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PD-L1 gene expression in Japanese lung cancer patients

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Abstract. An imbalance in immune regulation affects tumor-specific T-cell immunity in the cancer microenvironment and reshapes tumor progression and metastasis. Blockade of interactions of immune function mediates antitumor activity in preclinical models. In the present study, we investigated *programmed cell death 1 ligand 1 (PD-L1)* mRNA expression by real-time polymerase chain reaction (RT-PCR) using a LightCycler in surgically treated non-small cell lung cancer (NSCLC) cases. This study included 123 surgically removed NSCLC cases for mRNA level analyses. The *PD-L1/β-actin* mRNA levels showed no marked difference in lung cancer (131.398±421.596) and adjacent normal lung tissues (78.182±254092, P=0.1482). The tumor/normal (T/N) ratio of *PD-L1/β-actin* mRNA levels was more than 2 in 49 cases and more than 1 in 63 cases. No difference was found in the T/N ratio of *PD-L1/β actin* mRNA levels among factors inlcuding gender, age, smoking status and pathological subtypes. The T/N ratio of *PD-L1/β actin* mRNA levels was markedly higher in pathological T4 cases (15.811±36.883) compared to T1 cases (3.492±8.494, P=0.0235). However, the *PD-L1* mRNA status did not correlate with lymph node metastasis status. Thus, *PD-L1* may drive tumor invasion, while providing a candidate for blockade of its function as a strategy to antagonize the progression process in NSCLC.

Introduction

Lung cancer is a major cause of mortality from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy (1). Lung cancer was the leading indication for respiratory surgery (47.5%) in 2009 in Japan (2), with >30,000 patients undergoing surgery due to lung cancer at Japanese institutions during the same year (2). The clinical behavior of non-small cell lung cancer (NSCLC) is largely associated with its stage. Treatment of the disease by surgery is only achieved in cases at an early stage of NSCLC (3).

An imbalance in immune regulation affects tumor-specific T-cell immunity in the cancer microenvironment and reshapes tumor progression and metastasis (4). The lack of immunostimulatory activation may be harmful if it impairs immune responses against cancer (5). Several receptor-ligand interactions are known to trigger anti-apoptotic pathways that prevent activation-induced T-cell death (6,7). Programmed death 1 (PD-1) protein, a T-cell co-inhibitory receptor, and one of its ligands, programmed cell death 1 ligand 1 (PD-L1), are involved in the ability of tumor cells to escape the host's immune system. PD-L1 is selectively expressed in a number of tumors (8-10). The blockade of interactions between PD-1 and PD-L1 enhances the immune function *in vitro* and mediates antitumor activity in preclinical models (8,9). Recent studies have suggested that antibody-mediated blockade of PD-L1 (10) and PD-1 (11) induced durable tumor regression and prolonged stabilization of the disease in certain patients with advanced cancers, including NSCLC. In their study, Topalian *et al* (12) demonstrated that immunohistochemical (IHC) analysis detected no objective response in PD-L1-negative patients. However, 36% of the patients with PD-L1-positive tumors had an objective response, although the sample number for IHC was small (n=42). Thus, PD-L1 might be a critical factor in cancer immunotherapy.

In this study, we examined *PD-L1* mRNA expression in Japanese NSCLC and adjacent normal lung tissues, by real-time quantitative polymerase chain reaction (qPCR) using LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) (13) in surgically treated cases. The findings were compared to the clinicopathological parameters of the NSCLC and *PD-L1* gene status.

Patients and methods

Patients. The study group comprised NSCLC patients who had undergone surgery at the Department of Surgery, Nagoya City University Hospital (Nagoya, Japan) between 2006 and 2009. The tumor samples were immediately frozen and stored at -80˚C until they were assayed. Patient consent was obtained from the patients. The study was approved by the ethics

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committee of the university. The clinical and pathological characteristics of the 123 NSCLC patients for *PD-L1* mRNA gene analyses were as follows: 80 (65.0%) were male and 43 were female, 95 (77.2%) were diagnosed with adenocarcinomas, 79 (64.2%) were smoker and 44 (35.8%) were non-smoker, and 81 (65.9%) were pathological stage I (Table I).

PCR assay for PD-L1 gene. Total RNA was extracted from NSCLC and adjacent normal lung tissues using the Isogen kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. RNA concentration was determined by NanoDrop ND-1000 Spectrophotometer (Nano Drop Technologies Inc., Rockland, DE, USA). Approximately 10 cases were excluded for each assay since tumor cells were insufficient in number to extract tumor RNA. RNA (1 μg) was reverse transcribed by the first strand cDNA synthesis kit with 0.5 μg oligo(dT)$_{16}$ (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The reaction mixture was incubated at 25˚C for 15 min, 42˚C for 60 min, 99˚C for 5 min and at 4˚C for 5 min. The cDNA concentration was determined by a NanoDrop ND-1000 Spectrophotometer. Approximately 200 ng of each cDNA was used for PCR analysis. To ensure the fidelity of mRNA extraction and reverse transcription, the samples were subjected to qPCR amplification with the *β-actin* primers (Nihon Gene Laboratory, Miyagi, Japan) using LightCycler-FastStart DNA Master HybProbe Kit (Roche Diagnostics GmbH). The *PD-L1* qPCR assay reactions were performed using the LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics GmbH) in a 20 μl reaction volume. The primer sequences for *PD-L1* gene were: forward: 5'-CAAAGAATTTTGGTTGTGGA-3' and reverse: 5'-AGCTTCTCCTCTCTCTTGGA-3' (155 base pairs). The cycling conditions were as follows: initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec, annealing at 54˚C for 10 sec and extension at 72˚C for 7 sec.

Statistical analysis. Statistical analysis was carried out using the Student's t-test for unpaired samples and Wilcoxon's signed rank-sum test for paired samples. Correlation coefficients were determined using the Chi-square test. Fisher's PLSD test was used to adjust multiple comparisons. The overall survival of lung cancer patients was examined by the Kaplan-Meier method, while differences were examined by the log-rank test. The analysis was carried out using the StatView software package (Abacus Concepts, Inc., Berkeley, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PD-L1 mRNA status in Japanese lung cancer patients. The *PD-L1* gene status was quantified for 123 NSCLC samples and adjacent normal lung tissues. The *PD-L1/β-actin* mRNA levels showed no statistically significant difference in lung cancer (131.398±421.596) and adjacent normal lung tissues (78.182±254.092, P=0.1482). The tumor/normal (T/N) ratio of *PD-L1/ β-actin* mRNA levels was >2 in 49 cases and >1 in 63 cases. The T/N ratio of *PD-L1/β actin* mRNA levels did not correlate with gender (male vs. female, P=0.4539), age (age ≤65 vs. >65, P=0.5359), smoking status (smoker vs. non-smoker, P=0.3644) and EGFR mutations status (wild type vs. mutant patients, P=0.3976). The T/N ratio of *PD-L1/ β-actin* mRNA level did not correlate with pathological subtypes (adenocarcinoma vs. others, P=0.2543) and lymph node metastasis (P=0.3456). The T/N ratio of *PD-L1/β-actin* mRNA level showed a gradual increase in pathological T stages, and was markedly higher in pathological T4 cases (15.811±35.883) when compared to the T1 cases (3.492±8.494, P=0.0235). The T/N ratio of *PD-L1/β-actin* mRNA levels was markedly higher in pathological stage III-IV (13.359±29.768) compared to stage II cases (2.213±4.422, P=0.0345), likely the effect of advanced T statuses.

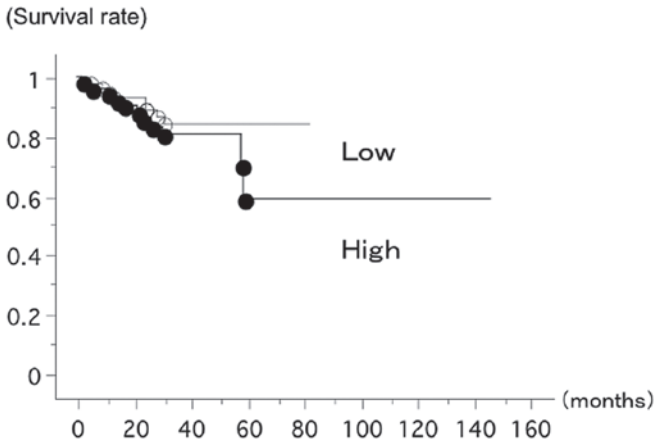

Figure 1. Overall survival of 123 lung cancer patients from Nagoya City University (Nagoya, Japan), with follow-up through July 31, 2012, was studied in reference to the *PD-L1* gene status. The survival of the patients (●) with a T/N ratio of *PD-L1/β-actin* mRNA level ≥1 (n=64, 8 deceased) and (○) those with a T/N ratio of *PD-L1/β-actin* mRNA level <1 (n=59, 11 deceased) showed no statistically significant difference (log-rank test, P=0.2336).

The overall survival of 123 lung cancer patients from Nagoya City University (Nagoya, Japan), with follow-up through July 31, 2012, was studied in reference to the *PD-L1* gene status. The survival of the patients with a T/N ratio of *PD-L1/β-actin* mRNA level ≥1 (n=64, 8 deceased) and those with a T/N ratio of *PD-L1/β-actin* mRNA level <1 (n=59, 11 deceased) showed no statistically significant difference (log-rank test, P=0.2336) (Fig. 1).

Discussion

In this study, we focused on one of the PD-1 ligands, PD-L1, to establish whether or not it might be a new molecular target for NSCLC. The results showed that *PD-L1* mRNA expression was correlated with tumor invasion in surgically resected NSCLC using LightCycler.

Human cancers harbor numerous genetic and epigenetic changes, generating neoantigens that are potentially recognizable by the immune system (14). Tumors develop multistep resistance systems, including local immuno-suppression, induction of tolerance and systemic dysfunction in T-cell signaling (15-18). In addition, tumors utilize several pathways to escape immune destruction. These observations generated intensive efforts to develop immunotherapeutic approaches for cancer, including immune-checkpoint-pathway inhibitors, such as anti-CTLA-4 antibody (19,20) and anti-PD-L1 therapy (11,12).

Table I. Clinicopathological parameters of 123 lung cancer patients.

Factors	*PD-L1*		
	No. of patients (n=123) (%)	T/N ratio of *PD-L1*/*β-actin* mRNA levels	P-value
Stage			
I	81 (65.9)	5.523±13.780	III-IV vs. II
II	20 (16.3)	2.213±4.422	0.0345
III-IV	22 (17.9)	13.359±29.768	
Tumor status			
pT1	56 (45.5)	3.492±8.494	T4 vs. T1
pT2	49 (39.8)	6.670±15.718	0.0235
pT3	6 (4.9)	12.231±24.958	
pT4	12 (9.8)	15.811±36.883	
Lymph node metastasis			
Negative	90 (73.2)	5.502±13.588	0.3456
Positive	33 (26.8)	8.798±24.337	
Age (years)			
≤65	59 (48.0)	5.382±10.094	0.5359
>65	64 (52.0)	7.295±21.597	
EGFR mutation			
Positive	28 (22.8)	7.412±21.261	0.3976
Negative	95 (73.6)	6.084±15.780	
Smoking			
BI=0	44 (35.8)	8.268±21.856	0.3644
BI>0	79 (64.2)	5.339±13.806	
Pathological subtypes			
Adeno	95 (77.2)	7.344±19.206	0.2543
Non-adeno	28 (22.8)	3.139±4.683	
Gender			
Male	80 (65.0)	5.536±16.039	0.4539
Female	43 (35.0)	7.969±19.000	

The mean age of the patients was 66.3±9.2 years. T/N, tumor/normal; NS, not significant; BI, Brinkman index; Adeno, adenocarcinoma.

PD-1 is a key immune-checkpoint receptor expressed by activated T cells that mediates immuno-suppressions. PD1 ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) are expressed by tumor and stromal cells (8,21-23). Thus *PD-L1* may also act as a molecule target for tumor progression in various types of cancer. *In vitro*, inhibition of the interaction between PD-1 and PD-L1 may enhance T-cell responses and mediate preclinical antitumor activity (8,9). Investigations into the role of anti-PD-1 antibody in advanced solid tumors are currently ongoing (24). Recent studies by Brahmer *et al* (11) and Topalian *et al* (12) have reported the safety and activity of anti-PD1 or PD-L1 immunotherapy in cancers including NSCLC. In NSCLC, 10% of patients exhibited a response to anti-PD-L1 antibody (11), while 18% of NSCLC patients exhibited a response to anti-PD-1 antibody (12). Notably, in the latter report (12), PD-L1 expression correlated with response. Of the limited number (n=42) of pretreatment tumor samples (12), none of the patients with PD-L1-negative tumors had an objective response. However, 36% with PD-L1-positive tumors had an objective response.

In our analysis, *PD-L1* expression correlated with tumor invasion. Tumor cells expressing PD-L1 might exhibit a high progression potential in NSCLC. However, only half of the tumors had >1 T/N ratio of *PD-L1* mRNA levels, while only one third of the tumors had >2 T/N ratio of *PD-L1* mRNA levels. Thus, potential of basing patient selection for the suppression of PD-L1 signaling on *PD-L1* expression in tumors requires prospective assessment. In addition, the development and validation of strategies to improve effective identification of the high-responder patient population with anti-PD-L1 strategies are important and likely to play a role in clinical practice.

In conclusion, PD-L1 might drive the tumor invasion of NSCLC in certain patient populations, while providing a

candidate for blockade of its function as a strategy to antagonize the progression process.

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