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Relationship between consolidation tumor ratio and tumor-infiltrating lymphocytes in small-sized lung adenocarcinoma

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Abstract

Background: Consolidation tumor ratio (CTR) is associated with cancer progression and histological invasiveness in lung adenocarcinoma (LAD). However, little is known about the association between CTR and immune-related factors, including tumor-infiltrating lymphocytes (TILs) density or tumor expression of programmed death ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase 1 (IDO1) in small-sized LAD.

Methods: This study included 258 patients with LAD (<3 cm) who underwent surgery. Patients were assigned to four groups: CTR = 0; 0 < CTR <0.5; $0.5 \leq$ CTR <1 (ground-glass opacity [GGO] group); and CTR = 1 (pure-solid group). CD4$^+$, CD8$^+$, and FoxP3$^+$ TIL density and PD-L1 and IDO1 tumor expression were assessed by immunohistochemistry.

Results: Among the GGO group, CD8$^+$ and FoxP3$^+$ TIL density increased significantly with increasing CTR ($p < 0.001$ and $p < 0.001$, respectively). Moreover, PD-L1 and IDO1 expression was significantly higher in the pure-solid group than in the GGO group ($p < 0.001$ and $p < 0.001$, respectively).

Conclusions: CTR was correlated with the abundance of CD8$^+$ and FoxP3$^+$ TILs in the GGO group. PD-L1 and IDO1 positivity rates were significantly higher in the pure-solid group than in the GGO group. Increased CTR may be correlated with immunosuppressive condition.

KEYWORDS
IDO1, lung adenocarcinoma, PD-L1, surgery, tumor-infiltrating lymphocytes

INTRODUCTION

Lung adenocarcinoma (LAD) is the most common histological type among patients with non-small cell lung cancer.[1] Widespread screening using low-dose helical computed tomography (CT) has led to a higher early detection rate of small-sized lung cancers.[2] The consolidation tumor ratio (CTR), which is an imaging feature of small-sized LAD, is the ratio of solid tumor diameter to total tumor diameter. LAD patients with ground-glass opacities (GGO) have been

reported to have a good prognosis, and the pathological features of most cases indicated minimal invasion.[3,4] CTR is an established radiological parameter for identifying pathological non- or invasive tumors on CT images.[4,5]

Programmed death ligand 1 (PD-L1) is an immune checkpoint molecule expressed on tumor cells and suppresses the immune response to tumor cells by binding to programmed death 1 expressed on the surface of T cells.[6] Additionally, indoleamine 2,3-dioxygenase 1 (IDO1) metabolizes tryptophan to kynurenine, and tryptophan deficiency

TABLE 1 Clinicopathological characteristics of patients with small-sized LAD

Characteristics	Number of patients (*n* = 258)
Age (mean), years	69 (29–85)
Sex	
Female	136 (52.7%)
Male	122 (47.3%)
Smoking history	
Never smoker	132 (51.2%)
Smoker	126 (48.8%)
CTR	
CTR = 0	31 (12.0%)
0 < CTR < 0.5	57 (22.1%)
0.5 ≤ CTR < 1	59 (22.9%)
CTR = 1	111 (43.0%)
Clinical T status	
Tis	31 (12.0%)
T1mi	27 (10.5%)
T1a	52 (20.1%)
T1b	90 (34.9%)
T1c	58 (22.5%)
Surgical procedure	
Sublobar resection	90 (34.8%)
Lobectomy	168 (65.2%)
Pleural invasion	
Positive	32 (12.4%)
Negative	226 (87.6%)
Vascular invasion	
Positive	45 (17.4%)
Negative	213 (82.6%)
Lymphatic invasion	
Positive	20 (7.8%)
Negative	238 (92.2%)
Histological subtype	
AIS/MIS/lepidic	73 (28.3%)
Others	185 (71.7%)
TILs (mean), cells/0.04 mm^2	
CD4$^+$ TILs	38.6 (0–104.6)
CD8$^+$ TILs	41.5 (0–114.2)
FoxP3$^+$ TILs	12.8 (0–49.4)
PD-L1 expression	
Positive (≥1%)	69 (26.7%)
Negative (<1%)	189 (73.3%)
IDO1 expression	
Positive (≥1%)	142 (55.0%)
Negative (<1%)	116 (45.0%)
Pathological nodal status	
N0	235 (91.1%)
N1–2	23 (8.9%)

(Continues)

TABLE 1 (Continued)

Characteristics	Number of patients (*n* = 258)
Driver oncogene	
Absent	83 (32.2%)
Present	84 (32.5%)
Unknown	91 (35.3%)

Abbreviations: AIS, adenocarcinoma in situ; CD, cluster of differentiation; CTR, consolidation tumor ratio; FoxP3, forkhead box protein P3; IDO1, indoleamine 2,3-dioxygenase 1; LAD, lung adenocarcinoma; MIA, minimally invasive adenocarcinoma; PD-L1, programmed cell death ligand 1; TILs, tumor-infiltrating lymphocytes.

suppresses T cell proliferation and induces regulatory T cell differentiation.[7,8] Therefore, PD-L1 and IDO1 are important factors that suppress antitumor immune responses. We previously reported that high expression of PD-L1 or IDO1 was correlated with poor prognosis in LAD.[9,10] A recent clinical study demonstrated that PD-L1 inhibitors, which are approved as a standard treatment for advanced lung cancer with high PD-L1 expression, significantly improved prognosis.[11]

Tumor-infiltrating lymphocytes (TILs) play a major role in the tumor immune response, and there are multiple phenotypes of TILs. CD4 is a surface antigen expressed on helper T cells that receive antigen presentation.[12] While CD8$^+$ TILs have a cytotoxic function, regulatory T cells expressing FoxP3 suppress the immune response.[13,14] Our previous study demonstrated that a low ratio of CD8$^+$ TILs and FoxP3$^+$ TILs is an independent poor prognostic factor of disease-free survival in stage IA LAD.[15]

A retrospective study has examined the relevance between CTR and prognosis or pathological characteristics in early lung cancer; however, the detailed analysis between CTR and the TILs or immunosuppressive factors has not been performed.[16] In this study, we examined the relationship between CTR and TILs and the expression of PD-L1 and IDO1.

METHODS

Patients

This retrospective study was reviewed and approved by the institutional review board of Kyushu University (approval no. 2019–232). The study included 365 patients with clinical stage 0/I LAD and a tumor diameter <3 cm who underwent surgical resection at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, between January 2003 and December 2016. We excluded 107 patients because of insufficient tumor tissue or missing data, incomplete resection, or receipt of neoadjuvant therapy, and a total of 258 patients was enrolled. Clinical stage was defined by the T (tumor), N (node), and M (metastasis) classification of the International Association for the Study of Lung Cancer guidelines (8th edition).[17] The clinicopathological
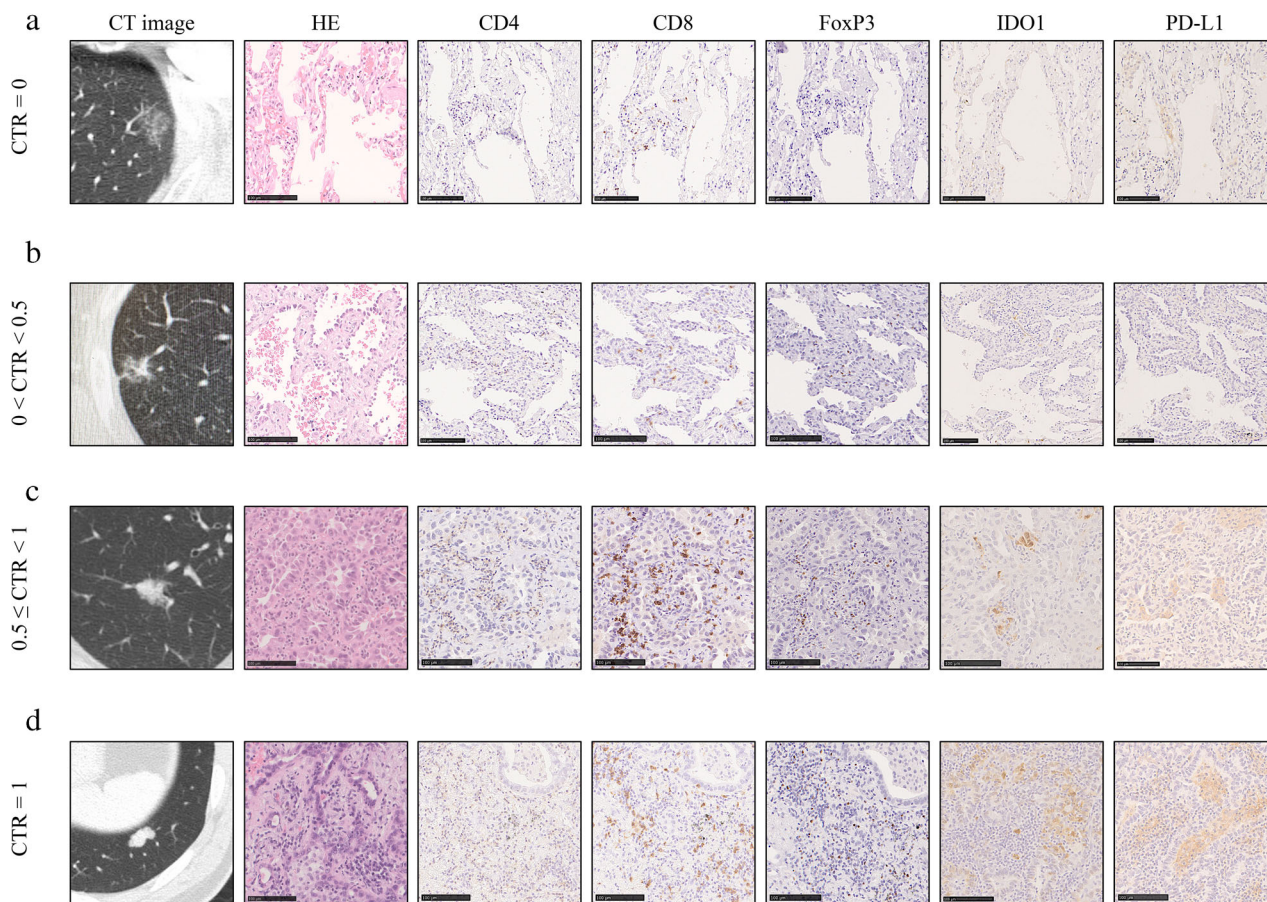
FIGURE 1 Representative images of computed tomography scans, H&E staining, and immunohistochemical staining of immune markers in resected lung adenocarcinoma specimens. (a–d) Specimens with consolidation tumor ratio (CTR) of 0 (a), 0.12 (b), 0.92 (c), and 1 (d). Scale bar: 100 μm

features analyzed were age at surgical resection, sex, smoking history, radiological whole tumor size, radiological solid component size, CTR, surgical procedure, pleural invasion, lymphatic invasion, vascular invasion, histological subtype, epidermal growth factor receptor (*EGFR*) and anaplastic lymphoma kinase (*ALK*). The *EGFR* status was examined in resected specimens with the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method[18]; and *ALK* gene rearrangement was tested by fluorescence in situ hybridization. Genetic alterations of *EGFR* or *ALK* were available in 167 patients. Clinical data were obtained from patient records.

Calculation of CTR

All patients underwent CT imaging in the supine position during inspiratory breath-hold using various multidetector row scanners (Aquilion 4, Aquilion 64, Aquilion ONE, Aquilion ONE Vision [Toshiba], SOMATOM Plus4 Volume Zoom [Siemens Medical Solutions, Malvern], Brilliance CT, and Brilliance iCT [Philips Healthcare]).

The diameter of consolidation in each tumor and the diameter of the whole tumor, including GGO, were measured

with axial two-dimensional CT data at 2-mm slice thicknesses, and the CTR was calculated. The CT images were evaluated by three of the authors (Y.O., K.T., and T.K.). When independent assessments disagreed, the images were viewed and discussed together to reach a final consensus. Patients were assigned to four groups of CTR = 0; 0 < CTR < 0.5; 0.5 ≤ CTR < 1; and CTR = 1. Additionally, tumors without GGO component were defined as the pure-solid group (CTR = 1) and the other tumors with GGO were defined as the GGO group (0 ≤ CTR < 1).

Immunohistochemical staining

Immunohistochemistry was performed for 258 LAD using formalin-fixed tissue sections. The detailed method has been described in previous reports (primary monoclonal antibodies: antihuman CD4 [1:100; ab213215, Abcam], antihuman CD8 [1:100; C8/144B; Dako], antihuman FoxP3 [1:100; 236A/E7; eBiosciences], antihuman PD-L1 [1:100; SP142; Spring Bioscience], and antihuman IDO1 [1:200; UMAB126; Origene Technologies).[9,10,15,19]

We selected hot spots within 0.04 mm² areas in five fields per section and counted the numbers of CD4⁺, CD8⁺,

T A B L E 2 Association between CTR and clinicopathological features

Characteristics	n	CTR = 0 (n = 31)	0 < CTR < 0.5 (n = 57)	0.5 ≤ CTR < 1.0 (n = 59)	CTR = 1 (n = 111)	p-value
Age						
<69	126	13 (41.9%)	29 (50.9%)	23 (39.0%)	61 (55.0%)	0.199*
≥69	132	18 (58.1%)	28 (49.1%)	36 (61.0%)	50 (45.0%)	
Sex						
Female	136	18 (58.1%)	37 (64.9%)	33 (55.9%)	48 (43.2%)	0.045*
Male	122	13 (41.9%)	20 (35.1%)	26 (44.1%)	63 (56.8%)	
Smoking history						
Never smoker	132	19 (61.3%)	34 (59.7%)	33 (55.9%)	46 (41.4%)	0.053*
Smoker	126	12 (38.7%)	23 (40.3%)	26 (44.1%)	65 (58.6%)	
Clinical T status						
Tis	31	31 (100%)	0 (0%)	0 (0%)	0 (0%)	<0.001**
T1mi	27	0 (0%)	24 (42.1%)	2 (3.4%)	1 (0.9%)	
T1a	52	0 (0%)	27 (47.4%)	17 (28.8%)	8 (7.2%)	
T1b	90	0 (0%)	6 (10.5%)	35 (59.3%)	49 (44.1%)	
T1c	58	0 (0%)	0 (0%)	5 (8.5%)	53 (47.8%)	
Surgical procedure						
Sublober resection	90	19 (61.3%)	25 (43.9%)	19 (32.2%)	27 (24.3%)	<0.001*
Lobectomy	168	12 (38.7%)	32 (56.1%)	40 (67.8%)	84 (75.7%)	
Pleural invasion						
Negative	226	30 (96.8%)	56 (98.3%)	56 (94.9%)	84 (75.7%)	<0.001*
Positive	32	1 (3.2%)	1 (1.7%)	3 (5.1%)	27 (24.3%)	
Vascular invasion						
Negative	213	31 (100%)	54 (94.7%)	53 (89.8%)	75 (67.6%)	<0.001*
Positive	45	0 (0%)	3 (5.3%)	6 (10.2%)	36 (32.4%)	
Lymphatic invasion						
Negative	238	31 (100%)	56 (98.3%)	58 (98.3%)	93 (83.8%)	<0.001*
Positive	20	0 (0%)	1 (1.7%)	1 (1.7%)	18 (16.2%)	
Histological subtype						
AIS/MIA/lepidic	73	30 (96.8%)	33 (58.9%)	10 (16.9%)	0 (0%)	<0.001*
Others	185	1 (3.2%)	24 (41.1%)	49 (83.1%)	111 (100%)	
Pathological nodal status						
N0	235	31 (100%)	56 (98.3%)	56 (94.9%)	92 (82.9%)	<0.001*
N1–2	23	0 (0%)	1 (1.7%)	3 (5.1%)	19 (17.1%)	

Abbreviations: AIS, adenocarcinoma in situ; CTR, consolidation tumor ratio; MIA, minimally invasive adenocarcinoma.

*p-value was estimated by Fisher's exact test.

**p-value was estimated by Wilcoxon rank sum test.

and FoxP3^{+} TILs; the results are presented as the average densities. As reported previously, PD-L1 and IDO1 expression on tumor cells was evaluated and the cutoff value for positivity was set at 1% of tumor cells.[9,10] Staining was evaluated by at least two investigators, one of whom was a pathologist.

Statistical analysis

Associations between CTR and clinicopathological factors were analyzed using Wilcoxon's rank sum test and Fisher's exact test; associations between CTR and TILs in the GGO group were analyzed by regression analysis, and differences in TILs between the GGO and the pure-solid groups were analyzed by Student's t-test. A Cochran–Armitage test was applied to investigate trends in PD-L1 and IDO1 positivity among the GGO group.

Comparisons of PD-L1 and IDO1 positivity between the GGO and pure-solid groups were performed using Fisher's exact test. All statistical analyses were performed with JMP Statistical Discovery software (version 14.0; SAS Institute), and a p-value <0.05 was regarded as statistically significant.

RESULTS

Clinicopathological characteristics of patients with small-sized LAD

The clinicopathological characteristics of the study cohort of 258 patients with clinical stage 0/I LAD (<3 cm) are shown in Table 1. The CTR = 0, 0 < CTR <0.5, 0.5 ≤ CTR < 1, and CTR = 1 groups consisted of 31 (12.0%), 57 (22.1%), 59 (22.9%), and 111 (43.0%) patients, respectively. The mean density (cells/0.04 mm^2) of CD4$^+$, CD8$^+$, and FoxP3$^+$ TILs was 38.6 (range, 0–104.6), 41.5 (range, 0–114.2), and 12.8 (range, 0–49.4), respectively. PD-L1 and IDO1 expression was positive (≥1% of cells) in tumors from 69 (26.7%) and 142 (55.0%) patients, respectively. A total of 235 patients (91.1%) had pathological N0. Figure 1 shows representative CT images and each immunohistochemistry images according to CTR (Figure 1a–d).

### Associations between clinicopathological characteristics and CTR

The associations between clinicopathological features and CTR are shown in Table 2. Clinical T factor increased in parallel with increasing CTR ($p < 0.001$, Wilcoxon rank sum test), and increased CTR was significantly associated with cancer progression. The proportion of patients with pleural, vascular, and lymphatic invasion also increased in parallel with increasing CTR (all $p < 0.001$; Fisher's exact test). The ratio of nonlepidic pattern to lepidic pattern histological subtype was significantly higher in patients with high CTR ($p < 0.001$).

Associations between CTR and the abundance of TILs

We next analyzed the associations between CTR and the density of TIL. In the CTR = 0, 0 < CTR <0.5, 0.5 ≤ CTR < 1, and CTR = 1 groups, the mean (± standard deviation) density of CD4$^+$ TILs was 41.3 ± 4.2, 43.0 ± 3.1, 40.3 ± 3.1, and 34.7 ± 2.2 per 0.04 mm^2, respectively (Figure 2a), while the mean density of CD8$^+$ TILs was 28.7 ± 4.8, 38.7 ± 3.6, 49.1 ± 3.5, and 42.5 ± 2.6 per 0.04 mm^2, respectively (Figure 2b), and the mean density of FoxP3$^+$ TILs was 6.3 ± 1.8, 9.6 ± 1.3, 17.1 ± 1.3, and 14.0 ± 0.9 per 0.04 mm^2, respectively (Figure 2c).

To determine whether the densities of TILs were significantly associated with CTR in the GGO group, we performed regression analysis of the cell densities of each TIL type in the CTR = 0, 0 < CTR < 0.5, and 0.5 ≤ CTR < 1 groups. We found that CTR was not significantly associated with the density of CD4$^+$ TILs ($p = 0.776$; Figure 2a);
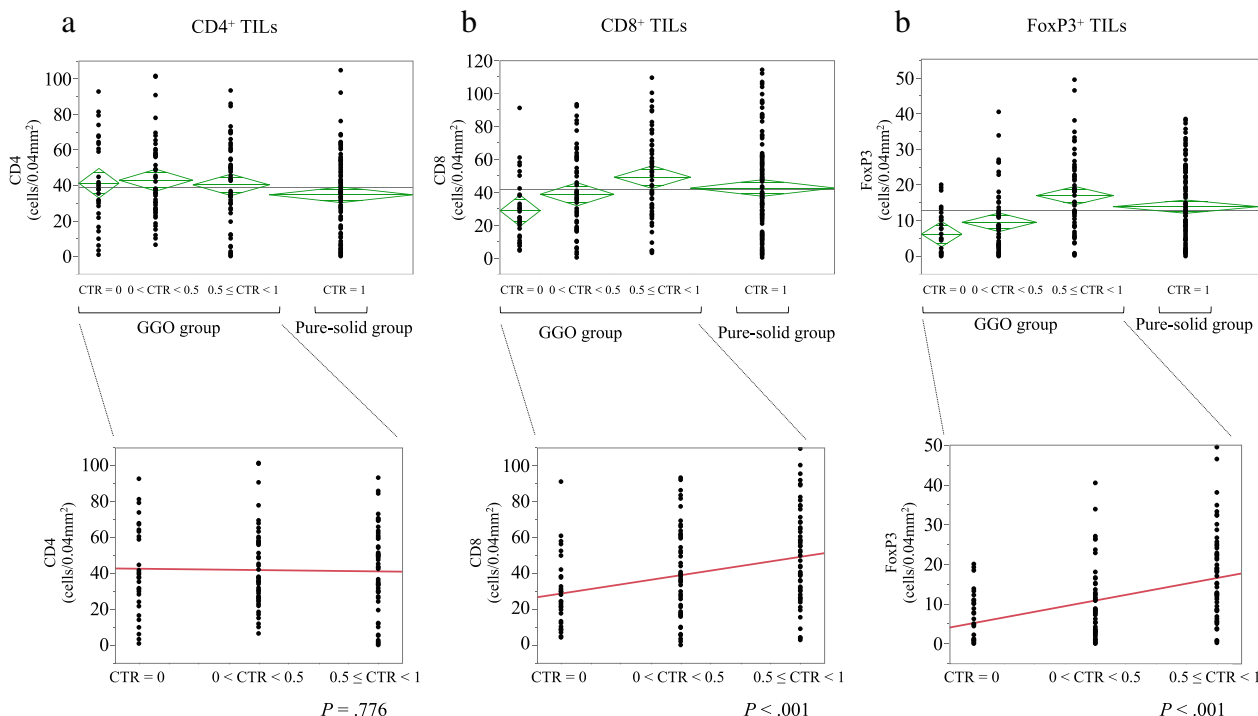
FIGURE 2 Correlations between consolidation tumor ratio (CTR) and tumor-infiltrating lymphocytes (TILs) in lung adenocarcinoma specimens. (a) CD4$^+$ TILs, (b) CD8$^+$ TILs, (c) FoxP3$^+$ TILs according to CTR. Data are presented as the mean (horizontal line in green diamond) and confidence interval (upper and lower points of green diamonds), with individual samples shown as solid black circles. N = 31, 57, 59, and 111, for CTR = 0, 0 < CTR < 0.5, 0.5 ≤ CTR < 1, and CTR = 1, respectively. $p = 0.776$, $p < 0.001$ and $p < 0.001$ for ground-glass opacity tumors trends in (a), (b), and (c), respectively
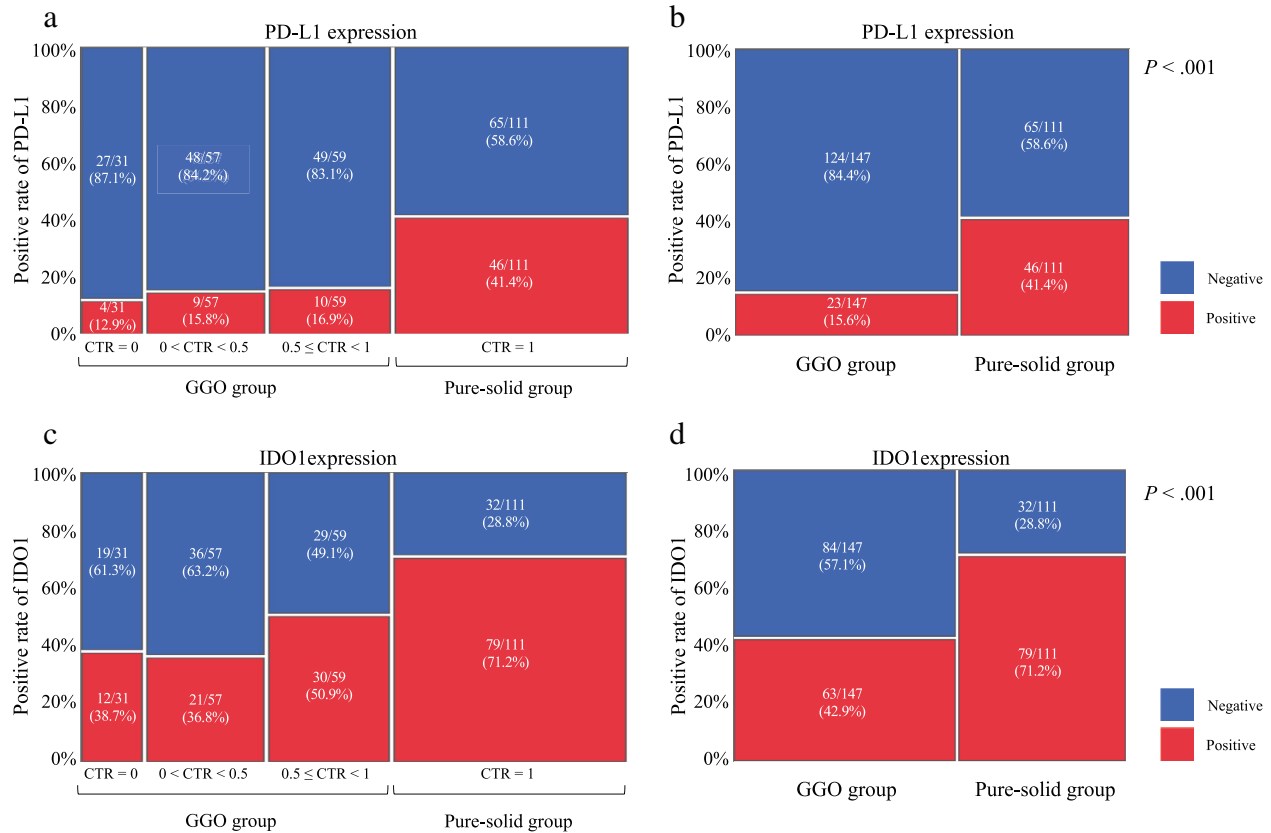
FIGURE 3 Correlation between positivity rates for programmed death ligand 1 (PD-L1) (a and b) and indoleamine 2,3-dioxygenase 1 (IDO1) (c and d) in ground-glass opacity (GGO) tumors compared with pure-solid tumors. $p < 0.001$ and $p < 0.001$ for PD-L1 (b) and IDO1 (d), respectively

however, it was significantly positively associated with the densities of both CD8$^+$ and FoxP3$^+$ TILs ($p < 0.001$ and $p < 0.001$, respectively; Figure 2b,c).

In contrast, when comparing TILs between the GGO and pure-solid groups, we observed the opposite trend. Thus, the CD4$^+$ TIL density was significantly lower in the pure-solid group than in the GGO group ($p = 0.022$; Figure S1a), whereas the density of CD8$^+$ and FoxP3$^+$ TILs was comparable between the GGO and pure-solid groups ($p = 0.618$ and $p = 0.116$, respectively; Figure S1b,c).

Associations between CTR and PD-L1 and IDO1 positivity

Next, we determined whether CTR was positively associated with the rate of PD-L1 and IDO1 tumor expression. The positivity rates of PD-L1 in tumors in the CTR = 0, 0 < CTR <0.5, 0.5 ≤ CTR < 1, and CTR = 1 groups were 12.9% (4/31), 15.8% (9/57), 16.9% (10/59), and 41.4% (46/111), respectively (Figure 3a), and the positivity rates of IDO1 were 38.7% (12/31), 36.8% (21/57), 50.9% (30/59), and 71.2% (79/111), respectively (Figure 3c). To determine whether a significant trend existed in the tumor expression of PD-L1 or IDO1 between the GGO groups, we performed a Cochran–Armitage

trend test. However, this analysis revealed no significant trend in either PD-L1 or IDO1 positivity between the CTR = 0, 0 < CTR < 0.5, and 0.5 ≤ CTR < 1 groups ($p = 0.628$ and $p = 0.188$, respectively; Figure S2a,b).

When PD-L1 and IDO1 positivity was compared between the pure-solid and the GGO groups, using Fisher's exact test we detected significantly higher PD-L1 and IDO1 positivity in the pure-solid group than in the GGO group ($p < 0.001$ and $p < 0.001$, respectively (Figure 3b,d).

Associations between driver oncogene and TILs and immunosuppressive molecules

We performed an exploratory analysis of the relationship between driver oncogenes including *EGFR* and *ALK*, and TILs and immunosuppressive molecules. Driver oncogene information was available in 167 patients (Figure S3). Of these, 84 patients had some driver oncogenes, all of which were *EGFR*. The group with driver oncogenes had significantly lower CTR than those without ($p = 0.004$); however, it was not associated with the abundance of CD4$^+$, CD8$^+$, and FoxP3$^+$ TILs, and the expression of PD-L1 and IDO1 ($p = 0.187$, $p = 0.737$, $p = 0.125$, $p = 0.108$, $p = 0.438$, respectively).

DISCUSSION

The present study evaluated the associations between the CTR of small-sized LAD and immune-related factors, including CD4^{+}, CD8^{+}, and FoxP3^{+} TIL abundance, and PD-L1 and IDO1 positivity. Our study revealed several findings of significant positive associations between the CTR and the density of CD8^{+} and FoxP3^{+} TILs among the GGO group, and significantly higher PD-L1 and IDO1 positivity in the pure-solid group compared with the GGO group. Our study implies that increased CTR might be correlated with immunosuppressive condition in antitumor immune response.

The current study showed that the density of CD8^{+} and FoxP3^{+} TILs was significantly higher in high CTR tumors than that in low CTR tumors among GGO tumors. These results may reflect the transition of the antitumor immune response as the tumor grows. LAD without solid component, which pathologically corresponds to adenocarcinoma in situ (AIS),[20] exhibits a low antitumor immune response.[21] As tumor grows, the antitumor immune response is triggered, and cytotoxic cells including CD8^{+} lymphocytes infiltrate into tumors. However, immunosuppressive cells including Foxp3^{+} lymphocytes also infiltrate into tumors as the tumor grows, which may cause an immunosuppressive state.[22] The gradual increases in the density of CD8^{+} and FoxP3^{+} TILs paralleling the CTR may be related to tumor growth.

In the present study, the positive rate of PD-L1 and IDO1 expression was significantly higher in the pure-solid group than in the GGO group (Figure 3), which was similar to a previous study.[16] Moreover, pure-solid group has been pathologically more invasive than GGO group (Table S1). In several studies, the survival of LAD without the GGO component has been reported to be worse than those with the GGO component. Additionally, PD-L1 and IDO1 on tumor cells was reported to be poor prognostic factors in several types of cancer, including LAD.[10,23,24] Our results suggest that these immunosuppressive factors might have been correlated with the poor prognosis of pure-solid LAD.

The differences of two types of LADs with or without GGO component are well known regarding to survival, pathological aggressiveness and the frequency of gene alterations. Our present and previous study demonstrated that pure-solid LAD was more pathological invasive and was associated with poor clinical outcome.[25] Moreover, a previous report revealed that the frequency of an epidermal growth factor receptor (EGFR) mutation in early-stage pure-solid LAD was significantly low than LAD with GGO component.[26] In our result, there were no significant differences in the abundance of CD8^{+} and FoxP3^{+} TILs between the pure-solid and the GGO groups (Figure S1), although CD8^{+} and FoxP3^{+} TILs increased significantly with increasing CTR among the GGO group. However, as previously described, the expression of both PD-L1 and IDO1 was significantly higher in the pure-solid group than in the GGO group (Figure 3); on the other hand, the expression of PD-L1 and IDO1 was statistically comparable among the GGO group (Figure S2). These findings might be associated with the differences in the biological characteristics between LAD with and without GGO component. Our exploratory analysis showed that TILs and the immunosuppressive molecules were comparable between the tumors with and without driver oncogene, although driver oncogene was associated with CTR (Figure S3). A previous study revealed that advanced LADs with EGFR wild-type had more CD8^{+} TILs and higher PD-L1 expression than those with EGFR mutation.[27] A few reports have shown the association between diver oncogenes and TILs in small-sized LAD.[27] Further analyses of larger numbers of patients are needed to clarify the association between CTR and immune condition in small-sized LAD.

The role of CD4^{+} TILs in the tumor micro environment was not evaluated in this study. TILs expressing CD4 are classified into some types of lymphocytes, such as T helper-1 (Th1), Th2, Th17, and regulatory T cells. CD4^{+} Th1 produces interferon-γ, which has a cytotoxic function to tumor cells; on the other hand, CD4^{+} Th17 secretes interleukin-4, interleukin-10, and interleukin-13, which assists in tumor progression.[28] To further research, the correlation between CD4^{+} TILs and CTR in small-sized LAD, cytokines and the surface antigen of CD4^{+} TILs needs to be assessed.

There were several limitations to this study. First, this was a retrospective analysis performed at a single institution. Validation cohort studies should be conducted for the same analysis. Second, there is no definitive assessment method for quantifying TILs in lung cancer.[29] Furthermore, the score of TILs may fluctuate in the evaluation of randomly selected areas in the entire tumor; however, the method of selecting the hot spots is considered to have a small variation in the score. Although several limitations exist, we report important findings that are necessary for understanding tumor progression and the accompanying changes.

In conclusion, high CTR in small-sized LAD was positively correlated with the abundance of CD8^{+} and FoxP3^{+} TILs, and PD-L1 and IDO1 positivity rates were significantly higher in the pure-solid group than in the GGO group. These features suggest that small-sized LAD with high CTR might be in a more immunosuppressive state than that with low CTR in small-sized LAD.

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