binds to WNT1 proteins and prevents Wnt signal activation [35]. Queimado et al. suggested that Wnt signal activation due to WIF1 downregulation might play a role in the development of PA [34], although the molecular mechanism of WIF1 downregulation was not detailed in their study.

LEF-1 is a nuclear transcription factor of Wnt/β-catenin signal pathway. Recently, Bilodeau EA et al. reported that coexpression of LEF-1 and nuclear β-catenin was present in basaloid salivary gland tumors and various odontogenic tumors [36]. As a diagnostic marker, the authors suggested that LEF-1 expression may be helpful to distinguish BCA and BCAC from other salivary gland tumor.

In conclusion, our results suggest that nuclear β-catenin immunoreactivity with appropriate criteria may be useful to distinguish BCA from morphologically similar salivary gland tumors, although pathologists should pay attention to unexpected nuclear β-catenin expression in a minor subset of ACCs. In addition to previously reported *CTNNB1* mutation in BCA, we revealed *APC* and *AXIN1* mutations in some population of BCAs and *CTNNB1* mutation in BCAC. These alterations in Wnt/β-catenin signaling pathway may play a role in the pathogenesis of BCA and BCAC.

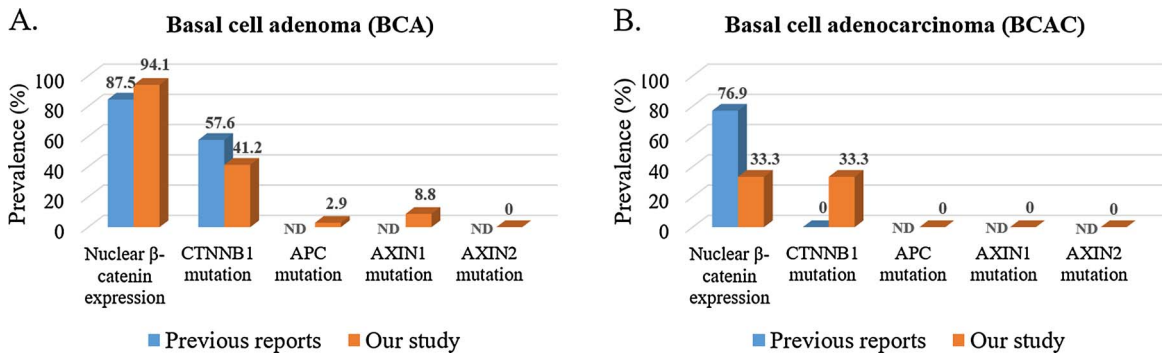

Fig. 5. Comparison between previous reports and our study about Wnt/β-catenin signal alterations in BCA (A) and BCAC (B). Blue and orange bars correspond to the data from previous studies and those from the current study, respectively. The prevalence of Wnt/β-catenin signal alterations in the previous reports is shown by summing up the number of "positive" cases in the three studies [13–15] included in Table 7. Abbreviation: N.D., not done.

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Competing interests

The authors declare that there are no potential conflicts of interest.

Ethics approval

This study was approved by the Ethics Committees of Kyushu University (no. 26–185, 29–100) and the National Kyushu Cancer Center (no. 2015-5).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2017.12.016.

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