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Original Article

Potential diagnostic and prognostic value of β-catenin and SF-1 in adrenocortical tumors

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Abstract: Objective: this study primarily evaluated the diagnostic and prognosis effect of β-catenin and SF-1 in adrenocortical adenoma (ACA) and adrenocortical carcinoma (ACC). Methods: immunohistochemistry was applied to assess the protein expression of β-catenin and SF-1 on tissue samples from patients with complete data, including 25 cases of ACAs and 23 cases of ACCs both exactly diagnosed by Weiss system. Results: 13 of 25 ACAs (52%) and 20 of 23 ACCs (87%) had a diffuse cytoplasm or nucleus accumulation of β-catenin. The difference of β-catenin expression between the two groups was statistically significant (P=0.01); Positive staining for SF-1 was observed in 88% (22/25) of the ACA group including 7 weak positive, 9 moderate positive and 6 strong positive, and 83% (19/23) of the ACC group including 2 weak positive, 2 moderate positive and 15 strong positive (P=0.486); overall survival of patients who present with positive staining results of β-catenin were shorter than negative ones, but the median OS time between SF-1 (positive) group and SF-1 (negative) group was not statistical significance (P>0.05). Conclusions: Firstly, β-catenin is a potential and promising cutoff between ACA and ACC, as well as reverse prognosis marker; Secondly, as though SF-1 isn't an ideal index to identify nature of ACT or evaluate prognosis of patients, SF-1 seems to be a specific hallmark of ACT; Thirdly, β-catenin (++)/SF-1(+/++/+++) is expected to be a potentially differential index between ACA and ACC.

Keywords: Adrenal cortical carcinoma, diagnosis, β-catenin, SF-1, immunohistochemistry, IHC

Introduction

Adrenocortical tumor (ACT) is a rare endocrine tumor derived from the adrenal cortex, especially adrenocortical carcinoma (ACC) that is rarer and more aggressive with a poorer prognosis. Its annual incidence is approximately 0.5 to 2 per million populations, and five-year survival rate varies from 16~38% [1]. Early radical surgical resection is theoretically considered as the only curable means for those patients with ACC until recently, but due to absent of clinically reliable laboratory indexes, imaging manifestations and pathological features now, it's extremely hard for clinical doctors to early identify ACC from ACT. Consequently, majority of patients either present with advanced stage of disease at the time of primary diagnosis, or are misdiagnosed as benign owing to localized lesions but long-term follow-up shows their tumors to be malignant, so that they are less likely to receive radical operation in time. Albeit molecular pathogenesis of ACT had made partly progress in the past couple of decades, there remains unchanged much in clinical aspects of diagnosis and treatment [2]. Therefore, the establishment of candidate diagnostic biomarkers to validly distinguish between benign and malignant ACT is a relatively pressing need.

Molecular studies have pinpointed that the activated Wnt signaling pathway played a pivotal role in pathogenesis of a variety of tumors, like colon [3], gastric [4] and liver carcinomas [5]. As a major intracellular mediator, β-catenin was translocated into nucleus would activate classic Wnt signaling pathway, the end results of which were transcription of target genes, and then cell proliferation or difference. Moreover, dysregulation of the Wnt/β-catenin was implicated in Epithelial-mesenchymal transition and tumorigenesis [6]. It is noteworthy that β-catenin was also associated with formation and function of the adrenal cortex [7]. Overexpression

of β-catenin has been immunohistochemically verified in 38% of adrenocortical adenoma (ACA) and 85% of ACC [8]. Steroidogenic factor-1 (SF-1) or adrenal 4 binding protein (Ad4BP), as a transcription factor belonging to the nuclear receptor family NR5, had a great effect on formation and growth of endocrine tissues and organs (pituitary, adrenal gland and gonad) and synthesis process of steroid hormone under physiological dosage [9]. Whereas, it could facilitate cell proliferation and suppress cell apoptosis, even gave rise to adrenal tumorigenesis under an increased dosage [10]. Similarly, overexpression of SF-1 has been reported in adrenocortical tumors [9, 11]. And high expression level of SF-1 protein was closer with the malignant in children [12].

As described, SF-1 involved in endocrine function is a marker specially reflecting endocrine organs and β-catenin involved in tumorigenesis is a marker reflecting nature of tumors. Accordingly, we reasonably suppose that research for index combined with two markers to judge nature of tumors and prognosis of disease is a significant work. However, many previous studies have just investigated correlation of β-catenin or SF-1 alone with malignant behaviors or prognosis in ACCs. And little is clear about whether the index is in line with our expectations or not. Therefore, we respectively detect β-catenin and SF-1 expression level in ACA and ACC through immunohistochemistry method in our study, in order to provide a theoretical basis for clinically identifying ACC from ACA as soon as possible.

Materials and methods

Tissue samples

Approved by institutional ethics review board, all tissue samples were collected between December 1995 and December 2012 from those patients who received adrenalectomy in Department of Urology in Ruijin Hospital affiliated to Medicine School of Shanghai Jiaotong University in China. All objectives included in our study were necessary to satisfy the follow conditions: First, there were kinds of complete data, including medical history, symptoms, signs, pathological and follow-up data; Second, Weiss system was proposed as a differential diagnosis standard between ACC and ACA, including nuclear grade, mitoses, atypical mitoses, clear cells, diffuse architecture, confluent necrosis, venous invasion, sinusoidal invasion and capsular infiltration. The total scores >3 were diagnosed as ACC and total scores <2 were diagnosed as ACA. However, a score of 2 or 3 were eliminated from this study. According to the above conditions, a total of 48 cases ACT included in this study are divided into two groups: 23 ACC and 25 ACA.

Immunohistochemistry (IHC)

After fixing in 10% neutral formalin and dehydrating in concentration gradient alcohol, samples were embedded in paraffin, then cut into 3 μm serial sections, which were dewaxed twice in xylene, rehydrated in alcohol and then rinsed with phosphate buffer solution (PBS) followed by treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. After antigen retrieval, the slides were incubated with 10% goat serum under constant temperature to block non-specific reactions for 10 min. Subsequently, sections were treated with polyclonal rabbit antihuman β-catenin antibody (1:100 dilution; Abcam, UK) and polyclonal rabbit antihuman SF-1 antibody (1:200 dilution; Abcam, UK) for 12 h at 4°C. After washed with PBS three times, the slides were incubated with secondary antibody EnVision TM kit (DAKO, USA) at 37°C for 30 min. Washed again with PBS and developed in diaminobenzidine (DAB) substrate; the slides were counter-stained in hematoxylin and dehydrated with ethanol and xylene before being mounted. The slides were incubated with PBS instead of primary antibodies was as negative control.

Evaluation of immunohistochemical results

Two pathologists who were blinded to the subtype of the tumors independently judged staining intensity and mounted for those sections. Yellowish-brown granules located in the cytoplasm or nucleus for β-catenin and in the nucleus for SF-1 were considered as positive, and lack of any evident yellowish-brown granules located in the cytoplasm or nucleus for β-catenin and in the nucleus for SF-1 was considered as negative.

Score of staining intensity was judged based on follow categories: score 0 (no staining), score 1 (weak), score 2 (moderate), score 3 (strong); Three random high-power fields (400×) were

Table 1. Clinical features of patients in our study

Parameter	ACC	ACA	*P*-value
Total number	23	25	
Gender			P>0.05
Male	9	9	
Female	14	16	
Age (year, x ± s)	47.91 ± 12.32	48.40 ± 12.98	P>0.05
Tumor location			P>0.05
Left	8	17	
Right	14	7	
Bilateral	1	1	
Primary tumor size			
<5.0 cm	3	15	
5.0~10.0 cm	16	9	
>10.0 cm	4	1	
Mean diameter	8.25 ± 3.78	4.93 ± 3.04	P<0.05
ENSAT stage			
I	1		
II	9		
III	11		
IV	2		
Follow-up (months, x ± s)	43.48 ± 21.99	129.64 ± 34.34	P<0.05

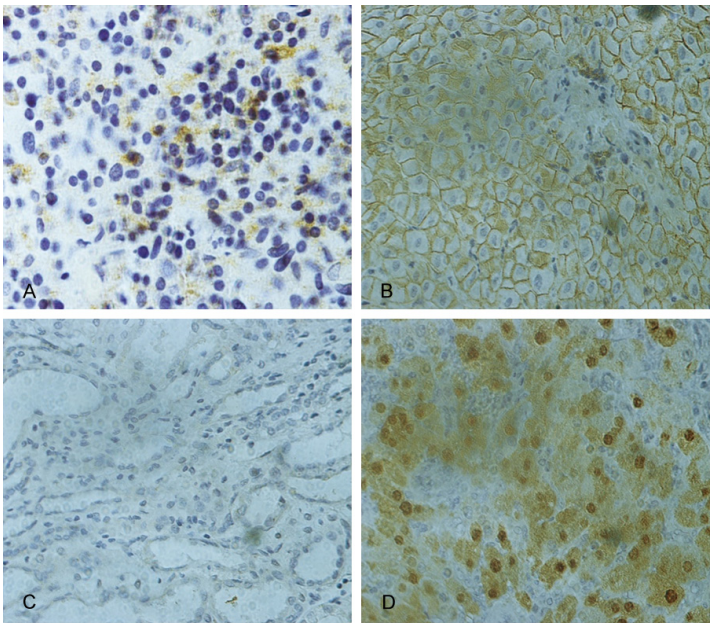

Figure 1. Expression of β-catenin in samples from adrenocortical adenomas (ACA) and adrenocortical carcinomas (ACC). (A) negative in ACC, (B) positive (cytoplasm) in ACC, (C) negative in ACA, (D) positive(cytoplasm and nucleus) in ACA. All images are 400×.

selected and then the number of positive-staining cells was amounted under a total of 200 cells, score of semi-quantitative as follow: score 0 (0-5%), score 1 (6~25%), score 2 (26~50%), score 3 (51~75%), score 4 (76~100%). Final score was determined according to the combination of these 2 variables and divided into: score 0 negative (-), otherwise positive including score 1~4 weak positive(+), score 5~8 moderate positive (++), score 9~12 strong positive (+++).

Statistical analysis

The data analyses were carried out using statistical software SPSS (version 21.0). All Clinicopathologic characteristics were expressed as mean ± standard deviation and their correlation was examined by student's t test or Pearson x^2-test. Pearson x^2-test, Fisher's exact test or non-parametric test was used to compare β-catenin and SF-1 expression level between ACC and ACA. Receiver operating characteristic curve (ROC) was drawn to evaluate effect of differential diagnostic test. Kaplan-Meier survival plot was generated to compare overall survival (OS) between ACC and ACA with the log-rank statistic. A *P*-value less than 0.05 were considered statistically significant.

Results

Clinicopathologic data

From 1995 to 2012 in Ruijin Hospital, we have identified 25 cases of ACAs and 23 cases of ACCs whose demographic features were summarized in **Table 1**. Gender distribution between two groups was not statistically significant (P>0.05). Difference of age at primary diagnosis was similar (P>0.05) between the ACAs (47.91 ± 12.32) and the ACCs (48.40 ± 12.98). The largest diameter of tumors in the benign and the malignant lesions was 4.93 ± 3.04 and 8.25 ± 3.78 respectively (P<0.05). Except with that, mean post-operation follow-up time differed between the ACAs and the ACCs (129.64 ± 34.34 vs 43.48 ± 21.99, P<0.05).

Table 2. Staining outcome of β-catenin and SF-1 in adrenocortical tumors

		β-catenin					
		ACA		Total	ACC		Total
		-	++		-	++	
SF-1	-	1	2	3	1	3	4
	+	3	4	7	0	2	2
	++	5	4	9	0	2	2
	+++	3	3	6	2	13	15
Total		12	13	25	3	20	23

β-catenin differentiates ACC from ACT alone

All slides were either negative or moderate positive for β-catenin. 13 of 25 ACAs (52%) and 20 of 23 ACCs (87%) had a diffuse cytoplasm or nucleus accumulation of β-catenin (**Figure 1**). The difference of β-catenin expression between the two groups was statistical significance (P=0.01). If β-catenin (++) was as a cut-off between ACA and ACC, the sensitivity was presumptively 87% and the specificity was 48% (**Table 2**).

Differentiates ACC from ACT alone

Frequency of SF-1 positive expression was observed in 88% (22/25) of the ACA group including 7 weak positive, 9 moderate positive and 6 strong positive, and 83% (19/23) of the ACC group including 2 weak positive,2 moderate positive and 15 strong positive (**Figure 2**; **Table 2**). The difference of SF-1 staining intensity between the two groups was not statistical significance (P=0.486). Similarly analyzed by logistics regression analysis (**Table 3**), we found that β-catenin (OR=6.167, P=0.014, 95% CI 1.447 to 26.271) was a superior biomarker than SF-1 (OR=0.639, P=0.617, 95% CI 0.111 to 3.698) to indicate malignant nature of ACT.

β-catenin and SF-1 are combined to differentiate ACC from ACT

Combining with two indexes, β-catenin(++)/SF-1(+++), β-catenin(++)/SF-1(++/+++) and β-catenin(++)/SF-1(+/++/+++) were observed in respectively 3, 7 and 11 of 25 ACAs, while 13, 15 and 17 of 23 ACCs (**Table 2**). Compared between difference of above indexes in ACA and ACC, correspondent *P*-values were P=0.001, P=0.01 and P=0.035, respectively. So area under curves (AUC) calculated by ROC were 0.732 (P=0.008), 0.686 (P=0.027) and 0.650 (P=0.076) respectively (**Figure 3**).

Kaplan-Meier survival analysis combined with β-catenin and SF-1

In our study, Kaplan-Meier survival analysis showed that the patients whose slides presented with β-catenin(++)/SF-1(+++) showed shorter overall survival than those whose staining results were non-β-catenin(++)/SF-1(+++) (P< 0.05, **Figure 4**). The median OS time of two groups was 178 (95% confidence interval (CI), 136.06-219.94 months) and 62 (95% CI, 49.16-74.84 months) respectively. Similarly, expression of β-catenin protein was negatively related to patients' OS (**Figure 4**). However, the median OS time between SF-1 (positive) group and SF-1 (negative) group was not statistical significance (P>0.05, 138 months vs 140 months, **Figure 4**).

Discussion

Unlike adrenocortical adenoma (ACA), adrenocortical carcinoma (ACC) is a rare but highly malignant epithelial tumors derived from adrenal cortex, characterized by dismal prognosis with 60% five-year survival for TNM stage I and II and less than 10% five-year survival for TNM stage III and IV [13]. However, that the pathologists quickly identify ACC form ACT still remains challenging due to the lack of specific pathologic characterization of ACC, which is common reason of misdiagnosis and missed diagnosis, even missed follow-up for suspicious malignant entities. At present, the most frequent methods used to diagnose ACT is Weiss system, while it presents with poor repeatability [14] and "grey zone" (nature of ACT with a score 2 or 3 was still controversial) [15]. Consequently, clinical workers starve for more effective diagnostic indexes, especially molecular biomarkers to guide routine work and improve prognosis of patients with ACTs.

In normal adrenal tissue, Wnt/β-catenin signaling pathway was deemed to be essential for its formation, development and function. Regardless of benign and malignant ACT, there was abnormal activation of this pathway [16-18]. In addition, β-catenin constitutive activation would lead adrenocortical cells to excessive proliferation and poor differentiation, even malignant transformation verified by murine animal model [19]. Therefore, we detected β-catenin expression level in ACT through immunohistochemistry analysis and its results showed that the difference of β-catenin expression

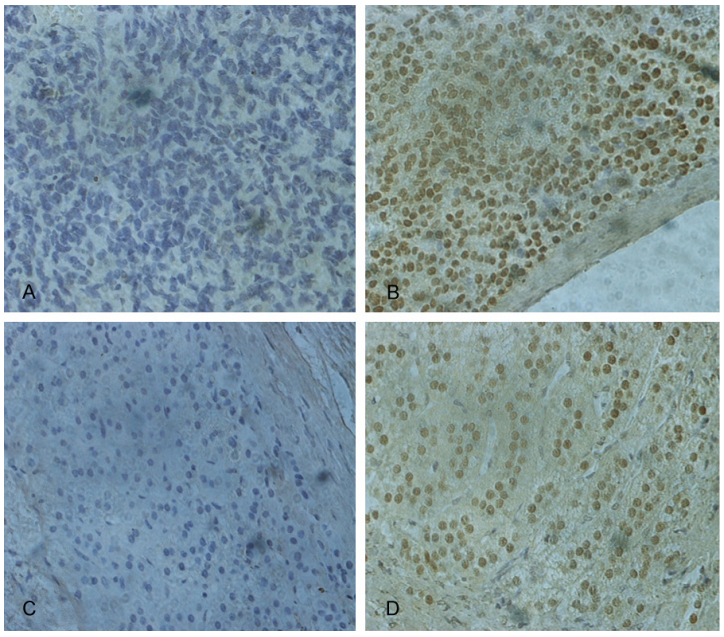

Figure 2. Expression of SF-1 in samples from adrenocortical adenomas (ACA) and adrenocortical carcinomas (ACC). (A) negative in ACC, (B) positive(nucleus) in ACC, (C) negative in ACA, (D) positive (nucleus) in ACA. All images are 400×.

Table 3. Results of logistics regression analysis

Variables	B	SE	Wald	*p*-value	OR	95% CI for OR
Constant	-1.006	0.989	1.033	0.309	0.366	
β-catenin	1.819	0.739	6.052	0.014	6.167	1.447 to 26.271
SF-1	-0.447	0.895	0.250	0.617	0.639	0.111 to 3.698

B, regression coefficient; SE, standard error; OR, odds ratio; CI, confident interval.

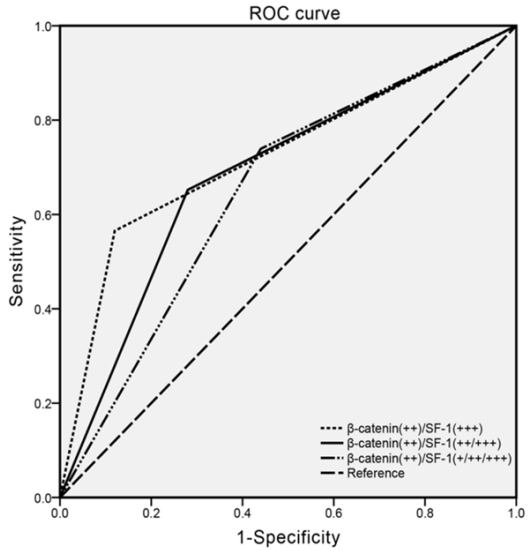

Figure 3. Receiver operating characteristic curve (ROC) for staining intensity of β-catenin and/or SF-1 as a predictor of malignant. β-catenin(++)/SF-1(+++), β-catenin(++)/SF-1(++/+++) and β-catenin(++)/SF-1(+/++/+++) respectively as diagnostic index of malignant, their corresponding AUC is 0.732 (P=0.008), 0.686 (P=0.027) and 0.650 (P=0.076). The number of β-catenin(++)/SF-1(++/+++) includes the total number of β-catenin(++)/SF-1(++) and β-catenin (++)/SF-1(+++).

between ACAs and ACCs was statistical significance (52% vs 87%, P=0.01), suggesting that β-catenin would be potentially used as independent diagnosis index for malignant ACTs (OR=6.167, P=0.014). Our results were similar to previous study by Tissier et al [8], in which positive β-catenin was observed in 38% ACAs (10/26) and 85% ACCs (11/13). On the other hand, we found patients who presented with β-catenin (++) has shorter potential life span (P=0.02) compared with β-catenin (-) groups, consistent with previous conclusion that β-catenin staining intensity was inversely associated with prognosis [20].

Recently, Sbiera et al [9] has indicated SF-1 as an adrenocortical specific biomarker appeared as 98.6% sensitivity and 100% specificity by analyzing 161 cases of ACCs and 73 cases of tumor tissues from non-steroidogenic organs and overexpression of SF-1 protein was inversely associated with poor prognosis. So, we tried to attest SF-1 as a highly potential differential diagnostic and prognostic index in ACT. Based on this study, however, we found that SF-1 alone was neither an appropriate differential diagnostic biomarker (OR=0.639, P=0.617), nor prognostic biomarker (P>0.05). So we also tried to analyse the causes. On the one hand, sample size or individual difference possibly led to no significant difference between SF-1 (positive) and SF-1 (negative) groups in this study. On the other hand, just similar to that hypothesis proposed by Galac S [21], we believed that difference between ACA and ACC possibly existed in function level instead of expression level of SF-1 protein. That is to say, its function to stimulate growth instead of ste-

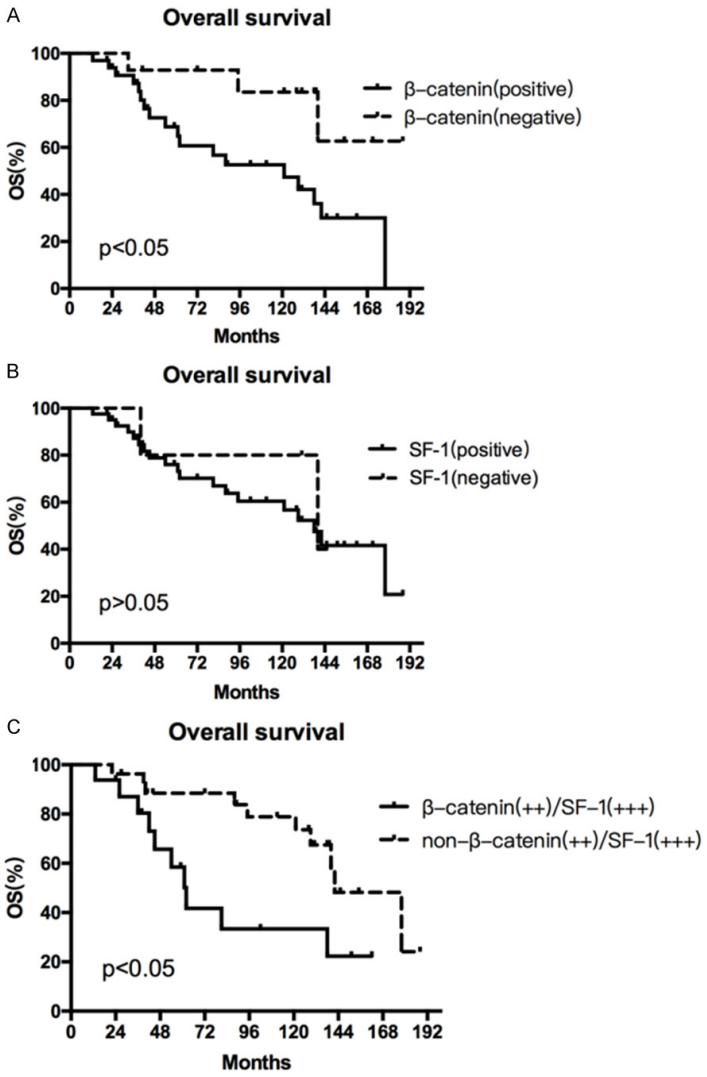

Figure 4. Kaplan-Meier curves showed overall survival distribution in patients. A. Represented those patients with β-catenin (positive) vs β-catenin (negative). B. Represented those patients with SF-1 (positive) vs SF-1 (negative). C. Represented those patients with β-catenin(++)/SF-1(+++) vs non-β-catenin(++)/SF-1(+++).

roid synthesis was negatively linked to degree of malignant or differentiation. Although our study didn't include normal adrenocortical tissue or non-steroidogenic tissue specimens resulting in impossibly estimating sensitivity and specificity of SF-1 as a diagnostic marker of ACT, this study showed that staining results of 85% (41/48) ACTs varied from weak positive to strong positive, possibly supporting SF-1 as a specific hallmark of ACT.

To our knowledge, there weren't any reports to evaluate whether the index combined with β-catenin and SF-1 staining results was promising as diagnosis and prognosis marker. As to differential diagnosis, we regarded respectively β-catenin(++)/SF-1(+++), β-catenin(++)/SF-1(++/+++) and β-catenin(++)/SF-1(+/++/+++) as cutoff between ACAs and ACCs, and found that AUC is 0.732 (P=0.008), 0.686 (P=0.027) and 0.650 (P=0.076) respectively, and sensitivity is 57%, 65% and 74%, respectively. Although AUC of test where β-catenin(++)/SF-1(+++) was as diagnosis index was higher than others, sensitivity was lower than others. Missed diagnosis is absolutely fatal so that we suggest that differential diagnostic criterion primarily satisfies high sensitivity at first. From two aspects of statistical analysis and high sensitivity, β-catenin(++)/SF-1(++/+++) is a possibly superior index.

In summary, we come to the following conclusions in this study: Firstly, β-catenin is a potential and promising cutoff between ACA and ACC, as well as reverse prognosis marker; Secondly, as though SF-1 isn't an ideal index to identify nature of ACT or evaluate prognosis of patients, SF-1 seems to be a specific hallmark of ACT; Thirdly, β-catenin(++)/SF-1(+/++/+++) is expected to be a potentially differential index between ACA and ACC. But owing to rarity of ACT, the number of available samples still remains the biggest stumbling block for research progress. Accordingly, we expect a large number of samples information to further verify our conclusion, then finally provide clinical workers with powerful evidence to differentiate ACAs and ACCs.

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Disclosure of conflict of interest

None.

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