CANCER DIAGNOSIS & PROGNOSIS
5: 32-41 (2025) doi: 10.21873/cdp.10409

Tumor β-Catenin Expression Associated With Poor Prognosis to Anti-PD-1 Antibody Monotherapy in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: Tumor intrinsic β-catenin signaling has been reported to influence the tumor immune microenvironment and may be a resistance mechanism to immune checkpoint inhibitors in various cancers. Patients and Methods: We studied the association between tumor β-catenin expression and survival in 50 patients with non-small cell lung cancer (NSCLC) treated with anti-programmed death-1 antibody monotherapy. Tumor β-catenin expression was evaluated by immunohistochemistry. Results: Patients with positive tumor β-catenin expression (20% of all patients) had worse progression-free survival and overall survival compared with those with negative tumor β-catenin expression. Patients with positive tumor β-catenin expression had reduced $CD8^+$ cell and $CD11c^+$ cell infiltration into tumor nests than those with negative tumor β-catenin expression. RT-PCR of tumor tissue revealed that patients with positive tumor β-catenin expression showed lower gene expression of CD8A, CD4, IFN-γ, BATF3, and CCL4. Knockdown of CTNNB1 tended to increase CCL4 expression, likely mediated by ATF3, in a lung cancer cell line with positive β-catenin expression. Conclusion: NSCLC patients with positive tumor β-catenin expression that were treated with anti-programmed death-1 antibody monotherapy had poor prognosis.*

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Key Words: Cancer immunotherapy, tumor immune evasion, beta-catenin, tumor-infiltrating lymphocytes, non-small cell lung cancer.

Lung cancer is the leading cause of cancer death worldwide. An estimated 1.8 million people died of lung cancer in 2020 globally (1). Immune checkpoint inhibitors (ICIs) have brought great benefits to patients with lung cancer and are now commonly used in its treatment. However, the 2-year overall survival rate of non-squamous non-small cell lung cancer (NSCLC) patients treated with pembrolizumab plus chemotherapy is only approximately 45.7% (2). One of the challenges in improving patient treatment is the lack of biomarkers other than programmed death-ligand 1 (PD-L1) expression in tumor tissue (3).

To identify patients who may benefit from treatment and to develop new therapeutic strategies, the identification of resistance mechanisms to ICIs is critical. Several studies have provided insights into the resistance mechanisms to ICIs in various cancers. For example, major histocompatibility complex (MHC) class I deficiency (4), deletions in the β2-microglobulin gene (5), and mutations in the interferon-γ signaling pathway (6-9) are well-known resistance mechanisms. Several studies have also shown that oncogene signaling can influence immune escape mechanisms (10-13).

Tumor intrinsic β-catenin signaling was reported to be a resistance mechanism to ICIs in an animal model of melanoma (14). β-Catenin signaling was shown to suppress the expression of C-C motif chemokine ligand 4 (CCL4) by activating transcription factor 3 (ATF3), resulting in the decreased recruitment of antigen presenting cells. We demonstrated that tumor β-catenin expression was associated with immune evasion in NSCLC (15). In lung cancer, tumor intrinsic β-catenin signaling has been reported to be associated with a tumor microenvironment that suppresses anti-tumor immunity (16). Whether tumor intrinsic β-catenin signaling is associated with resistance to ICIs in lung cancer has not been clarified. Moreover, whether β-catenin signaling suppresses the expression of CCL4 *via* ATF3 in lung cancer as in melanoma is unknown.

This study investigated the influence of tumor β-catenin expression on the survival of patients with NSCLC treated with

Table I. *Gene sets used for non-small cell lung cancer RT-PCR.*

Co-inhibition	Genes related to dendritic cells	Core T-cell signature genes	β-Catenin-mediated immuno-related genes
CD274	*ITGAX*	*CD8A*	*THBD*
CTLA4	*ITGAE*	*IFNG*	*BATF3*
HAVCR2		*CD4*	*IRF8*
LAG3			*CCL4*
PDCD1			*CCR5*

ICIs. Additionally, we studied the relation between tumor β-catenin expression and the tumor immune microenvironment. We further explored how β-catenin aids in the evasion of anti-tumor immunity in lung cancer cell lines. The main treatment strategies for advanced NSCLC without driver gene mutations are chemoimmunotherapy (chemotherapy + anti-PD-1/PD-L1 Ab ± anti-CTLA-4 Ab); we thus examined the effect of chemoimmunotherapy on this β-catenin related immune-resistant mechanism in lung cancer cell lines.

Patients and Methods

Patients. A total of 50 patients with recurrent NSCLC treated with anti-programmed death-1 (PD-1) antibody monotherapy at the Hospital of Fukushima Medical University between January 2016 and December 2019 were enrolled.

Immunohistochemistry. Paraffin-embedded NSCLC specimens were cut and stained using β-catenin (1:100; cat. no. UMAB15; OriGene Technologies, Rockville, MD, USA), CD8 (1:50; cat. no. C8/144B; Agilent Technologies, Santa Clara, CA, USA), or CD11c (1:200; cat. no. 2F1C10; ProteinTech Group, Chicago, IL, USA) in the way we have previously reported (15). Two investigators without knowledge of the clinicopathological data analyzed the micrographs for each sample. A light microscope (IX73; Olympus Corporation, Tokyo, Japan) with a CCD camera (DP73; Olympus Corporation) was used. As described in the previous study, specimens with tumor cells exhibiting only membranous staining were classified as having negative (normal) β-catenin expression, whereas specimens with tumor cells showing cytoplasmic staining were classified as having positive (abnormal) β-catenin expression (15). Infiltration of $CD8^+$ and $CD11c^+$ cells into tumor nests was evaluated as positive or negative (15).

PD-L1 expression in tumors was evaluated in 40 patients, randomly selected from the total 50 patients. The PD-L1 IHC 22C3 pharmDx immunohistochemistry assay, performed on the Dako Autostainer Link 48, was used for evaluation at SRL (Tokyo, Japan). The percentage of viable tumor cells with membrane staining was defined as the PD-L1 tumor proportion score (TPS) (17).

RT-PCR of NSCLC tumor tissues. Among the total 50 patients, seven patients were excluded because of insufficient specimens for RT-PCR and six were excluded from data analysis because of low gene expression. Finally, 37 patients were analyzed. The primer and probe sets for real-time PCR were from the TaqMan® Gene Expression Assay from Thermo Fisher Scientific (Waltham, MA, USA). Coinhibitory genes, genes previously reported to be associated with β-catenin-mediated immuno-related mechanisms (14), and genes associated with dendritic cells were examined in the 37 patients (Table I). Tumor sections were collected from unstained slides and deparaffinized, and total RNA was extracted using the RNeasy® FFPE Kit and the RNase Free DNase Set. RNA quality was assessed using a NanoPad DS-11 (DeNovix, Wilmington, DE, USA). Reverse transcription reactions were performed using the SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA amplification reactions were performed using the TaqMan® PreAmp Master Mix (Thermo Fisher Scientific). Real-time PCR was performed using the TaqMan® Universal Master Mix II, no UNG (Thermo Fisher Scientific). Ct values were calculated using the Fluidigm Real Time PCR Analysis 4.5.2 software (Fluidigm Corp, San Francisco, CA, USA). The average of the Ct values, measured in triplicate, was calculated, and the relative value for each sample was calculated using the $2^{-\Delta\Delta CT}$ method. A heatmap was created using Heatmapper (18).

Cell lines, cell culture, and treatments. The lung squamous cell carcinoma cell lines LK-2 (RCB1970) and RERF-LC-A1 (RCB0444) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. LK-2 cells were maintained in RPMI 1649 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). RERF-LC-A1 cells were maintained in MEM medium (Sigma-Aldrich) supplemented with 10% FBS (Thermo Fisher Scientific).

In some experiments, carboplatin (0.03 mg/ml, Fujifilm Wako Pure Chemical Corporation), paclitaxel (0.02 mg/ml, Fujifilm Wako Pure Chemical Corporation), nivolumab (0.01 mg/ml, Ono Pharmaceutical Co., Ltd., Osaka, Japan), and ipilimumab (3 ng/ml, Bristol Myers Squibb, Princeton, NJ, USA) were added to the culture.

siRNA knockdown. *CTNNB1* specific and control siRNAs were obtained from Thermo Fisher Scientific. Silencer™ Select Pre-Designed siRNA (ID: s437) was used for *CTNNB1*. For siRNA transfection, 3×10^5 tumor cells were plated in 6-well plates at a concentration of 1×10^5 per ml. Opti-MEM (Thermo Fisher Scientific) was mixed with 1.2 pmol siRNA and 1.5% RNAiMAX reagent (Thermo Fisher Scientific), and the mixture was added to the culture at a ratio of 1:5. After 36 h of culture, cells were harvested for RT-PCR.

RT-PCR. Total RNA was isolated from cell lines using TRIzol reagent (Thermo Fisher Scientific) and a PureLink RNA Mini Kit (Thermo Fisher Scientific). RNA quality was assessed using a

Table II. *Patient characteristics (n=50).*

	β-Catenin status		
	Positive	Negative	*p*-Value
No. of patients	10	40	
Age, years, median (range)	70 (51-85)	69 (46-85)	0.7535
Sex			
Male	7 (70%)	25 (63%)	0.7301
Female	3 (30%)	15 (37%)	
Histology			
Non-Sq	5 (50%)	36 (90%)	0.0101
Sq	5 (50%)	4 (10%)	
PD-L1 TPS			
<1%	3 (30%)	7 (18%)	0.7003
1%-49%	2 (20%)	15 (37%)	
≥50%	3 (30%)	10 (25%)	
NA	2 (20%)	8 (20%)	
ICI agent			
Pembrolizumab	5 (50%)	12 (30%)	0.2768
Nivolumab	5 (50%)	28 (70%)	
Treatment line with ICI, median (range)	2 (1-9)	2 (1-9)	0.2053

Data are shown as n (%) unless otherwise indicated. PD-L1: Programmed cell death-ligand 1; TPS: tumor proportion score; ICI: immune checkpoint inhibitor; Sq: squamous cell lung cancer; Non-Sq: non-squamous cell lung cancer; NA: not assessed.

Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific) and samples with a 260/280 nm absorbance ratio of 1.8 or greater were used for RT-PCR. One-step RT-qPCR was performed using a Taqman RNA-to-C_T 1-Step kit (Thermo Fisher Scientific). TaqMan Gene Expression Assays (Hs00355045_m1, Hs00231069_m1 and Hs99999148_m1; Thermo Fisher Scientific) were used to detect *CTNNB1*, *ATF3*, and *CCL4*. *GAPDH* (Hs02786624_g1; Thermo Fisher Scientific) was used as a normalization control. Reactions were run on a Step One Plus (Thermo Fisher Scientific). The $2^{-\Delta\Delta CT}$ method was used for quantitative evaluation of gene expression.

Statistical analysis. Statistical analyses were performed using GraphPad Prism v8.4.3 (GraphPad Software, Boston, MA, USA). Continuous variables were compared by two-tailed *t*-test. Categorical variables were compared by Fisher's exact test except for ICI agent comparison. ICI agent comparison was done by chi-square test. Progression-free survival and overall survival were defined as the time from the start of ICI administration to the occurrence of the event (disease progression or death). Survival was estimated using the Kaplan–Meier method, and survival curves were compared using log-rank tests.

Results

β-Catenin expression was more common in squamous cell carcinoma compared with non-squamous cell carcinoma. The characteristics of the 50 patients with NSCLC treated with ICI monotherapy are presented in Table II. Tumor β-catenin expression was positive in 20% of patients (n=10). The β-catenin-positive group had a higher proportion of patients with squamous cell carcinoma (50%) than the β-catenin-negative group (10%). There were no significant differences between the β-catenin-positive and -negative groups in age, sex, PD-L1 TPS, ICI drug administered, or line of treatment.

β-Catenin expression was associated with poor prognosis in NSCLC patients treated with ICI monotherapy. β-Catenin-positive NSCLC patients treated with ICI monotherapy had a significantly shorter progression-free survival than β-catenin-negative NSCLC patients (p=0.019, Figure 1A). The median progression-free survival was 76 days in β-catenin-positive NSCLC patients and 157 days in β-catenin-negative NSCLC patients. β-Catenin-positive NSCLC patients treated with ICI monotherapy also had a significantly shorter overall survival compared with β-catenin-negative NSCLC patients (p=0.002, Figure 1B). The median overall survival was 112 days in β-catenin-positive NSCLC patients and 620 days in β-catenin-negative NSCLC patients.

β-Catenin-expressing tumors are cold tumors. To investigate the relationship between tumor β-catenin expression and $CD8^+$ and $CD11c^+$ cell infiltration into tumor nests, we performed immunohistochemistry of CD8 and CD11c. β-Catenin-positive NSCLC patients had low levels of $CD8^+$ and $CD11c^+$ cells infiltrating into tumor nests (Table III). In the β-catenin-negative group, $CD8^+$ cell infiltration was found in 65% of patients and $CD11c^+$ cell infiltration was observed in 80% of patients. In contrast, in the β-catenin-positive group, $CD8^+$ cell infiltration was found in 20% of patients and $CD11c^+$ cell infiltration was

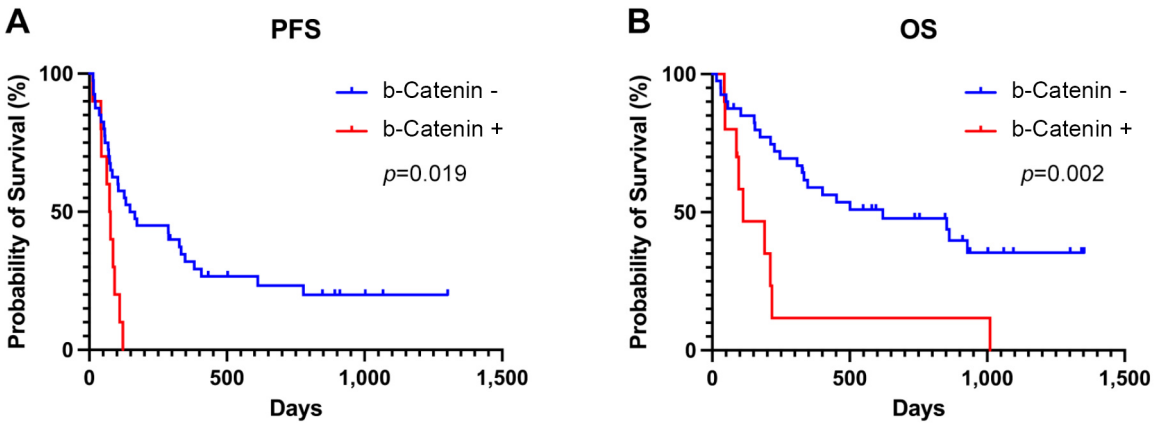

Figure 1. *Association between tumor β-catenin expression and survival. The β-catenin-positive cases showed significantly worse progression-free survival (A), and overall survival (B) compared with cases with negative β-catenin expression.*

Table III. *Association of β-catenin expression with tumor-infiltrating CD8⁺ cells and tumor-infiltrating CD11c⁺ cells in patients (n=50).*

	β-Catenin status		
Characteristic	Positive	Negative	*p*-Value
CD8⁺ cell infiltration into tumor			
+	2 (20%)	26 (65%)	0.015
–	8 (80%)	14 (35%)	
CD11c⁺ cell infiltration into tumor			
+	3 (30%)	32 (80%)	0.005
–	7 (70%)	8 (20%)	

Data are shown as n (%).

observed in 30% of patients. We next explored the relationship between β-catenin expression in tumors and the expression of coinhibitory genes, genes reported to be associated with β-catenin-mediated immuno-related mechanisms (14), and genes associated with dendritic cells (Table I) to examine how β-catenin influences anti-cancer immunity. As shown in Figure 2, β-catenin-positive expression was more common in cases with low expression of these genes.

CTNNB1 knockdown or chemotherapy plus ICIs showed a tendency to improve CCL4 expression in lung squamous cell carcinoma cell lines with β-catenin expression. To investigate whether β-catenin expression causes ATF3-mediated down-regulation of *CCL4* expression in NSCLC, as reported in melanoma by Spranger *et al*. (14), we knocked down *CTNNB1*, which encodes β-catenin, in LK-2 and RERF-LC-A1 lung squamous cell carcinoma cell lines by siRNA. Immunohistochemistry showed that LK-2 cells are positive for β-catenin protein expression, while RERF-LC-A1 cells are negative (Figure 3A and B). *CTNNB1* expression was significantly decreased by *CTNNB1*-specific siRNA in both LK-2 and RERF-LC-A1 cells (p=0.02, p=0.03, respectively, Figure 3C and D). *CTNNB1* knockdown had no significant impact on *ATF3* and *CCL4* in both cell lines, although *CTNNB1* knockdown showed a tendency to decrease *ATF3* and increase *CCL4* in LK-2 cells.

To explore the influence of clinically used chemotherapy + anti-PD-1 antibody + anti-CTLA-4 antibody, we added carboplatin + paclitaxel + nivolumab + ipilimumab to the cell lines. The results showed that *CTNNB1* expression was decreased in LK2 cells treated with the drug combination compared with that in control cells (p=0.04, Figure 3C), while *CTNNB1* expression in RERF-LC-A1 cells remained unchanged (Figure 3D). The drug combination had no significant effect on *ATF3* and *CCL4* though it showed a tendency to decrease ATF3 and increase CCL4 in both cell lines.

Discussion

We investigated the relationship between tumor β-catenin expression and anti-PD-1 antibody monotherapy outcome in NSCLC patients. Our results indicate that β-catenin-positive

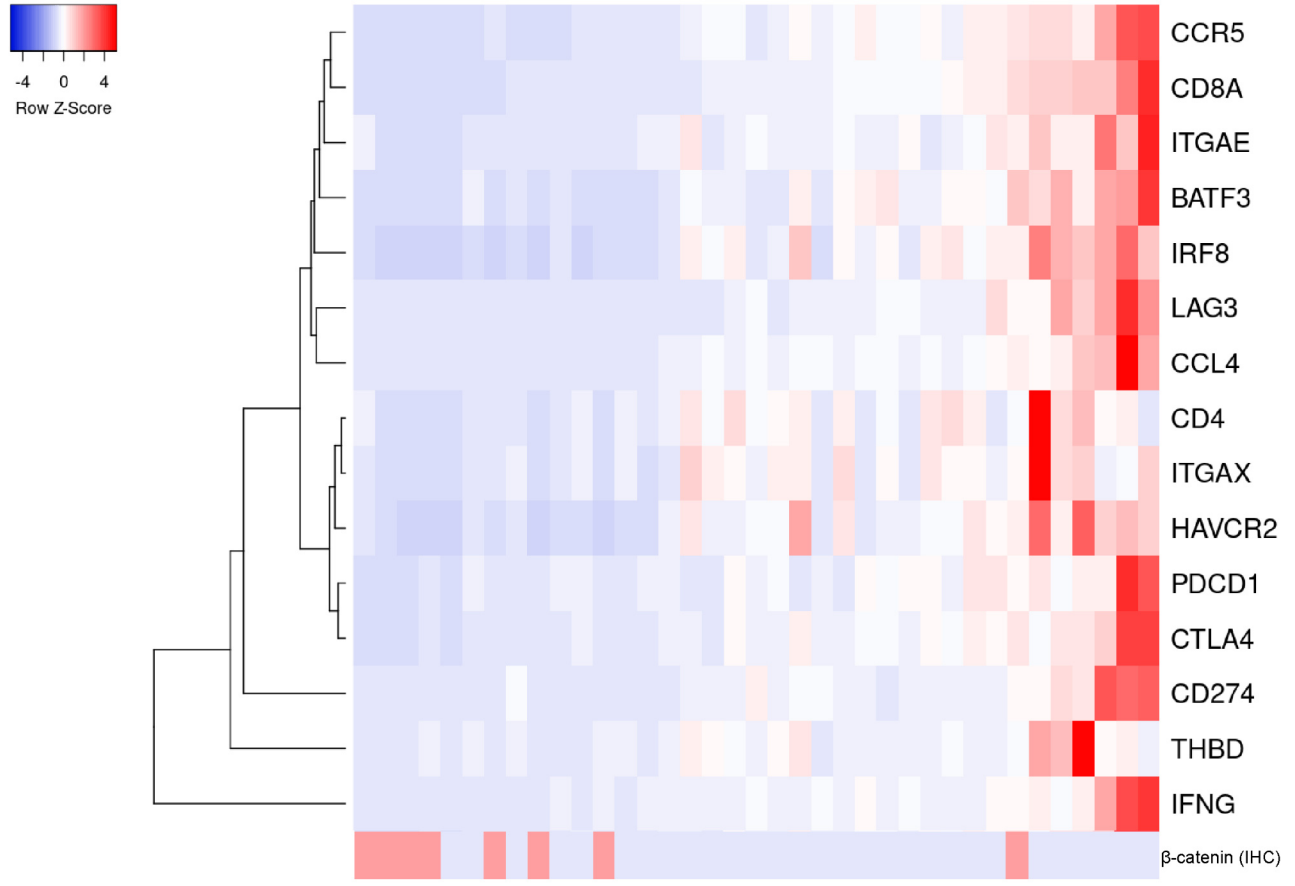

Figure 2. *Association between gene expression of tumor tissue and β-catenin expression (n=37). β-Catenin-positive non-small cell lung cancer tumors, as identified by immunohistochemistry, had lower expression of immune-related genes.*

NSCLC cases are poor responders to anti-PD-1 monotherapy. β-Catenin may be a predictive biomarker for anti-PD-1 antibody monotherapy for NSCLC patients and aid in selecting patients who are not expected to benefit from anti-PD-1 antibody monotherapy. Currently, PD-L1 expression is the only established biomarker for predicting anti-PD-1/L1 antibody efficacy. A previous study showed that β-catenin-positive NSCLC cases were often PD-L1 negative (15). In the present study, no significant relationship between β-catenin expression and PD-L1 TPS was found. Combining PD-L1 expression with β-catenin may help more accurately predict the response to treatment with anti-PD-1 antibody.

In this study, β-catenin expression was more common in squamous cell carcinoma, similar to the result of a previous study (15). Squamous cell carcinoma is less common than non-squamous cell carcinoma in NSCLC, and thus a prospective study with a validation cohort at multiple centers should be conducted to determine the potential for β-catenin as a predictive biomarker of anti-PD-1/L1 antibody monotherapy efficacy. Other biomarkers of ICI efficacy include TMB, which was shown in the KEYNOTE-158 trial to be a biomarker of treatment response to pembrolizumab in advanced solid tumors (19). TMB has been shown to be a potential biomarker for predicting the response to nivolumab alone or in combination with ipilimumab for NSCLC (20, 21). However, not all patients with a high TMB respond to anti-PD-1 antibody. In our previous study, we found that TMB was significantly higher in β-catenin-positive NSCLC patients than in negative NSCLC patients (15). One report showed that β-catenin is activated when TMB in tumor cells increases (16), suggesting that β-catenin may be a mechanism of resistance to ICI therapy in cancers with a high TMB. Moreover, β-catenin expression increases as the TMB increases (16), which supports our finding that more β-catenin-positive cases were found in squamous cell carcinomas of the lung, which generally have a higher number of gene mutations, than in lung adenocarcinomas (22). Other studies showed that gene mutations are involved

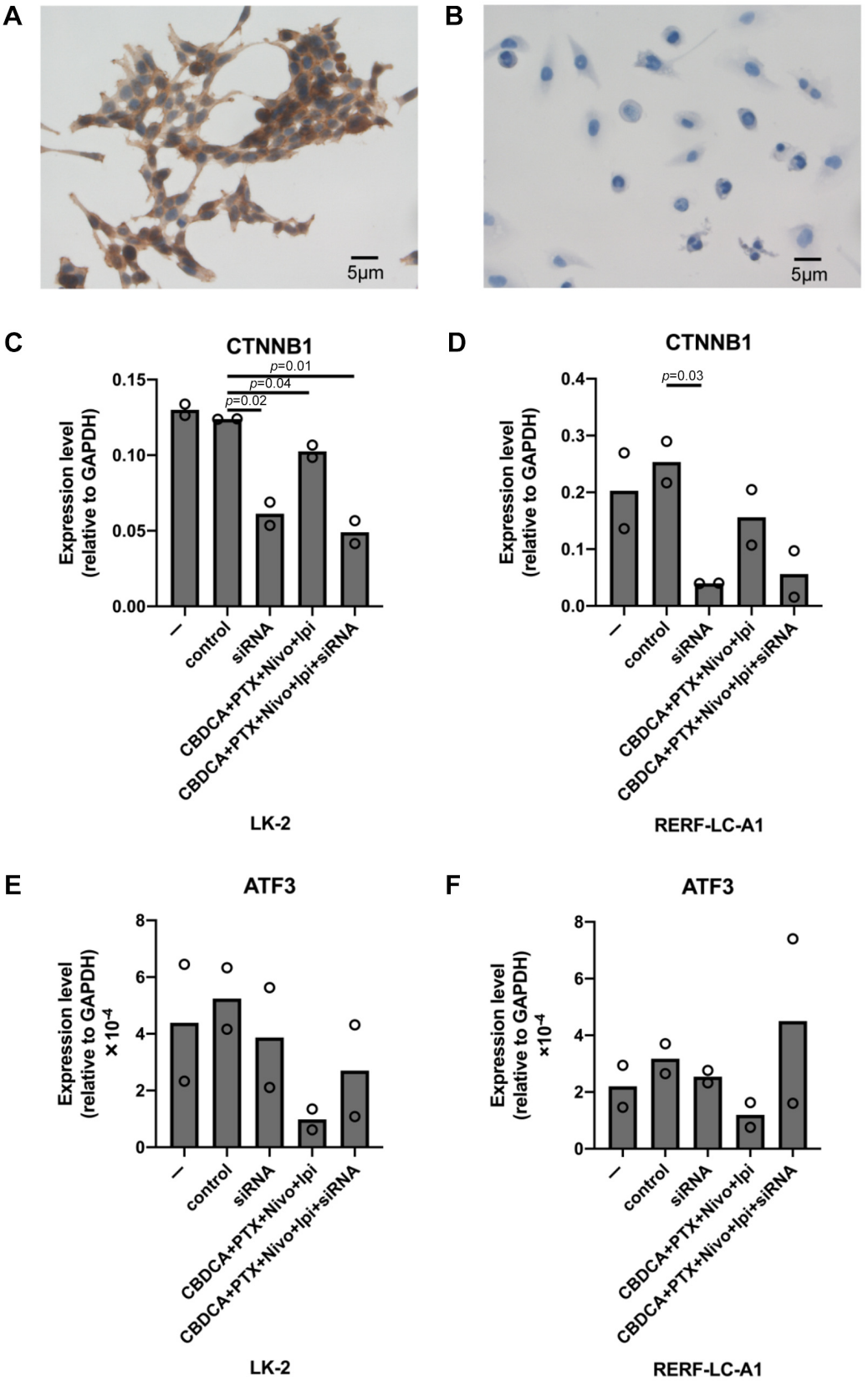

Figure 3. *Continued*

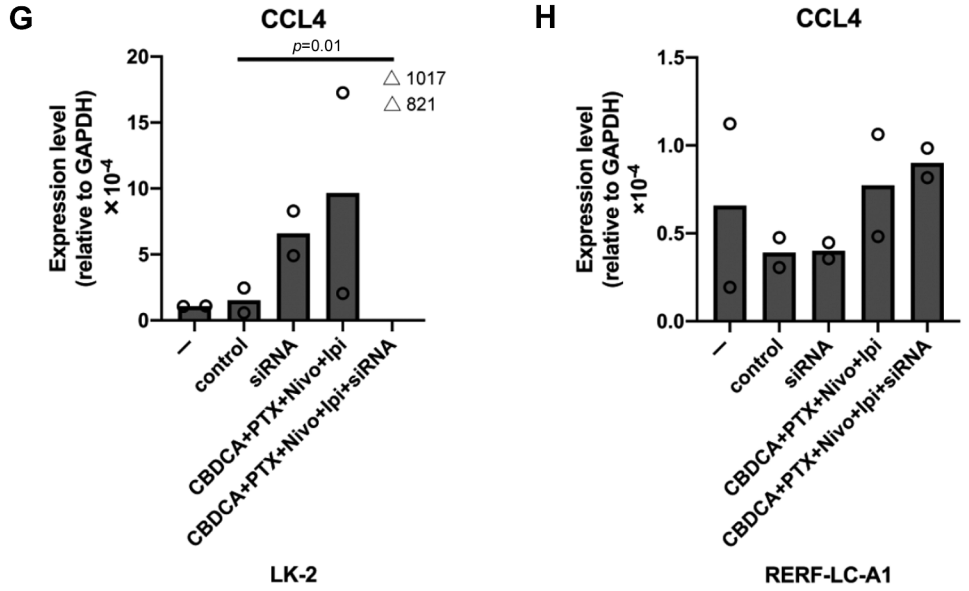

Figure 3. *The influence of CTNNB1 knockdown and chemotherapy plus immune checkpoint inhibitors in lung squamous cell carcinoma cell lines. Tumor cells were plated at a concentration of 1×10^5 per ml; cells were transfected with 1.2 pmol siRNA transfection and treated with vehicle or drug for 36 h in two replicates. CTNNB1 knockdown had no significant impact on ATF3 and CCL4 in both cell lines; however, in LK-2 cells, which are β-catenin-positive by immunohistochemistry (A), CTNNB1 siRNA decreased CTNNB1 expression (p=0.01, C) and tended to decrease ATF3 expression (E) and up-regulate CCL4 expression compared with controls (G). In RERF-LC-A1 cells, which are β-catenin-negative by immunohistochemistry (B), CTNNB1 specific siRNA decreased CTNNB1 expression (p=0.03, D) but had no impact on ATF3 expression (F) and CCL4 expression (H). Carboplatin (CBDCA) (0.03 mg/ml) + paclitaxel (PTX) (0.02 mg/ml) + nivolumab (Nivo) (0.01 mg/ml) + ipilimumab (Ipi) (3 ng/ml) had no significant effect on ATF3 and CCL4, though it showed a tendency to decrease ATF3 and increase CCL4 in both cells.*

in the acquired resistance to ICI (23). Cytotoxic T cells and IFN-γ induce cancer cells to acquire genetic instability (24). If ICI treatment increases the TMB, β-catenin may be involved in mechanisms of acquired resistance to ICIs.

We also explored the mechanism by which β-catenin is involved in ICI resistance in NSCLC. Our results also indicate that this may involve ATF3-CCL4-mediated inhibition of dendritic cell recruitment, as shown by Spranger *et al*. in melanoma (14). First, evaluation of the tumor microenvironment by immunohistochemistry of tumor tissue showed lower infiltration of $CD11c^+$ cells into tumor nests in β-catenin-positive cases than in β-catenin-negative cases. We reported that most $CD11c^+$ cells were $CD163^+$ M2 macrophages, but there were a few $CD11c^+$ and $CD163^-$ cells determined by flow cytometry of tumor-infiltrating lymphocytes in our previous study (15). CD11c is expressed by various types of antigen presenting cells (25, 26). The low intra-tumor infiltration of $CD11c^+$ cells is thought to be the result of reduced recruitment of dendritic cells to the tumor microenvironment from the decreased production of CCL4. Spranger *et al*. reported that in gene expression analysis, β-catenin-positive cases were also found among patients with low levels of BATF3 dendritic cells (14, 27). These results suggest that β-catenin suppresses dendritic cell recruitment *via* ATF3-CCL4 in lung cancer. The decreased invasion of CD8 T cells and decreased expression of CD8-related genes may be the result of this suppression. This may be followed by decreased expression of interferon-γ and coinhibitory molecules. Second, knockdown of *CTNNB1* in a β-catenin-positive lung squamous cell carcinoma cell line resulted in a tendency for decreased *ATF3* expression and increased *CCL4* expression. In the β-catenin negative lung squamous cell carcinoma cell line, knockdown of *CTNNB1* did not show such changes.

The results of this study suggest that knockdown of *CTNNB1* tended to recover the expression of *CCL4 via* decreased *ATF3*. Inhibition of Wnt/β-catenin signaling may overcome the resistance to ICIs in β-catenin-positive NSCLC. One study reported that β-catenin inhibition with CTNNB1 Dicer siRNA (DCR-BCAT) increased T-cell infiltration into tumors in syngeneic mouse-tumor models (28). Several clinical trials are ongoing on the combination of Wnt/β-catenin signaling inhibitor with ICIs in various cancers (29). Notably, in addition to *CTNNB1* siRNA, carboplatin, paclitaxel, nivolumab, and ipilimumab tended to recover the expression of *CCL4* in this study. We did not examine the precise

mechanism how these agents increase the expression of *CCL4*. However, these agents may suppress Wnt/β-catenin signaling because expression of *ATF3* is decreased in this experimental system. β-Catenin over-expression was reported to be associated with chemoresistance in various cancers (30-32). Further study is needed to clarify how these agents modulate Wnt/β-catenin signaling. Whether these agents have an effect on patients with β-catenin-positive NSCLC should be explored. Wnt/β-catenin signaling is activated in hepatocellular carcinoma (33). *CTNNB1* mutated hepatocellular carcinoma was reported to be resistant to ICI monotherapy (34). In hepatocellular carcinoma, the combination of ICI with anti-vascular endothelial growth factor (VEGF) antibody was shown to overcome this resistance. Anti-VEGF antibody combined with anti-PD-L1 antibody showed a clinical effect on hepatocellular carcinoma regardless of Wnt/β-catenin signaling activation (35). This combination therapy may work by suppression of regulatory T cells and myeloid cells rather than β-catenin (36).

Study limitations. We did not examine the relationship between β-catenin and gene expression downstream of β-catenin in gene enrichment analysis. However, a previous report showed that Wnt/β-catenin signaling activation correlated with high TMB and a non-inflamed tumor immune microenvironment with reduced *CCL4* expression in NSCLC mouse models (16). We also did not examine the precise mechanisms by which carboplatin, paclitaxel, nivolumab, and ipilimumab affect Wnt/β-catenin signaling.

Conclusion

This study revealed that β-catenin-positive NSCLCs are resistant to anti-PD-1 monotherapy. Down-regulation of CCL4 via the β-catenin-ATF3 axis is considered the mechanism and may result in the suppression of the recruitment of dendritic cells into tumor nests. While chemoimmunotherapy may overcome this resistance, further study is required to clarify whether chemoimmunotherapy has a favorable effect on β-catenin-positive NSCLCs.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, S.M. and H.S.; Data curation, S.M.; Formal analysis, S.M.; Funding acquisition, S.M.; Investigation, S.M.; Methodology; Project administration, S.M.; Resources, S.M., Y. O., H. Y., M. W., N.O., Y. M. and H.S.; Supervision, K.H. and H.S.; Validation, Y.O.; Visualization, S.M.; Writing – original draft, S.M.; Writing – review and editing, S.M. and H.S. All Authors have read and agreed to the published version of the manuscript.

Acknowledgements

The Authors are grateful to Ms. Yukiko Kikuta and Ms. Eiko Ohtomo for the technical support. The Authors also thank Gabrielle White Wolf, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Funding

This research was supported by a Grant-in-Aid for Young Scientists (JSPS KAKENHI Grant Number JP19K18222).

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Received October 28, 2024
Revised November 20, 2024
Accepted November 21, 2024