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Tumor PD-L1 and VEGF Expression, and CD8 T Cell Infiltration Predict Clinical Response to Immune Checkpoint Inhibitors in Non-small Cell Lung Cancer

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Abstract. *Background/Aim: We evaluated the efficacy of "the tumor immune microenvironment (TIME) classification" for predicting clinical response to immune checkpoint inhibitors (ICIs) in patients with non-small cell lung cancer (NSCLC). In addition, we aimed to evaluate the "modified TIME classification", which adds the vascular endothelial growth factor (VEGF) status to TIME. Materials and Methods: Programmed cell death receptor ligand-1 (PD-L1), CD8 T cell tumor-infiltrating lymphocytes (CD8+TILs) count and VEGF expression analyses were performed using immuno-histochemistry in 44 patients who had undergone ICI monotherapy. Results: Regarding TIME classification, type-I (PD-L1 high and CD8+TILs high) had a significantly higher response than the other types. Using the modified TIME classification, type-IA (PD-L1 high, CD8+TILs high, and VEGF low) had a significantly higher response than the other types. Conclusion: The modified TIME classification, which adds tumor VEGF expression to "the TIME classification", could be useful in predicting clinical response to ICI monotherapy.*

Cancer immunotherapy using immune checkpoint inhibitors (ICIs) is a standard therapy for many cancers, especially non-small cell lung cancer (NSCLC) (1). Many studies have attempted to identify predictors of clinical response to ICIs, such as 1) programmed cell death receptor ligand-1 (PD-L1) expression status, 2) the count of tumor-infiltrating lymphocytes (CD8 or CD4), 3) mismatch-repair (MMR) protein expression status or tumor mutation burdens (TMB), 4) oncogene mutations, 5) radiographic markers, and 6) clinical pathological features (2). Recently, Hu-Lieskovan S *et al*. reported that the TMB, PD-L1 status, and CD8 count were each associated with a benefit from ICI therapy in patients with NSCLC (3).

The tumor immune microenvironment (TIME) classification of tumors, which consists of four types based on PD-L1 expression status and CD8 T cell tumor-infiltrating lymphocytes (CD8+TILs) count, was proposed in 2015 as follows: type-I (high PD-L1 expression and high CD8+TIL count), type-II (low PD-L1 expression and low CD8+TIL count), type-III (high PD-L1 expression and low CD8+TIL count), and type-IV (low PD-L1 expression and high CD8+TIL count) (4). Following this concept, we previously studied PD-L1 expression status and CD8+TILs count in 170 lung adenocarcinoma patients who had undergone pulmonary resection as an initial treatment (5). We reported that patients with low PD-L1 expression levels and high CD8+TILs counts in their tumors had significantly better outcomes than patients with high PD-L1 expression levels and low CD8+TILs counts.

In the current study, we evaluated the association between the TIME classification (type I-IV) and clinical response to ICIs in patients with advanced or recurrent NSCLC. In addition, we conceived the concept of the "modified TIME classification (type I-IV, subgroup A or B)," which adds vascular endothelial growth factor (VEGF) status to the TIME classification. Here, we evaluated the efficacy of the modified TIME classification for predicting clinical response to ICI monotherapy in patients with advanced or recurrent NSCLC.

Materials and Methods

Patients, clinical specimens, and clinical efficacy. Fifty-four patients with histologically confirmed advanced or recurrent NSCLC who had undergone ICI monotherapy between April 2017 and September

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2020 at our hospital were included in this study. Of these, 10 patients were excluded for the following reasons: 7 patients received only one cycle of ICI administration, 2 patients received ICI re-administration and tissue samples were not available for one patient. The clinical information for all the patients was retrieved from the Hospital Information System. Clinical responses to ICI monotherapy were assessed according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) (6). The study was approved by the Ethics Committee of Kawasaki Medical School (No.3555: Approved on July 6, 2019), and all the procedures were conducted in accordance with ethical principles.

Immunohistochemical analysis and assessment

PD-L1 analysis. The 22C3 primary monoclonal antibody directed against PD-L1 was used in the PD-L1 IHC 22C3 pharmDx KIT (Dako North America Inc., Carpinteria, CA, USA). PD-L1 expression was immunohistochemically categorized as positive when staining of the tumor-cell membrane (at any intensity) was present. In this study, we defined a tumor proportion score (TPS) ≥10% as high PD-L1 expression (7). The TPS is determined as the percentage of PD-L1 positive stained tumor cells relative to the total number of tumor cells.

CD8+TIL and VEGF analyses. CD8+TIL and VEGF immunohistochemical (IHC) analyses were performed using an automated immunostainer (Nexes; Ventana, Tucson, AZ, USA). The following primary antibodies were used according to the manufacturer's instructions and according to a previously described protocol: a mouse monoclonal anti-CD8 antibody (1:50, clone C8/144B, DAKO; Agilent Technologies, Inc., Santa Clara, CA, USA), and VEGF (1:200, clone sc-152; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). To evaluate the CD8+TIL count, 5 digital high-power field (HPF) images of the tumor area were selected, and the absolute number of CD8+TILs in these images was determined. A CD8+TIL count of less than 50 was considered "low," while a count of more than 50 was considered "high" (8). For VEGF, the slides were scored according to the intensity of staining (grade 0, negative; grade 1, weak; grade 2, moderate; grade 3, high) and the percentage of positively stained cells (grade 0, 0%; grade 1, 1%-9%; grade 2, 10%-49%; and grade 3, 50%-100%). The *H*-score (0-9) was calculated as the product of the intensity and percentages. High VEGF expression was defined as an *H*-score ≥4 (9). All the slides were examined by two investigators (NY and SK) who were blinded to the corresponding clinicopathological data.

"TIME classification" and "modified TIME classification". The four types of tumors according to TIME classification based on the PD-L1 expression status and the CD8+TIL count have been previously proposed as follows: type-I (high PD-L1 expression and high CD8+TIL count), type-II (low PD-L1 expression and low CD8+TIL count), type-III (high PD-L1 expression and low CD8+TIL count), and type-IV (low PD-L1 expression and high CD8+TIL count) (4, 5). For the modified TIME classification, we added VEGF information and defined the following additional groups: group-A (VEGF low), and group-B (VEGF high). For example, Type-IA is defined as high PD-L1 expression, high CD8+TILs count, and low VEGF expression.

Statistical analysis. All the statistical analyses were performed using the SPSS statistical software package (version 23.0; SPSS, Chicago, IL, USA). Frequencies were compared using the chi-square test for categorical variables, and the Fischer exact test was applied for small samples. Survival curves were determined using the Kaplan-Meier method. Overall survival (OS) was calculated from the time of the first day of ICI therapy until death or the last follow-up visit (December 31, 2020), and progression-free survival (PFS) was calculated from the time of the first day of ICI therapy until death or disease relapse. A value of $p<0.05$ was considered to indicate statistically significant differences.

Results

Patient characteristics. Forty-four patients were included in the final analysis. The characteristics of these patients are summarized in Table I. The median age was 68 years (range=41-84 years). The histologic subtypes were non-squamous type in 31 patients (26 adenocarcinoma and 5 NSCC-NOS) and squamous cell carcinoma in 13 patients. Four of these patients had *EGFR* mutations. Regarding the ICIs that were used, nivolumab was administered in 11 patients, pembrolizumab in 25, and atezolizumab in 8. The best response was a partial response (PR) in 20 patients (45.5%), stable disease (SD) in 6 (13.6%), and progressive disease (PD) in 18 (40.9%). The overall response rate (ORR) was 45.5% (20/44). The 1-year and 2-year progression-free survival rates were 24.8% and 14.9%, and the 1-year and 2-year overall survivals rates were 66.7% and 42.4%, respectively.

Immunohistochemical analysis. Sixteen patients had tumors with high PD-L1 expression (TPS≥50%), 18 had tumors with intermediate expression (10%≤TPS<50 in 11 patients and 1%≤TPS<10% in 7 patients), and 10 had tumors with negative PD-L1 expression (TPS<1%). The minimum and maximum number of CD8+TILs per HPF were 3 and 330, respectively. Twenty-two patients had high CD8+TIL (≥50/HPF) tumors, and 22 patients had low CD8+TIL (<50/HPF) tumors. Fifteen patients had tumors with high VEGF expression, and 29 had tumors with low VEGF expression (Table II).

Representative sections showing PD-L1 expression, CD8+TIL counts, and VEGF expression are depicted in Figure 1. Case 1 was a TIME type-I squamous cell carcinoma that was further classified as modified TIME type-IA. Case 2 is a TIME type-II adenocarcinoma that was further classified as modified TIME type-IIB. Case 3 is a TIME type-IV adenocarcinoma that was further classified as modified TIME type-IVB.

Associations between ORR and TIME and modified TIME classifications. Figure 2 shows the ORRs according to the TIME or modified TIME classifications. The ORR of the TIME type-I group was 73.3% (11/15). The TIME type-II group did not contain any responders (0/10). The ORRs of the TIME type-III and TIME type-IV group were 50.0% (6/12) and 42.9% (3/7), respectively. When the patients were grouped according to the modified TIME classification,

Table I. *Patient characteristics (n=44).*

Variable	Number	%
Gender		
Male	32	72.7
Female	12	27.3
Histology		
Nonsquamous	31	70.5
Squamous	13	29.5
Method of obtaining specimen		
Biopsy	27	61.4
Surgically	17	38.6
Driver oncogene mutations		
EGFR	4	9.1
None	40	90.9
Previous therapy lines		
0	11	25.0
1	22	50.0
>2	11	25.0
ICI		
Nivolumab	11	25.0
Pembrolizumab	25	56.8
Atezolizumab	8	18.2
Best response		
PR	20	45.5
SD	6	13.6
PD	18	40.9

EGFR: Epidermal growth factor receptor; ICI: immune checkpoint inhibitors; PR: partial response; SD: stable disease; PD: progressive disease.

Table II. *Immunohistological features.*

Variable	Number	%
PD-L1		
<1%	11	25.0
1%≤TPS<10%	6	13.6
11%≤TPS<50	11	25.0
≥50%	16	36.4
CD8+TIL		
<50/HPF	22	50.0
≥50/HPF	22	50.0
VEGF		
H-score<4	29	65.9
H-score≥4	15	34.1

PD-L1: Programmed cell death receptor ligand-1; TPS: tumor proportion score; CD8+TIL: CD8 T cell tumor-infiltrating lymphocytes; HPF: high-power field; VEGF: vascular endothelial growth factor.

group A (low VEGF expression) had a significantly higher ORR than group B (high VEGF expression) (62.1% *vs*. 13.3%, p=0.003). The ORR of the modified TIME type-IA group was 83.3% (10/12), whereas that of the type-IB group was 33.3% (1/3). The ORR of the modified TIME type-IIIA group was 75.0% (6/8), whereas the type-IIIB group did not contain any responders (0/4).

Models for predicting best response to ICIs. Next, we evaluated the most useful combination of parameters for predicting clinical response to ICIs. When individual biomarkers were examined, PD-L1 expression (ORRs for high *vs*. low expression: 63.0% *vs*. 17.6%, p=0.005), CD8+TIL status (ORRs for high *vs*. low counts: 63.6% *vs*. 27.3%, p=0.033), and VEGF expression (ORRs for low *vs*. high expression: 62.1% *vs*. 13.3%, p=0.003) were significant factors for predicting the response to ICI treatment. When patients were grouped according to their TIME classifications, the type-I group (PD-L1 high and CD8+TIL high) had a significantly higher ORR than the other groups (ORR: 73.3% *vs*. 31.0%, p=0.011). When patients were grouped according to their modified TIME classifications, the type-IA group (PD-L1 high, CD8+TIL high, and VEGF low) had a significantly higher ORR than the other types (ORR: 83.3% *vs*. 31.2%, p=0.005) (Table III). These results showed that the modified type-IA classification was the most useful biomarker for predicting a response to ICI monotherapy.

Discussion

The success of ICIs in the treatment of patients with NSCLC is an important advancement in the history of cancer therapy (10). The hallmark of cancer immunotherapy is the maintenance of a tumor-specific immune response. However, this maintenance has only been achieved in select patients, highlighting the need for biomarkers to predict patient response and survival.

PD-1 is a key immune checkpoint receptor that is expressed in activated T cells. The interaction of PD-1 with PD-L1 inhibits T-cell activation and proliferation, leading to cancer cell immune evasion (11). It has been reported that PD-L1 expression is associated with a poor clinical outcome in patients with NSCLC (12, 13). In addition, PD-L1 over-expression is correlated with an improved response to ICI treatment (7, 14-16). Lymphocytic infiltration has been shown to be crucial in predicting tumor progression in many cancers. In addition, tumor-infiltrating T cells have been associated with a prolonged survival and reduced recurrence rates in patients with colorectal, ovarian, or breast cancer (17-19). An important biomarker of a tumor-associated immune response is cytotoxic T cells. Furthermore, the presence of CD8+TILs has been associated with a favorable outcome in NSCLC (20). In 2015, Michele *et al*. proposed and reported the efficacy of the TIME classification using PD-L1 expression and CD8+TIL counts for evaluating cancer treatment. Our study revealed that the TIME classification was useful for predicting the best response to ICI monotherapy. The type-I group had the highest response

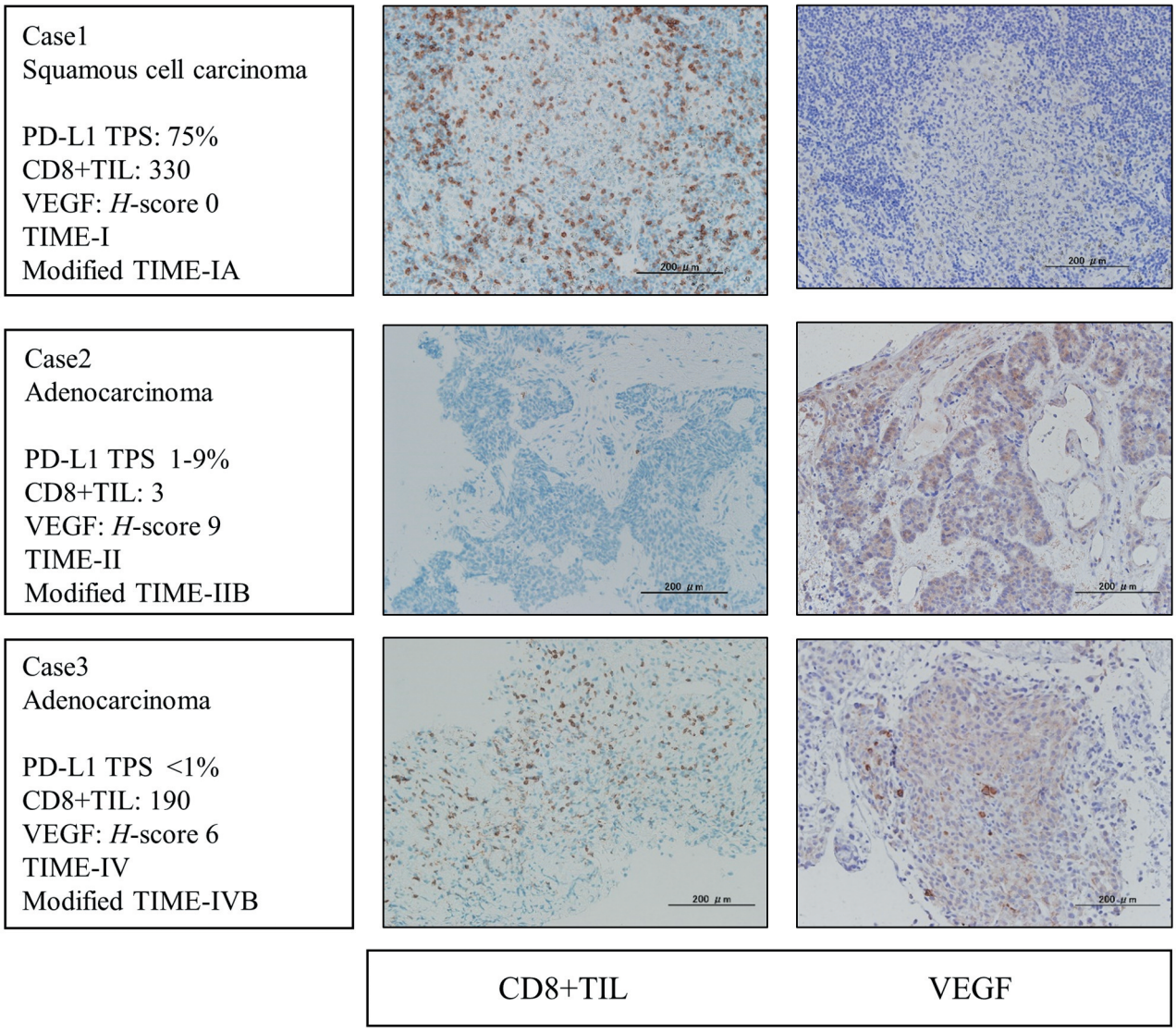

Figure 1. *Representative immunohistochemistry staining images. Case 1: Squamous cell carcinoma classified as the tumor immune microenvironment (TIME) type-I and modified TIME type-IA. Case 2: Adenocarcinoma classified as TIME type-II and modified TIME type-IIB. Case 3: Adenocarcinoma classified as TIME type-IV and modified TIME type-IVB.*

rate to ICI monotherapy. In contrast, the type-II group did not contain any responders to ICI monotherapy. Interestingly, most type-II tumors are known to carry an *EGFR* mutation or another driver oncogene mutation or fusion (5). These results confirmed the fact that ICIs are less effective in patients with *EGFR*-mutant adenocarcinoma.

In the current study, we proposed the "modified TIME" classification, which adds VEGF expression to the TIME classification. Angiogenesis is important for tumor progression, and VEGF is the most important factor in angiogenesis. In lung cancer, the addition of bevacizumab to a standard chemotherapy regimen conferred a significant improvement in survival in patients with non-squamous cell carcinoma (21). This result indicated that the suppression of angiogenesis enhanced the effect of the chemotherapy. In our current study using the modified TIME classification, patients with high tumor VEGF expression did not respond satisfactorily to ICI monotherapy. This result suggests that angiogenesis may influence the TIME. Regarding this point, Wallin *et al*. studied how VEGF blockade with bevacizumab enhanced the effect of ICIs in patients with metastatic renal cell carcinoma (22). They reported that the number of CD8+TILs increased after treatment with bevacizumab and ICIs, and they concluded that an anti-VEGF/anti-PD-L1 combination improved antigen-

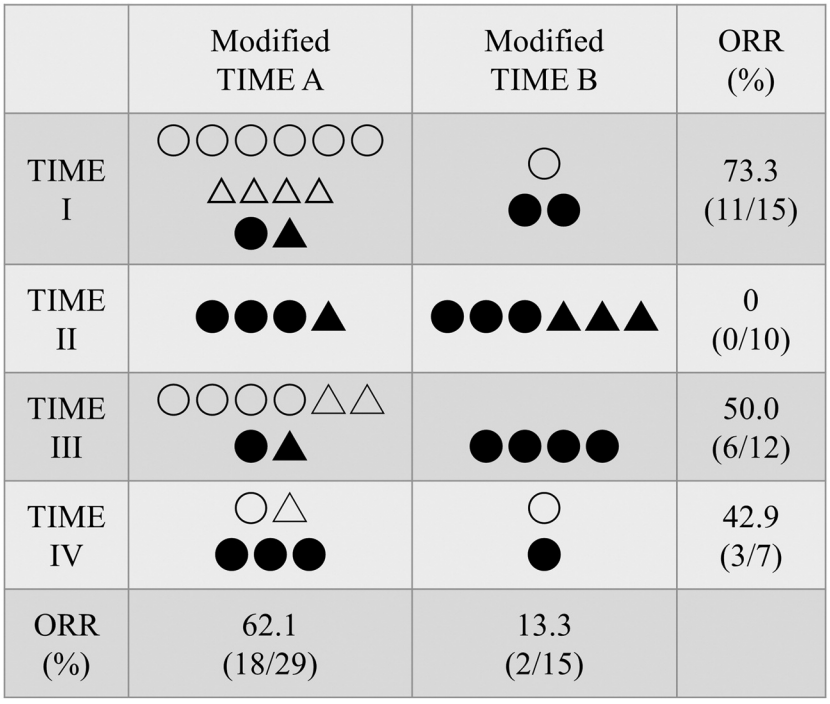

Figure 2. *Association between ORR and modified tumor immune microenvironment (TIME) classification. ○: Non-squamous case with partial response (PR), ●: non-squamous case with stable disease (SD) or progressive disease (PD), △: squamous case with PR, ▲ SQ: squamous case with SD or PD.*

specific T-cell migration. Similarly, in the IMpower 150 trial, the addition of atezolizumab to bevacizumab plus chemotherapy significantly improved survival, compared with bevacizumab plus chemotherapy alone, among patients with metastatic non-squamous NSCLC (23). Considering these results, angiogenesis is likely to play an important role in ICI therapy as well, and the efficacy of ICI therapy might be predicted with greater accuracy by including an evaluation of VEGF expression. In the future, the addition of bevacizumab to ICI therapy might improve treatment efficacy among patients with type-II and type-III (low CD8+TIL group) tumors, especially those with type-IIIB.

Our study has several limitations. First, the number of patients enrolled in the study was relatively small. Second, for the IHC analyses of PD-L1 expression, CD8+TIL counts, and VEGF expression, we used relatively standardized scoring systems. However, the PD-L1 scoring protocols differ for each of the ICIs presently available commercially. Although a standardized stromal CD8+TIL scoring system is currently available, a standardized CD8+TIL scoring protocol dose not yet exist for NSCLC.

In conclusion, an assessment of the TIME was useful for predicting the efficacy of ICI monotherapy. VEGF expression was a significant predictor of a response to ICI treatment. The

Table III. *Models for predicting best response using tumor characterictics.*

Characteristics	Number	ORR	*p*-Value
Single models			
PD-L1 expression			0.005
High (TPS≥10%)	27	63.0	
Low (TPS<10%)	17	17.6	
CD8+TIL count			0.033
High (≥50)	22	63.6	
Low (<50)	22	27.3	
VEGF expression			0.003
High (H-score≥4)	15	13.3	
Low (H-score>4)	29	62.1	
Dual models (TIME)			
PD-L1 and CD8+TIL			0.011
High + High (Type I)	15	73.3	
Others	29	31.0	
Triple models (modified TIME)			
PD-L1and CD8+TIL and VEGF			0.005
High +High +Low (Type IA)	12	83.3	
Others	32	31.2	

PD-L1: Programmed cell death receptor ligand-1; TPS: tumor proportion score, CD8+TIL: CD8 T cell tumor-infiltrating lymphocytes; HPF: high-power field; VEGF: vascular endothelial growth factor; TIME: tumor immune microenvironment.

modified TIME classification in which VEGF expression has been added might be more useful for predicting the response to ICI monotherapy.

Conflicts of Interest

The Authors state that there are no conflicts of interest to disclose in relation to this study.

Authors' Contributions

Study concept and design: KS, MN. Data acquisition: NY, SS, AI, KT, KK. Data analysis and interpretation: NY, KS. Manuscript preparation: NY, KS. Manuscript review: OT, OM, MN. All the Authors have read and approved the final manuscript.

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