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ORIGINAL RESEARCH

β-Catenin-driven adrenocortical carcinoma is characterized with immune exclusion

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Aim: Adrenocortical carcinoma (ACC) is characterized by overexpressed *CTNNB1*, which is reported to modulate immune exclusion. Cross talk between *CTNNB1* and cancer immunity in ACC remains unclear.

Materials and methods: In silico reproduction of TCGA-ACC dataset (N = 92) and external validation using tissue samples were performed (N = 16). Expression data of *CTNNB1*, PD-1, and PD-L1 were extracted in silico and tumor-infiltrating lymphocytes (TILs) were profiled using code provided by Tumor IMmune Estimation Resource (TIMER). In-house formalin-fixed paraffin-embedded ACC samples were processed using immunohistochemical (IHC) staining for *CTNNB1*, CD45, PD-1, and PD-L1.

Results: Increased *CTNNB1* expression was significantly associated with worsened overall survival (OS) ($P = 0.006$). $CD8^+$ cells were significantly associated with better OS ($P = 0.02$). Higher PD-L1 ($P = 0.019$), but not PD-1 expression ($P = 0.325$), was associated with better OS. *CTNNB1* overexpression was significantly associated with increased tumor purity ($r = 0.356$, $P = 0.002$) and fewer TILs ($r = -0.833$, $P = 0.029$), decreased infiltrating $CD8^+$ cells ($P = 0.033$), and increased infiltrating B cells ($P = 0.026$). *CTNNB1* expression was negatively correlated with PD-L1 expression ($r = -0.308$, $P = 0.006$) but not with PD-1 expression ($P = 0.067$), which were externally validated ($P = 0.032$ for PD-L1 and $P = 0.400$ for PD-1). The Cox regression model encompassing gender, B cells, $CD8^+$ cells, PD-L1, CTNNB1, and Ki-67 revealed that only Ki-67 overexpression remained significantly associated with OS ($P < 0.001$), while *CTNNB1* showed marginal significance ($P = 0.06$). *CTNNB1*-overexpressed patients were more likely to have cortisol excess ($P = 0.003$).

Conclusion: ACC with CTNNB1 overexpression is associated with poor prognosis and decreased immunity. Our findings suggest that CTNNB1-targeting therapy may overcome immune exclusion in ACC.

Keywords: adrenocortical carcinoma, CTNNB1, tumor-infiltrating lymphocyte, PD-L1, prognosis

Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive disease. Though curable at its early stage by surgery, there is a dearth of definitive treatment for advanced or metastatic disease.[1] Unfortunately, with the absence of specific cancer-related early symptoms, about 70% of patients are diagnosed with stage III or IV diseases. Driving genetic alterations have recently been identified owing to the second-generation sequencing technique, showing driver genes including *CTNNB1*, *TP53*, *CDKN2A*, *RB1*, *MEN1*, and newly identified *PRKAR1A*, *RPL22*, *TERF2*, and *CCNE1*.[2,3]

CTNNB1 encodes β-catenin, an essential component of the canonical Wnt/β-catenin pathway, which plays an important role in a variety of biological processes

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that involve cell growth and differentiation, including but not limited to cell–cell adhesion, development, and cardiac physiology.[4–6] Such biological advantage can also be taken in case of cancer. It has been estimated that ~10% of all tissue samples sequenced from all cancers display mutations in the *CTNNB1* gene.[7] Alterations such as gain or loss of functional mutation at different sites of the gene, as well as overexpression, result toward enhanced and/or increased gene product that vitalizes cancer cells. As one of the driver genes in ACC, *CTNNB1* mutation is reported to be present in up to 70% of cases and to exert pro-tumorigenic function via activation of Wnt/β-catenin pathway.[8]

Recent development in immune checkpoint inhibitors (ICIs) appears promising, despite moderate response rate. Of note, anti-PD-1/PD-L1 treatment has been proven efficacious in several types of solid and hematological tumors, including melanoma of the skin, non-small-cell lung cancer, kidney cancer, bladder cancer, head and neck cancers, and Hodgkin's lymphoma.[9] Five PD-1/PD-L1 blockade agents and one anti-CTLA4 agent are now US FDA approved and are being tested further in more types of cancer for safety and efficacy. Identifying more cancer types that can potentially benefit from ICI and developing sensitive and specific biomarkers for potential responder are the two current hotspots for research work.

Our previous findings shed light on a possible immune-modulating role of *CTNNB1* in ACC.[10,11] In the current study, we aim to evaluate association between CTNNB1 expression and cancer immunity in silico followed by external validation. Our findings may not only complement the current understanding of biology of ACC but also contribute to development of novel targeted and immune therapies for ACC.

Materials and methods

Data mining of TCGA-ACC dataset

The TCGA ACC (Provisional) dataset was selected on the cBioportal online platform. The *CTNNB1* mRNA expression level of all cases was queried. The clinical information including age, gender, tumor stage, excessive hormone status, survival period, and follow-up time was extracted. To obtain the immunity profile of TCGA-ACC dataset, an online analytical tool named "Tumor IMmune Estimation Resource (TIMER)" was used. TIMER is a web resource for systematical evaluations of the clinical impact of different immune cells in diverse cancer types.[12] The immune estimate of TCGA-ACC dataset was obtained and plotted from TIMER platform. The gene module on TIMER provided the correlation of selected gene expression with various immune infiltration levels. The survival module illustrated the Kaplan–Meier curve and provided the Cox regression analysis between overall survival (OS) and clinical factors, immune infiltrates, or gene expression level.

Patients and ACC specimen

Sixteen tumor samples from ACC patients, who had undergone surgical resection between January 2009 and August 2017, were included. HE-stained sections were reviewed by an independent pathologist for confirmation. Clinicopathological parameters such as age, gender, stage, metastasis status, and Ki-67 score were extracted from the patient medical records and pathological reports. Endocrine activity was not included as over half of the cases were incidentalomas and diagnosis of ACC was made only after resection. Survival data were not collected as over half of the patients were lost to follow-up. Signed informed consent was obtained from each patient whose sample was analyzed in the current study and the study protocol was approved by the local ethical committee (Huashan Institutional Review Board).

Immunostaining and pathological evaluation

A standard immunohistochemical (IHC) protocol was followed and 2 μm unstained tumor sections from formalin-fixed paraffin-embedded tissue were deparaffinized in xylene and rehydrated through graded alcohols. After antigen retrieval, the sections were stained with primary antibodies, PD-L1 (28-9; Abcam; dilution: 1:200), CD45 (EP322Y; Abcam; dilution: 1:250), and β-catenin (E247; Abcam; dilution: 1:250), followed by antibody localization using the Dako Envision + HRP-labeled polymer (DAKO). Staining was visualized by 5-minute incubation with diaminobenzidine.

Positivity for PD-L1 and PD-1 was defined as positive staining in both tumor cells and tumor-infiltrating lymphocytes (TILs). Expression of PD-L1 on tumor cell was scored as 0 (absence of staining), 1 (1%–10% of staining), 2 (10%–50% of staining), and 3 (>50% of staining). The extent of staining in TILs was recorded as absent (0), focal (1), mild (2), moderate (3), and strong (4) with score 0 or 1 considered negative. IHC expression of β-catenin in tumor cell was evaluated and scored as percentage of cytoplasmic and/or nuclear positivity multiplied by intensity (weak, 1; moderate, 2; and strong, 3), as described previously. All sections were reviewed by two independent pathologists until consensus was made.

Statistical analysis

The chi-square test was used to compare categorical variables. The Student's *t*-test and Mann–Whitney tests were used for continuous parametric variables. The Spearman's correlation coefficient was applied to measure the correlation

between two groups. The Cox regression model was used to test the contribution of individual factor to survival in a multivariate analysis. The *P*-value of <0.05 was accepted as statistically significant.

Results

Patient characteristics

The TCGA-ACC cohort included 92 cases. For comparison of the *CTNNB1* expression in patients with different clinicopathological parameters, we studied distribution of the cases within top and bottom 30% of *CTNNB1* expression. Demographic and clinicopathological parameters of those patients are summarized in Table 1. Female patients and hormone excess were associated with higher *CTNNB1* expression. Demographic and clinicopathological parameters of the 16 ACC patients in the validation cohort are summarized in Table 2. Expression of *CTNNB1* was not associated with any parameter in this cohort.

Prognostic role of CTNNB1, TILs, and PD-1/PD-L1 in ACC

Overactive canonical WNT signaling featuring gain-of-function alteration in CTNNB1 has been identified as one of the driver genetic events in ACC. When *CTNNB1* expression level was divided into top 30% versus lower 30%, we found that higher *CTNNB1* expression was significantly associated with worsened OS (Figure 1A). Accordingly, increased infiltrating $CD8^+$ cells (top 30% vs bottom 30%) was significantly associated with improved prognosis (Figure 1B). We then explored whether PD-L1 expression was associated with survival in TCGA cohort and found that higher PD-L1 expression was significantly associated with improved survival (Figure 1C), while PD-1 expression was not (Figure 1D).

In order to elucidate the independent impact of CTNNB1 expression on survival, we included factors unevenly distributed among patients with different *CTNNB1* expressions (Table 3). We found that Ki-67, the only clinically validated prognostic marker, remained the only significant factor in the multivariate model, leaving marginal significance for *CTNNB1* expression. Effects of $CD8^+$ cells, B cells, or gender on survival was lost in the Cox model (Table 3).

Association of CTNNB1 with cancer immunity in ACC

Cancer-intrinsic β-catenin has been shown to play a role in immune modulation.[13] We therefore hypothesized that immunity in ACC could be linked to *CTNNB1* expression. We analyzed correlation between *CTNNB1* expression and TIL abundance indicated by decreased tumor purity. We found

Table 1 Correlation between clinicopathological parameters and top and bottom 30% expression of CTNNB1, reproduced from TCGA ACC cohort

	CTNNB1		*P*-value
	Overexpression	Downexpression	
Age (years), median (min–max)	40 (14–69)	45 (22–77)	0.897
Gender			0.002
Male	1	9	
Female	14	5	
Endocrine activity			0.025
+	11	6	
–	2	8	
NA	2	1	
Stage (AJCC)			0.193
I	0	3	
II	8	9	
III	3	1	
IV	4	2	
Metastasis			0.361
No	11	13	
Yes	4	2	
Necrosis			0.357
Absent	2	4	
Present	12	10	
NA	1	1	
Mitosis/10 HPF (mean ± SD)	29.1 ± 31.8	8.9 ± 7.2	0.116

Abbreviations: AJCC, American Joint Committee on Cancer; NA, not accessible; HPF, high-power field; ACC, adrenocortical carcinoma.

Table 2 Correlation between clinicopathological parameters and CTNNB1 expression of ACC samples

	CTNNB1			*P*-value
	N	Expression (mean)	SD	
Gender				0.587
Male	10.00	2.00	0.82	
Female	6.00	2.00	0.63	
Stage (AJCC)				0.38
I	3.00	2.67	0.58	
II	8.00	1.75	0.71	
III	0.00	0.00	0.00	
IV	5.00	2.00	0.71	
Metastasis				0.657
No	10.00	2.00	0.82	
Yes	2.00	1.50	0.71	
Necrosis				>0.99
Absent	5.00	2.00	0.71	
Present	11.00	2.00	0.77	
Mitotic rate > 5/50 HPF				0.51
Absent	9.00	2.10	0.78	
Present	7.00	1.90	0.69	
	Mean	SD	Correlation coefficient	*P*-value
Age (years)	44.7	0.73	0.16	0.079
Ki67	8.2	5.30	0.61	0.62
Weiss score	3.9	1.20	0.09	0.085

Abbreviations: AJCC, American Joint Committee on Cancer; HPF, high-power field; ACC, adrenocortical carcinoma.

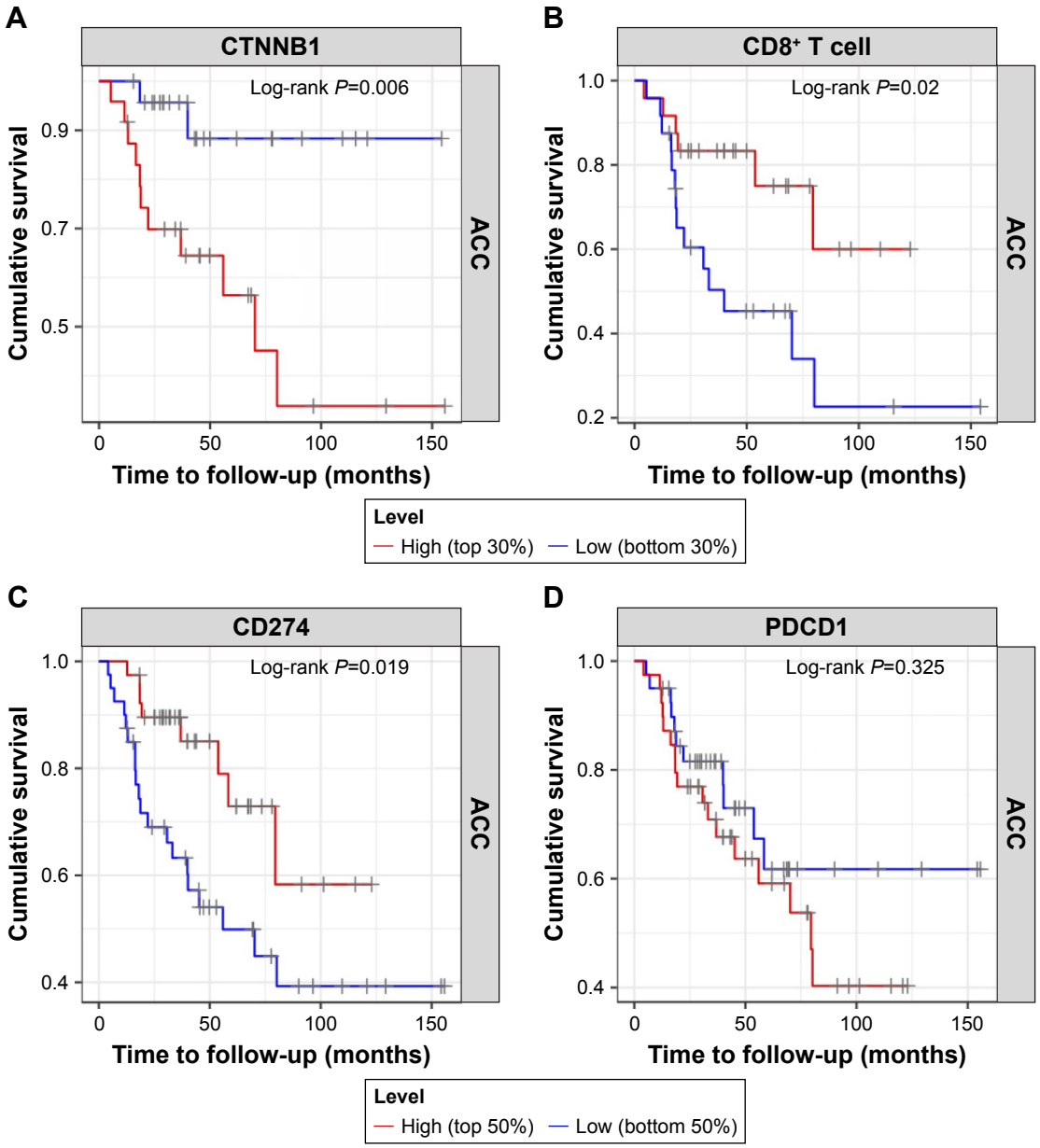

Figure 1 Prognostic roles of CTNNB1 and immune-related factors in adrenocortical carcinoma (ACC), reproduced from TCGA-ACC cohort, showing (**A**) significantly worsened overall survival (OS) in patients with top 30% versus bottom 30% of CTNNB1 expression; (**B**) OS in patients with top 30% versus bottom 30% of $CD8^+$ count; (**C**) OS in patients with top 50% versus bottom 50% of PD-L1 (CD274) expression; and (**D**) OS in patients with top 50% versus bottom 50% of PD-1 (PDCD1) expression.

Table 3 Cox regression analysis of factors contributing to overall survival

	Co-efficient	HR	95% CI lower	95% CI upper	*P*-value
Gender	0.045	1.046	0.392	2.79E+00	0.928
B cell	−20.471	0	0	4.32E+28	0.642
$CD8^+$ T cell	13.69	882,417.115	0.051	1.52E+13	0.107
PD-L1	−0.257	0.774	0.514	1.16E+00	0.218
CTNNB1	0.361	1.435	0.985	2.09E+00	0.06
MKI76	0.781	2.184	1.626	2.93E+00	<0.001

Abbreviations: HR, hazard ratio; CI, confidence interval.

that higher *CTNNB1* expression was significantly associated with increased purity (Figure 2A). Cases with higher *CTNNB1* expression showed significantly decreased infiltration of $CD8^+$ cells (Figure 2B, left panel) and increased infiltration of B cells (Figure 2B, right panel). *CTNNB1* expression was also negatively correlated with decreased PD-L1 level and was not correlated with PD-1 expression (Figure 2C). Expressions of CTNNB1 and Ki-67 were not correlated in the TCGA cohort, nor was the correlation observed in our validation cohort (Figure 2D, Table 2). Owing to the rarity

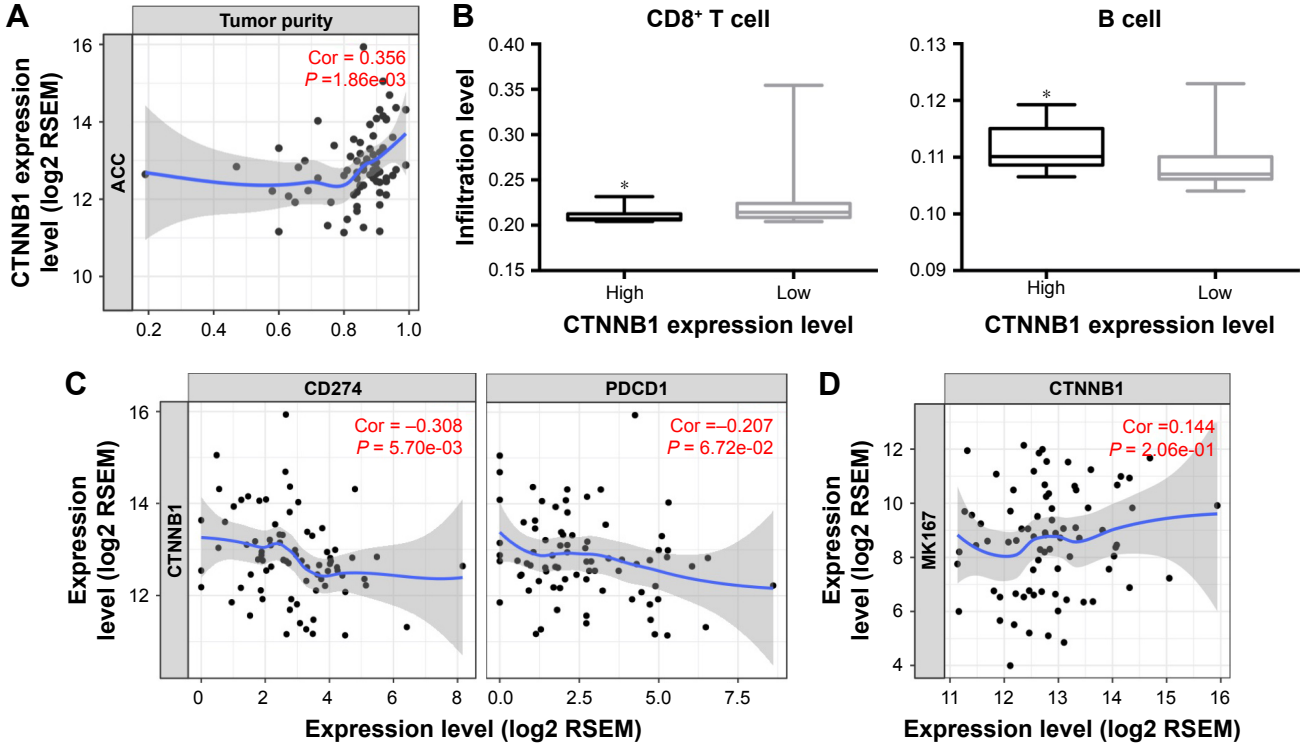

Figure 2 Correlation between CTNNB1 expression and immunity in ACC, reproduced from TCGA-ACC cohort, showing (**A**) correlation between CTNNB1 expression and tumor purity; (**B**) counts of $CD8^+$ T and B cells between patients with top 30% versus bottom 30% of CTNNB1 expression; (**C**) correlation between expressions of CTNNB1 and PD-L1 (CD274) or PD-1 (PDCD1); (**D**) correlation between expressions of CTNNB1 and Ki-67 (MKI67) (*$P < 0.05$).
Abbreviations: ACC, adrenocortical carcinoma; RSEM, RNA-seq by expectation-maximization.

of the disease, we collected 16 samples of ACC as validation cohort. Using continuous IHC expression (Figure 3A–D), we validated that CTNNB1 expression was significantly associated with decreased PD-L1 expression ($r = –0.752$, $P = 0.002$) and was not associated with PD-1 expression ($P = 0.302$). Indeed, PD-1 was barely expressed in ACC (Figure 3D).

CTNNB1 and PD-1/PD-L1 in ACC

As higher PD-L1 was associated with worsened prognosis in many cancers, we hypothesized that our contradictory findings were due to increased TILs with concomitant increased PD-L1 expression in ACC. Of note, increased purity was significantly associated with decreased PD-L1 expression (Figure 4A). Specifically, PD-L1 expression was associated with increased $CD8^+$ cells, neutrophils, and B cells (Figure 4B–D). In our validation cohort, we found that *CTNNB1* expression was significantly correlated with decreased TILs ($r = –0.650$, $P = 0.006$). Also, TIL abundance was significantly associated with PD-L1 ($r = –0.635$, $P = 0.015$) but not with PD-1 expression ($P = 0.303$). Nonetheless, we used a combined scoring system including PD-L1 positivity in both tumor cells and TILs, and we observed majority of the tumor cells expressed PD-L1 (Figure 4B).

CTNNB1 was associated with increased cortisol level

Over 50% of ACC cases were characterized with hormone excess. There is a dearth of study showing association between *CTNNB1* and hormone status. Given the aforementioned findings, we hypothesized that decreased TILs in ACC with higher *CTNNB1* expression could result from excessive hormone levels, especially cortisol. We found that cases with excessive hormone demonstrated significantly higher *CTNNB1* expression (Figure 5A). In the analyses with breakdown of hormone types, we found that excessive cortisol-containing cases showed significantly higher *CTNNB1* expression (Figure 5B). Cases with excessive hormone types other than cortisol demonstrated *CTNNB1* expression level not significantly different from cases without ("None" in Figure 5B, data not shown). There were three patients in our validation cohort who exhibited excessive cortisol and data for the rest were missing.

Discussion

While the prognostic role of *CTNNB1* expression in ACC has been reported in several studies,[14,15] its role in cancer immune

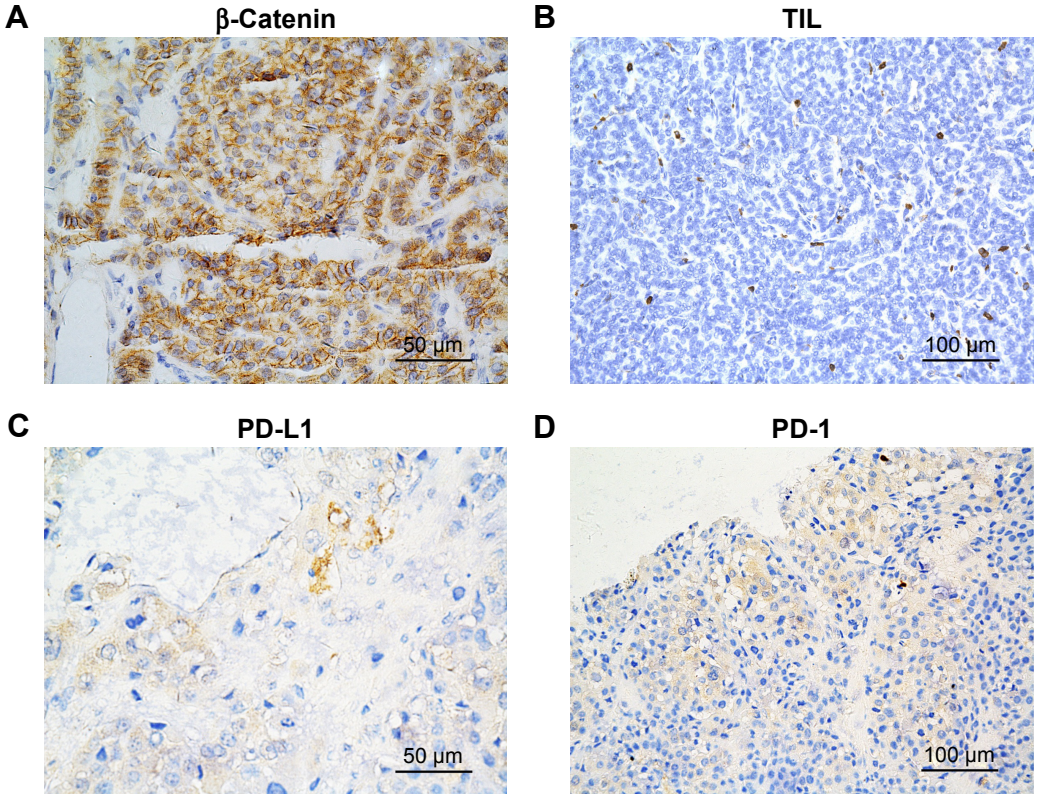

Figure 3 External validation using ACC samples from our institute showing representative IHC staining of (**A**) CTNNB1 (β-catenin, 400×); (**B**) TILs (200×); (**C**) PD-L1 (400×); and (**D**) PD-1 showing very limited staining (200×).
Abbreviations: ACC, adrenocortical carcinoma; IHC, immunohistochemical; TILs, tumor-infiltrating lymphocytes.

modulation remains unstudied. We have here demonstrated that higher *CTNNB1* expression is not only associated with poor OS but also with impaired immune response, both revealed in silico and in our in-house validation cohort. Previous study has demonstrated that PD-L1 positivity in either tumor cell membrane or TIL is not significantly associated with higher stage at diagnosis, higher tumor grade, excessive hormone secretion, or OS in ACC.[16] The discrepancy with our findings could result from the dichotomized designation for positivity therein.

We suggest that the most important finding in the current study is the negative association between *CTNNB1* and TIL counts, including decreased infiltration of $CD8^+$ cells. This possibly puts ACC in a newly recognized cancer entity in which the immune escape is β-catenin-driven. While Wnt/β-catenin pathway plays an important role in T-cell immunity, the regulatory mechanism has long been studied solely within T cell itself.[17] How cancer-intrinsic β-catenin is involved in immune exclusion is a newfound that has recently come into view.[18] Spranger et al have presented the

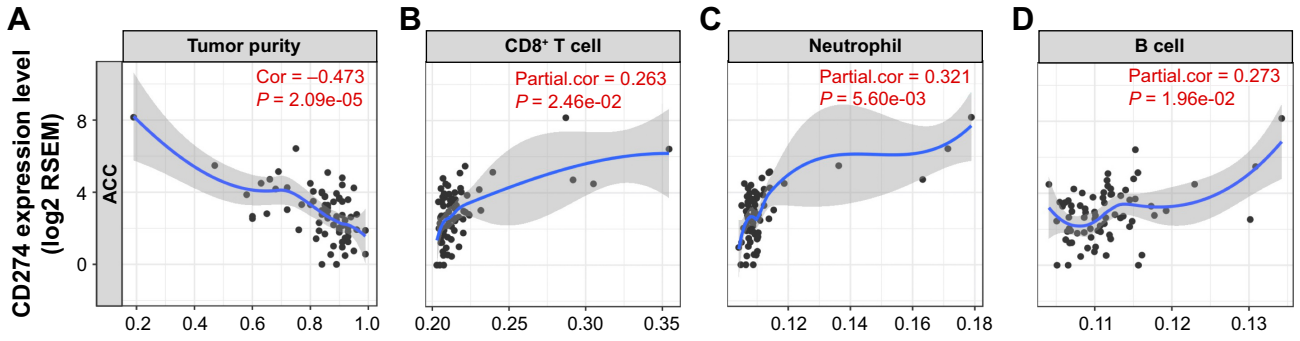

Figure 4 Correlation between PD-L1 (CD274) expression and immunity in ACC, reproduced from TCGA-ACC cohort, showing (**A**) correlation between PD-L1 expression and tumor purity, and correlation between PD-L1 expression and (**B**) $CD8^+$ T-cell count, (**C**) neutrophil count, or (**D**) B-cell count.
Abbreviations: ACC, adrenocortical carcinoma; RSEM, RNA-seq by expectation-maximization.

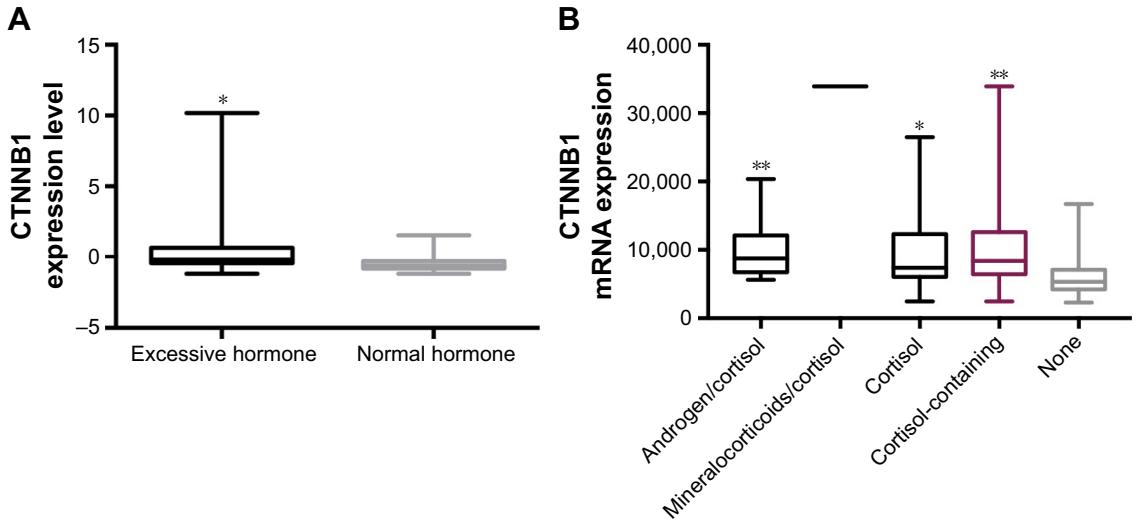

Figure 5 Relation between CTNNB1 expression and hormone excess in ACC, reproduced from TCGA-ACC cohort, showing (**A**) CTNNB1 expression level in cases with or without hormone excess, and (**B**) CTNNB1 expression level in cases with or without cortisol excess (*$P < 0.05$; **$P < 0.01$).
Abbreviation: ACC, adrenocortical carcinoma.

first evidence of an inverse relationship between melanoma intrinsic β-catenin signaling and intratumoral T-cell infiltration and have used autochthonous mouse models to prove the causal effect by *CTNNB1*.[13,19] Despite the rapid development of ICIs, only a limited percentage (<~40%) of patients with a certain type of cancer respond to the therapy and there are certain tumors that do not even respond at all. Understanding the mechanisms leading to a non-T-cell-inflamed microenvironment are crucial for the development of novel treatment modalities to expand the fraction of patients benefiting from immunotherapy. Here we have for the first time revealed this correlation in both TCGA and external validation cohorts in ACC. The findings not only hold promise to optimize ICI in ACC but can also be extrapolated to other cancers which are β-catenin-driven.

ACC is also characterized with hormone excess. Our findings indicate that *CTNNB1* overexpression is associated with excessive cortisol, the most common type of hormone excess. We, however, did not include the hormone status in the Cox model, considering that mixed hormonal types could confound the interpretation. How *CTNNB1* is associated with hormone excess, in particular cortisol excess, warrants further study.

$CD8^+$ T cells play critical role in cancer immunity. Brownlie et al has reported resistance to TGF-β suppression and improved antitumor responses in $CD8^+$ T cells lacking PTPN22.[20] De Meulenaere et al has reported that $CD8^+$ T lymphocytes constitute an independent prognostic marker in patients diagnosed with oropharyngeal squamous cell carcinoma.[21] In our study, $CD8^+$ cells are significantly associated with improved OS. Also, lower $CD8^+$ counts are identified in cases with higher *CTNNB1* expression. Both findings support the anticancer role of $CD8^+$ cells in ACC.

Another intriguing finding in the current study is increased B-cell counts in ACC with higher *CTNNB1* expression. Recent studies suggest that B cells likely play a dual role in cancer immunity.[22] Distinct subsets of B cells dynamically help shape the tumor microenvironment in both a pro- and antitumorigenic manner. On one hand, B cells exert protumorigenic effect via contributing to an angiogenic and proinflammatory microenvironment, and by directly or indirectly suppressing T-cell activation.[23] On the other hand, B cells exert anticancer effect via production of cytokines coordinating other immune cells, particularly through enhancement of cytotoxic T-cell activity.[24] The sophisticated coordination between TIL subtypes can be reflected in our finding that B cells are increased in *CTNNB1* overexpressed cases while not contributing to prognosis. Though it appears that B cells exert pro-tumorigenic role in ACC, our results solely serve as an observation in a cross-sectional perspective.

The implication of our findings lies in two aspects. First, we have provided clue for a pro-tumorigenic pathway in ACC with *CTNNB1* activation. How CTNNB1 is mechanistically associated with immune escape warrants further elaboration. Second, association between *CTNNB1* and immunity supports application of WNT inhibitor in ACC; for instance, dual inhibition of *CTNNB1* and PD-L1 using a novel compound BBI-801, which is well tolerated with no signs of toxicity observed.[25] Our study has limitations. First, the in silico reproduction should be validated in a larger

cohort. However, as a rare disease, multicenter involvement is required for validation with enough power. Second, the heterogeneous detection method for *CTNNB1* and PD-L1 in TCGA, our cohort, and other reports makes it biased for horizontal comparison of the results. In vitro studies also provide limited translational merit as autochthonous mouse model for ACC is lacking. In a rare and lethal disease like ACC, perhaps only patients enrolling the trial could answer the question.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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