博 士 学 位 論 文

PD-L1 発現を有する肺癌の臨床病理学的特徴

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Clinical and pathologic features of lung cancer expressing programmed cell death ligand 1 (PD-L1)

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論文題目

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Clinical and pathologic features of lung cancer expressing programmed cell death ligand 1 (PD-L1)

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Abstract

Background: Programmed cell death 1 (PD-1) negatively regulates antigen receptor signaling upon binding by either of its ligands, programmed cell death ligand 1 or 2 (PD-L1/2). Blockade of this interaction with either PD-1 or PD-L1 antibodies has been successful in the treatment of human cancer, especially melanoma and non-small cell lung cancer. PD-L1 expression has been proposed as a predictor of tumor response. However, the relationships between PD-L1 expression and various clinicopathological characteristics remain unclear.

Materials and Methods: PD-L1 expression was examined in 220 non-small cell lung cancer specimens that were consecutively resected at our hospital after validating the E1L3N antibody immunohistochemical assay by comparing IHC and RT-PCR data for lung cancer cell lines. We evaluated the relationships between PD-L1 positivity, several clinical factors and the immunohistochemical expression of epithelial-mesenchymal transition (EMT), cancer stem cell and proliferative markers.

Results: PD-L1 was expressed in 22% of lung adenocarcinomas and 60% of squamous cell lung cancers. There was no significant association between PD-L1 expression and clinicopathological features in squamous cell lung cancer. However, in patients with lung adenocarcinoma, PD-L1 expression was significantly correlated with solid subtype histology, vimentin expression, increased Ki-67 labeling index and poor prognosis by multivariate analysis.

Conclusion: PD-L1 expression was associated with high proliferative activity and the EMT phenotype in adenocarcinoma but not in squamous cell

carcinoma of the lung. PD-L1 expression was a significant poor prognostic factor in patients with lung adenocarcinoma.

Keywords: Lung cancer, PD-L1, EMT, prognostic factor, adenocarcinoma

1. Introduction

Programmed cell death 1 (PD-1) negatively regulates antigen receptor signaling upon binding by either of its ligands, programmed cell death ligand 1 or 2 (PD-L1/2)[1]. PD-1 is expressed mainly on T-cells, while its ligands, PD-L1 and PD-L2, are expressed on the surface of various cells, including normal lymphoid cells and non-lymphoid cells [1]. PD-L1 and PD-L2 are also expressed in various types of cancer cells, resulting in tumor cell evasion from immune surveillance [1]. Hence, blockade of the interaction between PD-1 and PD-L1/2 by anti-PD-1 or anti-PD-L1 antibodies has been successful in the treatment of various types of human cancer, including melanoma and non-small cell lung cancer [2, 3].

Several studies have correlated PD-L1 expression on tumor cells with poor prognosis in various types of human cancer, including ovarian, skin, colon, bladder, breast, and pancreatic cancer as well as head and neck squamous cell carcinoma and renal cell carcinoma [1]. In the case of lung cancer, the correlation between PD-L1 expression and prognosis remains controversial [4-9]. In addition, clinicopathologic features of PD-L1-positive lung cancer have not been fully defined.

In the present study, we examined consecutive surgical specimens from our patients with lung cancer for PD-L1 expression after validating the PD-L1 immunohistochemical assay using lung cancer cell lines. We then correlated the results with various clinicopathological features, including prognosis, histologic subtype, proliferative activity, epithelial-mesenchymal transition (EMT) markers, and cancer stem cell (CSC) markers, some of which we examined in our previous study [10], to gain further insights.

2. Materials and Methods

2.1. Cell lines

The human NSCLC cell lines H1781, H3255, H1650, H596, PC9, H2228, H1975, HCC827 and HCC78 were cultured in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). The H1781, H2228 and H596 cell lines were purchased from ATCC (Rockville, MD, USA), and PC9 cells were purchased from IBL (Fujioka, Japan). The HCC827, H3255 and HCC78 cell lines were generous gifts from Dr. A.F. Gazdar (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas), and the H1975 and H1650 cell lines were generous gifts from Dr. S. Yano (Division of Medical Oncology, Cancer Research Institute, Kanazawa University).

2.2. Patients

A total of 224 patients underwent pulmonary resection for primary lung adenocarcinoma or squamous cell carcinoma from January 2007 to April 2009 in the Department of Surgery, Kinki University Hospital. This study was approved by the Institutional Review Board of kindai University Faculty of Medicine (No.24-253). Four patients were excluded from the study due to an insufficient amount of tumor-containing tissue. Of the remaining 220 patients, 55 had squamous cell carcinoma, and 165 had adenocarcinoma [1 minimally invasive adenocarcinoma, 153 invasive adenocarcinomas (3 lepidic, 58 papillary, 66 acinar, 25 solid, and 1 micropapillary predominant), and 11 invasive mucinous adenocarcinomas] according to the World Health Organization (WHO) classification published in 2015 [11]. Fifty-seven percent of the patients were male, and the median age was 65 years (range 39-85). Forty-five percent of the patients were never smokers. Sixty-five

percent of the patients presented with pathological stage I disease, 20% with stage II, 15% with stage III and 1% with stage IV disease according to the 7th version of the UICC TNM staging manual. Most of the patients (95%) underwent standard resection, i.e., lobectomies or more, and 37% of the patients received postoperative adjuvant chemotherapy.

2.3. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell lines using a mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, USA). RNA (10 μg) was reverse transcribed using ReverTra Ace qPCR RT Kits (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Approximately 1 μg of each cDNA sample was used for each PCR analysis. Real-time PCR was performed using Power SYBR Green Master Mix (Life Technologies, Carlsbad, USA) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, USA). The 18S rRNA housekeeping gene was used as a control. The primer sequences and the cycling conditions for amplifying the PD-L1 gene have been previously described [12]. The PD-L1 expression level in each cell line was calculated relative to PD-L1 expression in H2228 cells.

2.4. Cell block construction

We used the sodium alginate cell block method as described previously to construct cell blocks of cultured cells [13]. Briefly, one milliliter of 15% neutral buffered formalin was added to the cell pellets, which were incubated for three hours at room temperature. After centrifugation, one milliliter of 1% sodium alginate (Wako, Osaka,

Japan) was added to the pellet. After another centrifugation step, 200 µl of potassium chloride solution was added, and the resultant gel was embedded in paraffin.

2.5 Tissue microarray (TMA) construction

Formalin-fixed, paraffin-embedded (FFPE) NSCLC tissues were aligned to make tissue microarrays (TMAs). TMAs (2 mm diameter) were constructed by collecting tissue cores from two regions of the predominant histological subtype in the tumor area of each FFPE sample using a tissue microarrayer (type KIN-2, Azumaya, Tokyo, Japan).

2.6. Immunohistochemistry (IHC) of cell lines and TMA samples

TMA blocks and cell blocks were sectioned at a thickness of 4 µm, and immunohistochemical staining was then performed using a PD-L1 antibody (#13684, also known as E1L3N; 1:1600, Cell Signaling Technology, Danvers, MA). Briefly, sections were de-paraffinized using xylene followed by ethanol and then washed with distilled water (DW). For antigen retrieval, sections were boiled in Target Retrieval Solution (pH 9.0, 1:10, DAKO, Glostrup, Denmark) for 15 min at 121 °C. After the sections were rinsed with distilled water, endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 10 min at room temperature. The sections were then washed with DW and phosphate-buffered saline (PBS) and incubated in Serum-Free Protein Block (DAKO) for 60 min at room temperature. The slides were incubated overnight at 4 °C with various primary antibodies. To visualize the antigen, a peroxidase-labeled secondary antibody (REAL EnVision Detection Reagent Peroxidase Rabbit/Mouse, DAKO) was applied to the sections for 30 min at

room temperature. The sections were then rinsed in PBS, stained with DAB (liquid

DAB + substrate, DAKO) and counterstained with hematoxylin.

Two independent observers (M.S. and S.S.) examined the stained slides in a

blinded fashion. The intensity score (IS) was based on PD-L1 membrane staining: 0,

no staining; 1, weak and incomplete staining; 2, moderate to complete staining; or 3,

strong and complete, homogenous staining. We then calculated the H-score by

multiplying the IS by the percentage of positive cells [14]. Because an intra-tumor

heterogeneity of PD-L1 expression was anticipated, we independently analyzed two

cores for each tumor specimen. When scores of the two cores were different, we

adopted the higher scores for each tumor.

We also used IHC data regarding ALDH1A1, P-glycoprotein, E-cadherin,

vimentin, p53 and Ki-67 expression from our previous study [10]. The following

antibodies were used: ALDH1A1, ab52492 (Abcam, Cambridge, UK); P-glycoprotein,

ab3366 (Abcam); E-cadherin, 3195S (Cell Signaling Technology); vimentin, 5741S

(Cell Signaling Technology); Ki-67, DO-7 (Dako) and M7240 (Dako). Cut-off values for

positivity for these proteins were as follows: 10% for ALDH1A1, P-glycoprotein, and

vimentin; 90% for E-cadherin; and 15% for Ki-67.

2.7. Statistical analyses

The relationship between PD-L1 mRNA expression by RT-PCR and PD-L1

protein expression by IHC was analyzed using Pearson's product-moment correlation

coefficient. Clinical and pathological characteristics of PD-L1-positive tumors were

compared with those of PD-L1-negative tumors using the χ^2 test. The Mib-1 index was

calculated and compared between tumors with and without PD-L1 expression using

Student's t test. Factors with $P \leq 0.10$ in the univariate analysis were included in the

multivariate analysis using the logistic regression model. The overall survival (OS) of patients with PD-L1-positive and PD-L1-negative tumors was estimated using the Kaplan-Meier method, and differences were compared using the log-rank test. The Cox proportional hazards model was used to determine prognostic factors. Differences were considered statistically significant at a two-sided P-value of ≤0.05. All statistical analyses were performed using JMP software (Version 11.2.0, 2013 SAS Institute, Inc.).

3. Results

3.1. Correlation between PD-L1 mRNA expression and staining pattern in lung cancer cell lines

Positive immunostaining for PD-L1 was observed at the membrane and/or in the cytoplasm of tumor cells. Representative images of the PD-L1 staining patterns observed in tumor specimens are shown in Figure 1A. PD-L1 mRNA and protein expression in each cell line is shown in Supplementary Table 1. There was a 54-fold difference in PD-L1 expression between the lowest expressor (H1781) and the highest expressor (HCC78). The IHC intensity ranged from 1+ to 3+, and the percent positive ranged from 4.4% to 100%, resulting in an H-score range of 4.4 to 300. We noted a strong correlation between PD-L1 mRNA expression and H-score (R=0.95) (Figure 1B), suggesting the validity of this assay system.

3.2. Immunohistochemical detection of PD-L1 in lung cancer specimens

Representative images of the PD-L1, ALDH1A1, vimentin, and Ki-67 staining patterns observed in tumor specimens are shown in Figure 2A-H. Some tumors showed spacial heterogeneity in PD-L1 expression. However, in 202 cases (92%),

differences of H-score in two cores were 10 or less. Six adenocarcinomas showed a significant heterogeneity with H-score difference being 40 or more. Four of them contained solid and papillary subtypes. The other two were a pure solid subtype and a papillary subtype each. About two-thirds of the samples did not show any PD-L1 staining. In those with PD-L1 staining, the H-scores were nearly evenly distributed, without any appreciable peaks. Therefore, we set the cutoff H-score at 5. According to this definition, 70 of the 220 samples (32%) were positive for PD-L1 expression. We noted that the positivity in squamous cell carcinoma samples (33/55, 60%) was significantly higher than that in adenocarcinoma samples (37/165, 22%) ($P<0.0001$).

3.3. Relationship between PD-L1 expression and several clinicopathological features (Table 1)

Because PD-L1 positivity was markedly different between squamous cell carcinoma and adenocarcinoma samples, we decided to analyze factors that affect PD-L1 expression individually according to histologic type. In adenocarcinoma, the univariate analysis using the chi square test revealed that PD-L1 expression was significantly correlated with solid predominant histological subtype ($P<0.0001$), high p53 expression ($P=0.0044$), low E-cadherin expression ($P=0.0001$), high vimentin expression ($P<0.0001$), high Ki-67 expression ($P<0.0001$), low ALDH1A1 expression ($P=0.0001$), and high P-glycoprotein expression ($P=0.003$). In the multivariate analysis using logistic regression analysis, solid predominant subtype ($P<0.001$), high vimentin expression ($P=0.002$), high Ki-67 expression ($P=0.012$), and low ALDH1A1 expression ($P=0.002$) were significantly and independently associated with PD-L1 expression (Table 2).

On the contrary, in squamous cell carcinoma, there were no significant associations between PD-L1 expression and any of the abovementioned clinicopathological factors (Table 1).

3.4. Relationship between survival, PD-L1 expression and other variables in NSCLC cases

Next, we evaluated the prognostic effect of PD-L1 expression. In squamous cell carcinoma, although there was a trend of longer survival among patients with PD-L1 expression, this difference did not reach statistical significance (P=0.10, Figure 3A). On the other hand, in adenocarcinoma, patients with PD-L1 expression experienced significantly shorter survival compared with those without PD-L1 expression (P=0.0051 log-rank test, P=0.010 univariate Cox, Figure 3B). Advanced pathological stage and the Ki67 index were also identified as poor prognostic predictors by the univariate Cox model. When factors with P<0.1 in the univariate model were entered into the multivariate analysis, PD-L1, age and pathologic stage emerged as independent prognostic factors (Table 3).

4. Discussion

We detected PD-L1 expression in 60% of lung squamous cell carcinoma samples and 22% of lung adenocarcinoma samples. The reported percent positivity of PD-L1 expression in lung cancer has been variable, ranging from 20% to 70% [4-9, 15, 16, 24]. The reasons for these discrepancies may be multifactorial. Different distributions of histologic subtypes or different antibodies and cutoffs [17] may account for some of the discrepancies. Certain studies might not have validated their IHC assay system by using other assays, as we did in this study. Because each

laboratory set an original cut-off and used various antibodies, standardization of immunohistochemistry assay is strongly awaited

We found that PD-L1 expression in lung adenocarcinoma was significantly correlated with several clinicopathologic features, including solid subtype and high proliferative activity. PD-L1 was expressed in 65% of solid predominant tumors, while only 16% of non-solid predominant tumors were PD-L1 positive. These associations have been separately reported in previous publications. Zhang et al. found that lung adenocarcinomas with positive PD-L1 staining were more likely to be of the solid predominant subtype [8]. Correlations between PD-L1 and Ki67 expression have been reported in breast cancer [18].

We also found that PD-L1 expression was associated with EMT features, such as increased vimentin expression and down-regulation of E-cadherin, although E-cadherin did not attain statistical significance in the multivariate analysis. Recently, Chen et al. showed that activation of ZEB1, a well-known activator of EMT, represses miR-200 and thus leads to PD-L1 expression [19]. EMT is known to be closely linked to stemness in cancer [20]. For example, in mammary carcinoma, the induction of EMT promotes the generation of CD44^{high}CD24^{low} CSCs that can form mammospheres, and similarly defined CSCs isolated from tumors express EMT markers [21]. In our study, the CSC marker P-glycoprotein showed a positive correlation with PD-L1 expression in the univariate analysis but not in the multivariate analysis. However, ALDH1A1, another CSC marker, showed the opposite association, i.e., PD-L1 was positive in 12% and 38% of ALDH1A1 high and normal tumors, respectively. We do not have a plausible explanation for this discrepancy.

We found that PD-L1 expression was associated with poor prognosis in patients with lung adenocarcinoma. This observation is reasonable considering that EMT has

been closely associated with the acquisition of aggressive traits, such as invasion and migration, resulting in greater metastatic potential [20]. In addition, PD-L1 expression helps cancer cells evade attack by immune cells [1]. However, the prognostic impact of PD-L1 expression in lung cancer remains controversial. Whereas Zhang et al. [8], Azuma et al. [9], Mu et al. [5], Mao et al. [24] and Chen et al. reported that PD-L1 expression is a poor prognostic marker, Yang et al. and Velcheti et al. reported that it is a positive prognostic marker. Differences in PD-L1 assay systems, such as different antibodies and distributions of histologic subtypes, may have partly attributed to these discrepancies. Indeed, in our patients, the prognostic impact of PD-L1 differed between adenocarcinoma and squamous cell carcinoma cases.

In the recent phase III trials of lung cancer comparing the PD-1 antibody nivolumab with docetaxel, PD-L1 appeared to select patients with adenocarcinoma who are likely to benefit from nivolumab treatment [22]; however, in those with squamous cell carcinoma, the benefit of nivolumab was universal and independent of PD-L1 expression [23]. This fact may reflect the different roles of PD-L1 in squamous cell carcinoma and adenocarcinoma of the lung. In conclusion, our comprehensive analysis revealed that PD-L1 expression was associated with high proliferative activity and the EMT phenotype in adenocarcinoma, which might lead to a poor prognosis. If PD-1 expression is a negative prognostic factor as well as a positive predictive factor for PD-1/L1-targeted therapies, there could be a great benefit for patients with PD-L1 expression, as in the case of HER2-positive breast cancer treated with HER2-targeted therapy.

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Conflict of interest statement

T Mitsudomi has received lecture fees from Astra-Zeneca, Boehringer-Ingelheim, Chugai, Taiho, and Pfizer and research funding from Astra-Zeneca. He also took part in an advertisement for MSD, Boehringer-Ingelheim, Chugai, and Taiho. The remaining authors have no conflicts to disclose.

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