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ORIGINAL ARTICLE

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Enhancement of anti-PD-L1 antibody plus anlotinib efficacy due to downregulation of PD-L1 in the micro-conduit endothelium within the tumor: a randomized double-blind trial

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ABSTRACT

Objective: The possible enhancing effect of anlotinib on programmed death receptor ligand (PD-L1) antibody and the efficacy-predicting power of PD-L1 in micro-conduit endothelium, including lymphatic endothelial cells (LECs) and blood endothelial cells (BECs), were determined to identify patients who would benefit from this treatment.

Methods: PD-L1 positivity in LECs, BECs, and tumor cells (TCs) was assessed using paraffin sections with multicolor immunofluorescence in an investigator's brochure clinical trial of TQB2450 (PD-L1 antibody) alone or in combination with anlotinib in patients with non-small cell lung cancer. Progression-free survival (PFS) with different levels of PD-L1 expression was compared between the two groups.

Results: Among 75 patients, the median PFS (mPFS) was longer in patients who received TQB2450 with anlotinib [10 and 12 mg (161 and 194 days, respectively)] than patients receiving TQB2450 alone (61 days) [hazard ratio ($HR_{10\ mg}$ = 0.390 (95% confidence interval {CI}, 0.201–0.756), P = 0.005; $HR_{12\ mg}$ = 0.397 (0.208–0.756), P = 0.005]. The results were similar among 58 patients with high PD-L1 expression in LECs and TCs [159 and 209 *vs.* 82 days, $HR_{10\ mg}$ = 0.445 (0.210–0.939), P = 0.034; $HR_{12\ mg}$ = 0.369 (0.174–0.784), P = 0.009], and 53 patients with high PD-L1 expression in BECs and TCs [161 and 209 *vs.* 41 days, $HR_{10\ mg}$ = 0.340 (0.156–0.742), P = 0.007; $HR_{12\ mg}$ = 0.340 (0.159–0.727), P = 0.005]. No differences were detected in the mPFS between the TQB2450 and combination therapy groups in 13 low/no LEC-expressing and 18 low/no BEC-expressing PD-L1 cases.

Conclusions: Mono-immunotherapy is not effective in patients with high PD-L1 expression in LECs and/or BECs. Anlotinib may increase efficacy by downregulating PD-L1 expression in LECs and/or BECs, which is presumed to be a feasible marker for screening the optimal immune patient population undergoing anti-angiogenic therapy.

KEYWORDS PD-L1; lymphatic endothelial cell; blood endothelial cell; anlotinib; progression-free survival

Introduction

Defective immune cell function coupled with immunosuppressive factors in the tumor microenvironment (TME) results in cancer cells that evade recognition and destruction in patient with lung cancer. Given that patients with low tumor immune escape have a longer disease-free survival (DFS)[1], immune checkpoint inhibitors (ICIs) have achieved long-term survival in patients with advanced non-small cell lung cancer (NSCLC)[2] and inoperable stage III NSCLC[3]. However, a

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significant number of patients do not respond to therapy or rapidly develop drug resistance[4,5].

Abnormal microvasculature in the TME prevents immune effector cells from entering the tumor and creates a high lactic acid environment causing hypoxia, which inhibits effector T cell function. Therefore, anti-angiogenic therapy is introduced to reverse the immunosuppressive status and enhance tissue perfusion to promote the delivery of T cells and immune effector molecules[6]. Indeed, such therapy is beneficial to patients[7,8]. Nevertheless, the final analysis of the authoritative IMpower 150 trial revealed that the median overall survival (mOS) was only slightly prolonged in the atezolizumab-bevacizumab-carboplatin-paclitaxel (ABCP) group compared to the atezolizumab-carboplatin-paclitaxel (ACP) group[9], indicating that anti-angiogenic therapy (bevacizumab) is not effective in all patients. It is therefore important to determine the population that gains maximum benefit by combining anti-angiogenesis and immune therapies.

The unsatisfactory efficacy of bevacizumab may be due to bypassing activation of intracellular signaling, including fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), caused by single-target anti-angiogenic drugs when inhibiting the vascular endothelial growth factor (VEGF) pathway[10], which confers more active malignant biological behavior in the cells. Additionally, cancer cells induce high expression of PD-L1 in blood endothelial cells (BECs), thereby blocking and inactivating $CD8^+$ lymphocytes in the tumor and leading to tumor immune escape[11]. Notably, lymphatic endothelial cells (LECs) in tumors also inhibit T-cell activation *via* high PD-L1 expression, which helps tumor cells escape the immune system[12]. Therefore, it is crucial to find some effective means to decrease PD-L1 expression in the endothelium of tumor-associated micro-conduits. Anlotinib, a novel orally administered receptor tyrosine kinase inhibitor, has shown promising efficacy in clinical trials by inhibiting the activation of pro-angiogenic signals (VEGFR, PDGFR, and FGFR)[13-15]. Moreover, anlotinib improves the immune microenvironment by downregulating PD-L1 expression in BECs *in vivo*[11]. A clinical study using the combination of anlotinib and the programmed death PD-1 antibody, sintilimab, as the first-line treatment for advanced NSCLC revealed good efficacy[16]. We hypothesized that the mechanisms underlying the reinforcement of ICIs may be attributed to the downregulation of PD-L1 in the micro-conduit endothelium inside the tumor (**Figure 1**). Therefore, we collected pre-therapeutic tissue specimens from consented participants in a clinical study [phase Ib clinical study of the safety, pharmacokinetics, and efficacy of TQB2450 injection (PD-L1 antibody) combined with anlotinib in patients with advanced NSCLC at the Tianjin Medical University Cancer Hospital (Approval No. E2019211)] for multitargeted immunofluorescence staining of PD-L1 in micro-conduit endothelium (BECs and LECs) inside tumors and tumor cells (TCs). The association between the expression of PD-L1 and efficacy was determined to gain insight into the synergistic effect of anlotinib with PD-L1 antibody and the efficacy-predicting power of PD-L1 in the micro-conduit endothelium.

Materials and methods

Study design and inclusion & exclusion criteria

The trial was named "Efficacy and Safety of TQB2450 alone or with Anlotinib in Previously Treated Advanced Non-Small Cell Lung Cancer (NSCLC): A Multicenter, Randomized, Double-blind Clinical Trial"[17]. The enrolled patients were randomized into 3 groups at a 1:1:1 ratio, as follows: group 1, TQB2450 (1200 mg) + placebo; group 2, TQB2450 (1200 mg) + anlotinib (10 mg); and group 3, TQB2450 (1200 mg) + anlotinib (12 mg). The protocol was TQB2450 intravenous drips once every 3 weeks and anlotinib or placebo orally once daily on days 1–14 per cycle.

The primary inclusion criteria were as follows: 1) Eastern Clinical Oncology Group (ECOG) score of 0–1 and expected survival > 3 months; 2) histologically confirmed EGFR/anaplastic lymphoma kinase (ALK) wild-type stage IIIb-IV NSCLC; 3) patients who have failed or are intolerant to at least first-line standard chemotherapy, and according to the solid tumor efficacy evaluation standard Response Evaluation Criteria in Solid Tumors (RECIST) 1.1[18], present at least one measurable lesion; and 4) patients with a tumor proportion score (TPS) ≥1 (considered PD-L1 positive; ROCH/SP263). The primary exclusion criteria were as follows: 1) patients previously treated with anlotinib and/or immunotherapy against PD-1/PD-L1; 2) patients with central squamous lung cancer or at risk for macro-hemoptysis (> 50 mL/day); and 3) an arterial/venous thrombotic event within 6 months prior to randomization, such as a cerebrovascular accident (including transient ischemic attack, cerebral hemorrhage, and cerebral infarction), deep venous thrombosis, and pulmonary embolism.

The study protocol was approved by the Institutional Review Board of each institution. The study complied with the Good Clinical Practice guidelines and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent before enrollment (E2019211).

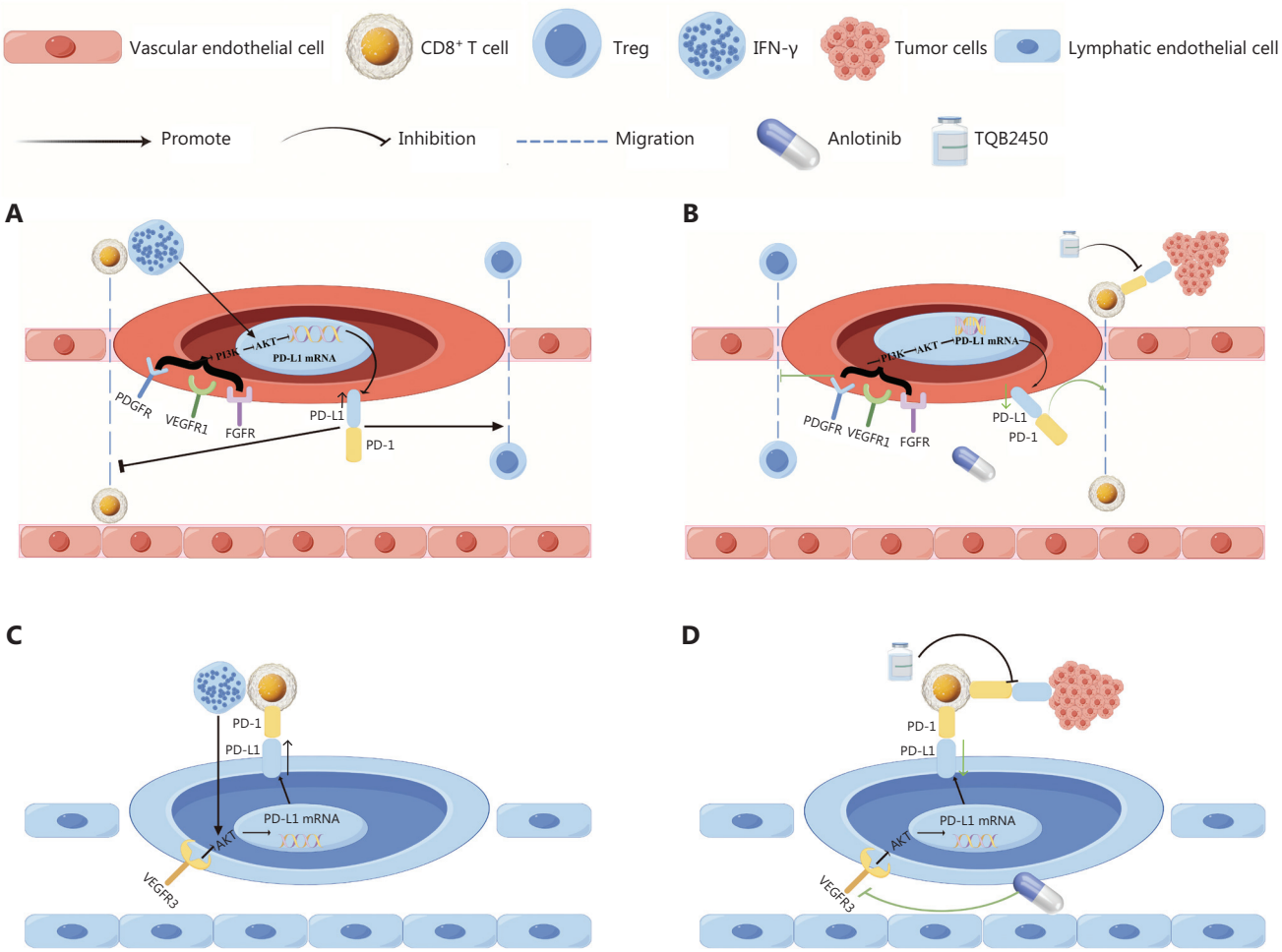

Figure 1 Schematic representation of the scientific hypothesis. (A) High PD-L1 expression in vascular endothelial cells is induced by interferon (IFN)-γ released from lymphatic cells in adjacent tumor tissue. PD-LI subsequently binds PD-1 in T cells in vessels, inhibits the migration of $CD8^+$ T cells from blood vessels to tumor tissue, and promotes the migration of Treg cells into tumors. High PD-L1 expression in vascular endothelial cells forms an "immune barrier" in vascular endothelial cells and an immunosuppressive microenvironment. (B) Anlotinib downregulates PD-L1 expression in vascular endothelial cells *via* inhibiting the VEGFR1-PI3K-AKT pathway which removes the "immune barrier." Subsequently, $CD8^+$ T cells easily pass through the vascular wall and enter the tumor, in which anti-PD-L1 (TQB2450) impedes the binding of PD-L1 in tumor cells to PD-1 in $CD8^+$ T cells and acts synergistically with anlotinib. (C) High expression of PD-L1 in lymphatic endothelial cells is induced by IFN-γ released from lymphatic cells *via* the AKT pathway. Subsequently, PD-L1 binds PD-1 in $CD8^+$ T cells in adjacent tumor tissues. $CD8^+$ T cells in the peritumoral stroma area are then inactivated, thus interfering with the destruction of $CD8^+$ T cells to tumor cells. (D) Anlotinib downregulates PD-L1 expression in lymphatic endothelial cells *via* inhibiting VEGFR3 signaling and decreasing the binding of PD-L1 to PD-1 in $CD8^+$ T cells, thereby restoring the lethality of $CD8^+$ T cells to PD-L1+ tumor cells.

This study was conducted in accordance with the principles of the Declaration of Helsinki. The protocol was approved by the Ethics Committee of each participating center [Shanghai Chest Hospital (Approval No. 202006), Tianjin Medical University Cancer Institute and Hospital (Approval No. E2020197), Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital (Approval No. 2019038), Jilin Cancer Hospital (Approval No. 201904-027-03), Gansu Provincial Cancer Hospital (Approval No. A201902270003), and Tianjin Chest Hospital (Approval No. 2020-002-02)]. Written informed consent was obtained from all patients.

Procedures

The efficacy was evaluated every 2 cycles (6 weeks) during treatment, excluding an evaluation every 9 weeks (i.e., 63 days) when the patient was in post-trial observation according to RECIST 1.1 and iRECIST criteria[19], and classified

as follows: CR, complete remission; PR, partial remission; SD, stable disease; PD, disease progression; iCR, immune complete remission; iPR, immune partial remission; iSD, immune stable disease; iUPD, immune unconfirmed progression; iCPD, immune confirmed progression. DCR, disease control rate (defined as the proportion of patients whose tumors had shrunk or remained stable, including CR, PR, and SD. PFS, progression-free survival was defined as the time from randomization of patients to the onset of disease progression.

After completing treatment, patients were followed for a minimum of 28 days to monitor the adverse events (AEs) [severe AEs (SAEs) were collected until 90 days after the end of treatment unless the participant started new antitumor therapy between days 29 and 90 after the end of treatment].

Multiplex immunofluorescence staining

All the specimens were serially sectioned (5 μm thick). Paraffin-embedded tissue sections were collected from archived specimens or wax blocks obtained before treatment in the Pathology Department. To ensure the feasibility of the staining results, tissue sections were collected within 12 months for a concomitant study (Ethical No. E2019211). Thereafter, the sections were subjected to multitarget immunofluorescence staining (cell nuclei were stained with DAPI). The specific steps were as follows: ① conventional dewaxing with xylene, ethanol, and distilled water; ② microwave antigen repair; ③ blocking antibodies; ④ primary antibody (**Table 1**) incubation for 30 min at room temperature (A reliable method to measure PD-L1 expression in TCs and LECs/BECs was confirmed. SP263 was used for the detection of PD-L1 expression in tumor cells. However, no data were available regarding application of SP263 on LECs and BECs. Therefore, we used CST13684[20], another widely used antibody, to re-assess the level of PD-L1 protein expression in cells, and verified that the results with the two antibodies were consistent with PD-L1 protein expression in cells); ⑤ secondary antibody incubation for 10 min at room temperature; ⑥ fluorescence staining to amplify the signal; ⑦ mounting (covering the slide with anti-fluorescent burst blocker and a coverslip); and ⑧ image acquisition [with PerkinElmer inForm image analysis software (version 2.4.0) for multispectral image decomposition]. Five randomly selected tumor tissue areas without bleeding, necrosis, or detachment were scanned in the entire field of view, tumor area, and stroma area. The results were confirmed by immunohistochemistry stain unless the specimen was too small.

Image analysis

Cells were accurately identified and enumerated with InForm image analysis software (version 2.4; PerkinElmer, Waltham, MA, USA). A reasonable threshold was set to identify positive cells. For enumeration, the number of $CK^+PD\text{-}L1^+/CK^+$ cells was defined as PD-L1 expression-positivity for TCs. The same method was used to calculate PD-L1 expression-positivity for BECs and LECs[21].

Delineation of the positive staining threshold

Pathologists interpreted the staining images and set an appropriate threshold for each case using the score function of the information (in which each case had a matching threshold).

We adopted the commonly used receiver operating characteristic (ROC) curve[22] to calculate the cut-off values for the expression of PD-L1. PFS measurements were obtained using imaging findings to determine the condition (progression or stability) at the end of the follow-up period.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 (SPSS, Chicago, IL, USA). PFS was estimated using the Kaplan-Meier

Table 1 Antibodies used for multi-color immunofluorescence staining

Antibody description	Item	Dilution ratio	Implication
CK	Ab215838	200×	Tumor cells
CD34	CST3569	100×	Blood endothelial cells
D2-40 (Podoplanin)	Ab77854	100×	Lymphatic endothelial cells
PD-1	CST13684	50×	Programmed cell death-Ligand 1

method. The TQB2450 and combination treatment groups were compared using the log-rank test. The disease control rate for each group was compared using Pearson χ^2 or Fisher's exact test when appropriate. PD-L1 levels and clinicopathologic data were analyzed using the Fisher's exact test[23]. Comparisons between groups were performed using ANOVA. Differences in the survival rates were compared using the log-rank method. A $P < 0.05$ was considered statistically significant.

Results

PD-L1 cut-off values determined in different cells and the correlation between PD-L1 expressed in different cells

Between 8 August 2018 and 29 March 2021, 101 patients were enrolled in this trial. Eligible pathologic sections were obtained from 75 patients with consent (**Figure 2**), including primary lesions, liver metastases, adrenal metastases, and metastatic lymph nodes. The average level of PD-L1 expression in TCs, BECs, and LECs were (median ± quartile) 26.97 ± 34.78%, 17.32 ± 29.62%, and 13.78 ± 30.12%, respectively. The expression value of PD-L1 was used as a variable in the ROC curve analysis to estimate the sensitivity, specificity, and areas under the ROC curves. The optimal cut-off value was obtained from the maximum value of Youden's index (sensitivity + specificity − 1). As shown in **Figure 3**, the cut-off values were 2.65%, 4.59%, and 2.75%, respectively. A positive correlation was observed between the rate of PD-L1 expression in BECs and LECs (r = 0.411, $P = 0.000$).

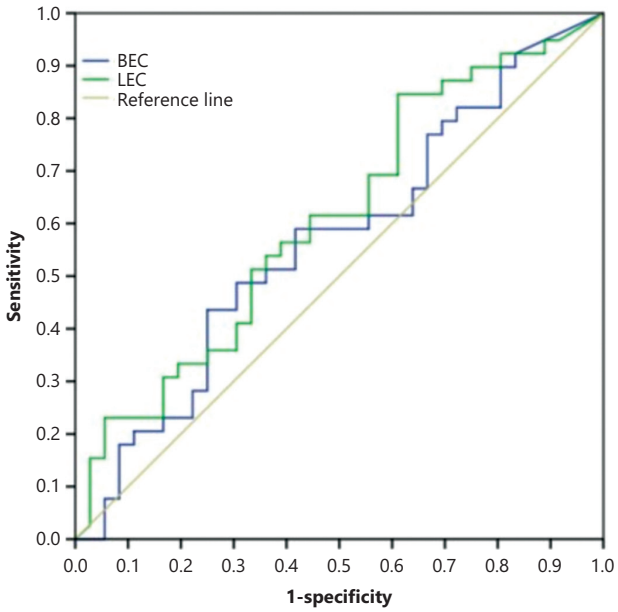

Figure 3 Receiver operating characteristic curve of PD-L1 expression in the blood and lymphatic endothelial cells.

Correlation between PD-L1 expression and short-term efficacy and PFS

Seventy-five patients were randomly assigned to 3 groups (TQB2450, 23 patients; TQB2450 plus anlotinib 10 mg, 25 patients; and TQB2450 plus anlotinib 12 mg, 27 patients).

Baseline characteristics were generally well-balanced among the three treatment arms (**Table 2**). The median follow-up period was 452 days [95% confidence interval (CI), 305–598]. Anlotinib plus TQB2450 demonstrated promising

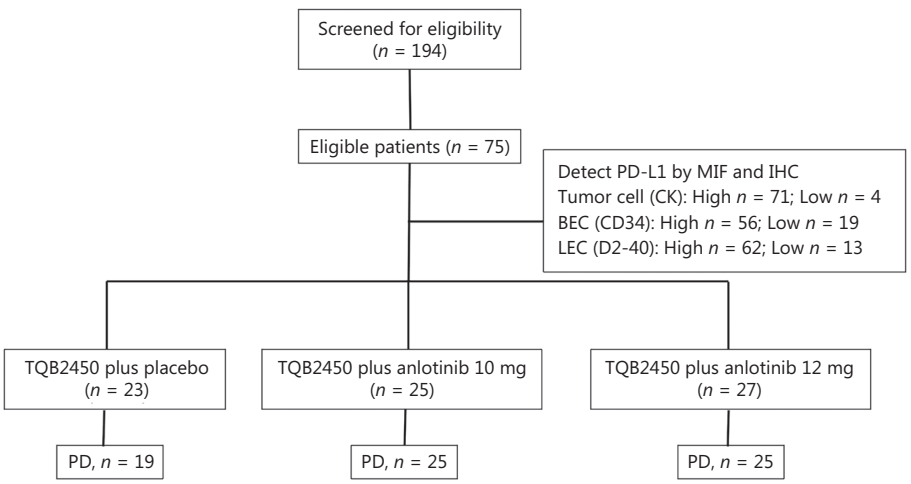

Figure 2 Study flowchart: full analysis set (FAS).

Table 2 Baseline clinicopathologic features on 3 therapeutic arms

Characteristics	T-QB2450 (*n* = 23)	T-QB2450 + anlotinib 10 mg (*n* = 25)	T-QB2450 + anlotinib 12 mg (*n* = 27)	*P* value
Median age (range), y	60	62	63	
Gender, *n* (%)				0.262
Male	21 (91.3)	19 (76)	20 (74)	
Female	2 (8.7)	6 (24)	7 (26)	
Age group, *n* (%)				0.328
≤ 60 y	13 (56.5)	9 (36)	11 (41)	
> 60 y	10 (43.5)	16 (64)	16 (59)	
Tobacco use history, *n* (%)				0.938
Never	5 (21.7)	7 (26)	7 (26)	
Current	3 (13.0)	5 (19)	3 (11)	
Previous	12 (52.2)	9 (33)	13 (48)	
Unknown	3 (13.0)	4 (15)	4 (15)	
Pathologic type, *n* (%)				0.119
Squamous cell carcinoma	8 (34.8)	7 (28)	5 (19)	
Adenocarcinoma	15 (65.2)	14 (56)	21 (77)	
Others	0 (0)	4 (16)	1 (4)	
TNM stage, *n* (%)				0.568
IVA	10 (43.5)	10 (40)	8 (30)	
IVB	13 (56.5)	15 (60)	19 (70)	

antitumor activity in patients with advanced NSCLC. The PFS was significantly longer than TQB2450 plus placebo, which is consistent with the overall results of this study[23]. The median progression-free survival (mPFS) was 61, 161, and 194 days in the TQB2450 plus placebo, TQB2450 plus anlotinib (10 mg), and TQB2450 plus anlotinib (12 mg) groups, respectively [$HR_{10\ mg}$ = 0.390 (95% CI, 0.201–0.756), *P* = 0.005; $HR_{12\ mg}$ = 0.397 (95% CI, 0.208–0.756), *P* = 0.005]. Fifty-eight patients had high PD-L1 protein expression in TCs (29.69 ± 19.82%) and LECs [28.49% ± 24.11% (TC^HLEC^H)]. Thirteen patients presented with high PD-L1 protein expression in TCs (37.25 ± 27.70%) and low/no PD-L1 expression in LECs [0.96 ± 0.85% (TC^HLEC^L)]. Fifty-three patients had high PD-L1 protein expression in TCs and BECs [34.54 ± 18.19% and 28.58% ± 16.92% (TC^HBEC^H)]. Eighteen patients had high PD-L1 protein expression in TCs (17.97 ± 11.85%) and lor/no PD-L1 expression in BECs [1.48 ± 1.41% (TC^HBEC^L)]. Furthermore, PD-L1 protein expression in TCs was low (1.32 ± 0.896%) in 4 patients ($TC^LBEC/LEC^{H\&L}$). The mPFS was significantly prolonged for the anlotinib-combined treatment compared to the TQ-B2450 only group among the TC^HLEC^H groups [82 days for 0 mg, 159 days for 10 mg, 209 days for 12 mg; $HR_{10\ mg}$ = 0.445 (95% CI, 0.210–0.939); $HR_{12\ mg}$ = 0.369 (95% CI, 0.174–0.784)]. The DCR was also significantly improved [12 mg *vs.* 10 mg *vs.* 0 mg (95% *vs.* 100% *vs.* 73%), respectively; *P* = 0.015]. Similar results were obtained for the TC^HBEC^H patients (**Table 3**, **Figure 4**). Nevertheless, no significant difference was detected in the DCR and mPFS between TQB2450 alone and combined therapy in the TC^HLEC^L and TC^HBEC^L groups (**Table 3**). Moreover, no significant differences in mPFS were detected between the TC^HLEC^H [186 (95% CI, 133–238) days] and TC^HLEC^L groups [126 (95% CI, 118–133) days; HR = 0.908, (95% CI, 0.403–2.048)]. Similar results were obtained in the TQ-B2450 alone group [82 (95% CI, 49–114) *vs.* 55 (95% CI 14–95); HR = 1.410 (95% CI, 0.485–4.904)] and in BECs when grouped according to PD-L1 expression. Notably, patients with BEC^H exhibited poorer efficacy (88 *vs.* 158 days, **Figure S1**) in the TQB2450 monotherapy group.

Table 3 Efficacy in various PD-L1 expression types

Groups	T^H + LEC^H			T^H + LEC^L			T^L + LEC^L		
	TQB2450 (*n* = 15)	TQB2450 + An 10 mg (*n* = 21)	TQB2450 + An 12 mg (*n* = 22)	TQB2450 (*n* = 5)	TQB2450 + An 10 mg (*n* = 3)	TQB2450 + An 12 mg (*n* = 5)	TQB2450 (*n* = 3)	TQB2450 + An 10 mg (*n* = 1)	TQB2450 + An 12 mg (*n* = 0)
DCR (%)	73 (11/15)	100 (21/21)	95 (21/22)	100 (5/5)	100 (3/3)	100 (5/5)	67 (2/3)	100 (1/1)	
P value	-	0.012*	0.053#	-	-	-	-	-	-
			0.323†			-			-
PFS (days) (95% CI)	82 (49–114)	159 (106–211)	209 (143–274)	55 (14–95)	452 (338–682)	126 (53–198)	42	168	
HR (95% CI)	-	0.445* (0.210–0.939)	0.369# (0.174–0.784)	-	0.115* (0.011–1.144)	0.425# (0.107–1.689)	-	-	-
			0.832† (0.450–1.539)			3.476† (0.366–32.996)			-
P value	-	0.034*	0.009#	-	0.065*	0.224#	-	0.725*	-
			0.558†			0.278†			-
Groups	T^H + BEC^H			T^H + BEC^L			T^L + BEC^L		
	TQB2450 (*n* = 14)	TQB2450 + An 10 mg (*n* = 18)	TQB2450 + An 12 mg (*n* = 21)	TQB2450 (*n* = 6)	TQB2450 + An 10 mg (*n* = 6)	TQB2450 + An 12 mg (*n* = 6)	TQB2450 (*n* = 3)	TQB2450 + An 10 mg (*n* = 1)	TQB2450 + An 12 mg (*n* = 0)
DCR (%)	71 (10/14)	100 (18/18)	95 (20/21)	100 (6/6)	100 (6/6)	100 (6/6)	67 (2/3)	100 (1/1)	-
P value	-	0.015*	0.049#	-	-	-	-	-	-
			0.348†			-			-
PFS (days) (95% CI)	41 (2–79)	161 (104–217)	209 (145–272)	55 (24–85)	41 (30–51)	126 (119–132)	42	168	-
HR (95% CI)	-	0.340* (0.156–0.742)	0.340# (0.159–0.727)	-	0.631* (0.164–2.31)	0.519# (0.142–1.889)	-	-	-
			0.995† (0.520–1.904)			0.838† (0.238–2.953)			-
P value	-	0.007*	0.005#	-	0.503*	0.320#	-	0.725*	-
			0.987†			0.784†			-

*TQB2450 + An 10 mg *vs.* TQB2450; #TQB2450 + An 12 mg *vs.* TQB2450; †TQB2450 + An 12 mg *vs.* TQB2450 + An 10 mg. An, anlotinib; CI, confidence interval; DCR, disease control rate; HR, hazard ratio; PFS, progress free survival; T^H, PD-L1-positivity on cells ≥ 2.65%; T^L, PD-L1-positivity on cells < 2.65%; LEC^H, PD-L1-positivity on lymphatic endothelial cells ≥ 2.75%; LEC^L, PD-L1-positivity on lymphatic endothelial cells < 2.75%; BEC^H, PD-L1-positivity on vascular endothelial cells ≥ 4.59%; BEC^L, PD-L1-positivity on vascular endothelial cells < 4.59%.

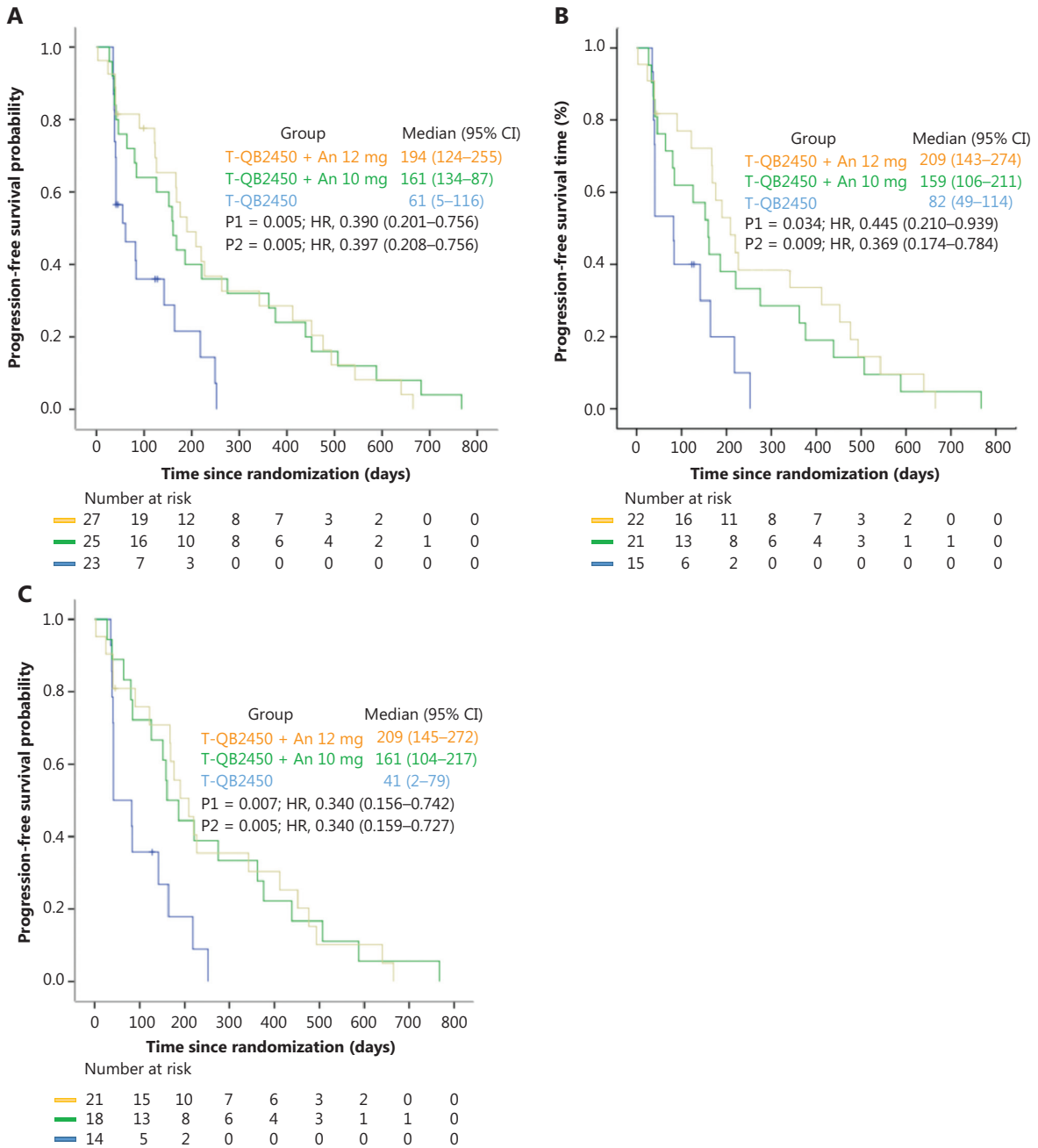

Figure 4 Kaplan-Meier survival curves of progression-free survival (PFS) in the TQB2450 and TQB2450 + anlotinib groups. (A) For all patients in the study (75 cases). (B) For patients with high PD-L1 protein expression in LECs and TCs (58 cases). (C) For patients with high PD-L1 protein expression in BECs and TCs (53 cases). LECs, lymphatic endothelial cells; TCs, tumor cells; BECs, blood endothelial cells; An, anlotinib. P1: TQB2450 *vs.* TQB2450 + An 10 mg, P2: TQB2450 *vs.* TQB2450 + An 12 mg.

Discussion

In recent years immunotherapy using ICIs has gained immense attention in cancer therapy[24] because ICIs activate effector T cells and enhance infiltration[25]. However, the efficacy of single-agent ICIs is limited because the tortuous tumor vasculature prevents immune cells from infiltrating the tumors. Anti-angiogenic therapy is an effective approach to improve the TME by targeting VEGF and VEGFR, which promotes normalization of the tumor vascular system and the delivery of T

cells as well as immune effector molecules[26]. Hence, immunotherapy in combination with anti-angiogenic drugs enhances treatment efficacy. Nevertheless, a significant number of patients have poor treatment efficacy. Therefore, it is necessary to elucidate the underlying causes and screen the population that will benefit from this therapy. The IMmotion150 study reported that the addition of bevacizumab to atezolizumab in first-line therapy was associated with benefits among patients with higher levels of PD-L1 expression who received a combination of the four drugs. In contrast, the JAVELIN Renal 101 study[27], which was presented at the 2019 annual ASCO meeting, concluded that the level of PD-L1 expression did not determine PFS benefit. Therefore, biomarkers that accurately predict the efficacy of immunotherapy plus anti-angiogenic therapy warrant clarification.

In contrast to the literature that focuses on PD-L1 in cancer cells, a few studies have shown that the expression of PD-L1 on tumor-associated micro-conduit endothelial cells limit the infiltration and activity of Teff cells and enhance infiltration of Treg cells[28] by binding with PD-1 on T cells, which leads to the immune evacuation of tumor cells and a poor prognosis[29]. Therefore, some effort has been made in assuaging and even removing the barrier imposed by high PD-L1 expression on BECs to improve immune therapeutic efficacy.

As reported by Su[30], normalization of vasculature enhances $CD8^+$ T cell infiltration in tumors. However, given the transient nature and uncertainty of vascular normalization the clinical significance is negligible[31]. A more practical approach was elucidated in our previous study. Specifically, low-dose anlotinib downregulates PD-L1 in BECs and $CD8^+$ T cells easily enter tumors, which achieves longer function.

Notably, the endothelium of lymphatic capillary responds more actively to the microenvironment of local lesions compared to a passive response to vascular vessels[32]. Tumor-associated LECs express higher PD-L1 than LECs in naïve animal skin, while tumor-associated BECs constitutively express PD-L1 independently in the graft-tumor context. Additionally, the study gained insight regarding the mechanism underlying inhibition of PD-L1 in LECs among $CD8^+$ T cells, i.e., $CD8^+$ T cells must be proximal to lymphatic vessels for dependent crosstalk between the IFN-γ secreted by infiltrating T cells and PD-L1 in LECs. Accordingly, $CD8^+$ T cells are closely proximal to the lymphatic vasculature in tumors. This finding indicates that PD-L1 in LECs, unlike the immune barrier for Teff cells in BECs, exerts local direct suppressive function in $CD8^+$ T cells existing inside tumors *via* binding. However, no practical clinical countermeasures have been recommended to date which decrease PD-L1 in the endothelium of micro-conduits to remove the immune barrier and diminish direct immune suppression. Fortunately, anlotinib enhances the infiltration and activity of $CD8^+$ T cells in tumors due to its direct downregulation of PD-L1 in BECs by inhibition of VEGF-PI3K-AKT signaling[11]. Similarly, we also confirmed that anlotinib suppresses VEGF signaling in LECs[33] involved in the regulation of PD-L1 expression. Taken together, anlotinib downregulates PD-L1 expression in tumor-associated micro-conduits to reinforce the infiltration of active $CD8^+$ T cells. Therefore, this study focused on the connection between PD-L1 expression in tumor-associated LECs and BECs, and the efficacy of the combined therapy of PD-L1 antibody and anlotinib.

To ensure the expression of PD-L1 in LECs and BECs, double staining (CD34 and D2-40) was performed. CD34 and D2-40 are predominantly expressed in the vascular and lymphatic endothelium, respectively. Therefore, there is a consensus that D2-40 signifies the lymphatic endothelium and CD34 represents the vascular endothelium, especially since only one marker stains positive. Clearly, vascular or lymphatic conduits can be identified using hematoxylin and eosin staining. However, the majority of tissues obtained by biopsy were too small after immunohistochemical staining to confirm the histologic types of NSCLC. As expected, some conduits with D2-40 and PD-L1 staining presented with high PD-L1 expression on LECs (**Figure 5A**), while other vessels, such as venules, revealed double $D2\text{-}40^+$ and $CD34^+$ staining (**Figure 5B, C**). D2-40 stain is positive in some vascular vessels [high endothelial venules (HEVs)] and the podoplanin (D24-0) maintains high endothelial venule integrity[34]. Accordingly, the $CD34^+$ BEC shedding band was demonstrated in the area without D2-40 stain in the vessel wall and formed debris compound in channels due to the loss of maintenance by podoplanin for integrity of endothelial cells (**Figure 5B**). Therefore, vessels in **Figure 5B and C** could represent HEVs. All immunofluorescence staining results were confirmed using immunohistochemistry staining (**Figure S2**). In HEVs, lymphocytes transmigrate through endothelial cells into adjacent tumor tissue[35,36] and PD-L1 in BECs could be a barrier that inactivate effector lymphocytes, baffle the migration, and cause apoptosis. Relying on the above detection platform, we present the relevant findings based on a phase Ib clinical study of TQB2450 in combination with anlotinib in patients with NSCLC.

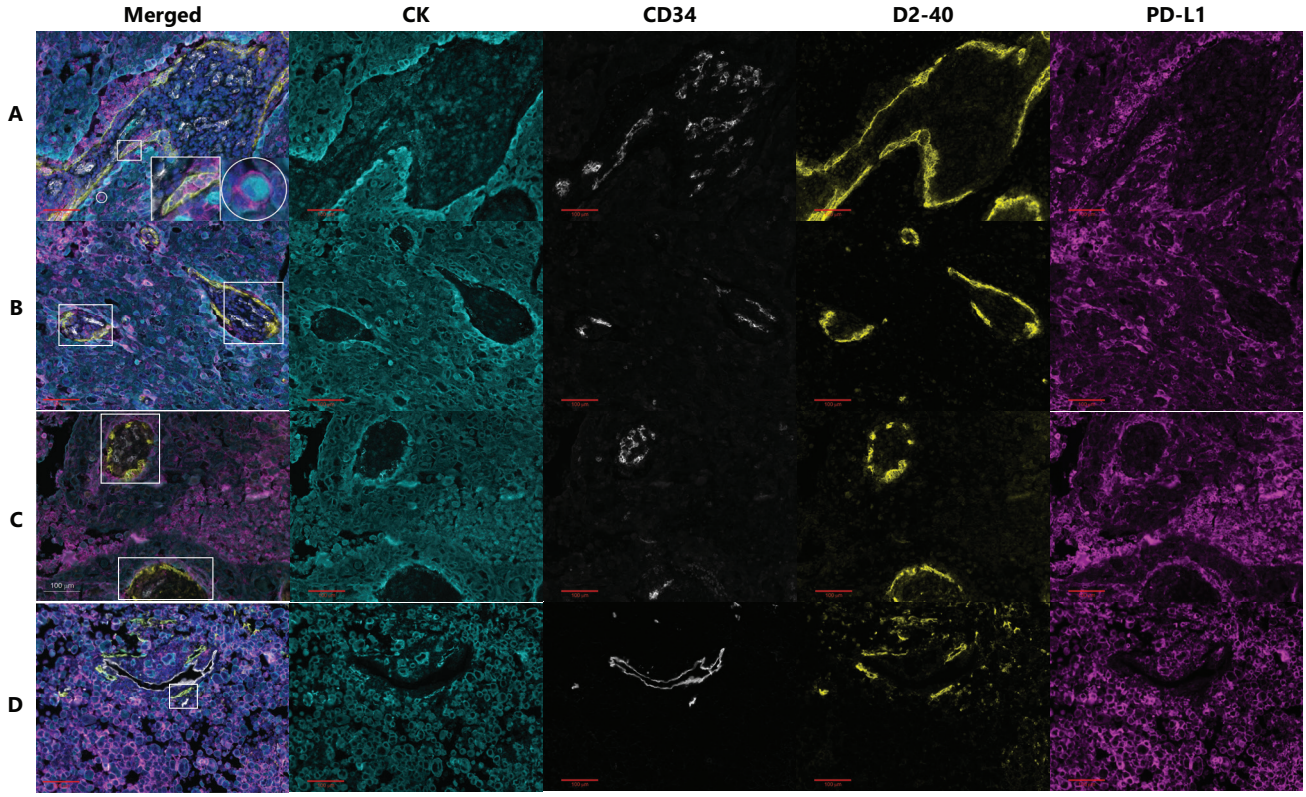

Figure 5 Expression of PD-L1 in different cells (200×). Staining: PD-L1, magenta; D2-40, yellow; CK, cyan; DAPI, blue; CD34, white. (A) Expression of PD-L1 protein in TCs and LECs. (B) Expression of PD-L1 protein in BECs with or without D2-40 staining, shed BECs without D2-40 staining (with some debris compound in the channel), and "bare wall" of venule without a BEC cover. (C) Expression of PD-L1 protein in TCs and BECs with CD34 and D2-40 staining. (D) Expression of PD-L1 protein in TCs, and no expression in LECs and BECs. TCs, tumor cells (CK^+); LECs, lymphatic endothelial cells ($D2\text{-}40^+$); BECs, blood endothelial cells ($CD34^+$). Scale bar = 100 μm.

In the present study patients with high PD-L1 expression in TCs, LECs, and BECs revealed poor short-term efficacy for single immunotherapy, a better DCR for anti-PD-L1 plus anlotinib treatment, and a longer mPFS for the TQB2450 + anlotinib (10 mg) and TQB2450 + anlotinib (12 mg) groups than the TQB2450 alone group (**Table 3**). These T^HBEC^H tumors can be classified as an immune exempt type, indicating that abundant immune cells may be amassed in the microvasculature around the tumor. However, the PD-L1 protein in BECs prevent $CD8^+$ lymphocytes from penetrating into the lesion. In the TQB2450 monotherapy group, patients with BEC^H exhibited poorer efficacy than patients with BEC^L (**Figure S1**), further validating our hypothesis that high expression of PD-L1 in BECs can form an immunologic barrier, which effects the efficacy of immunotherapy. In contrast, among T^HLEC^H tumors, high PD-L1 in LECs within peritumoral stroma inactivated proximal Teff cells in the tumor active zone. However, such a negative effect could also be offset by anlotinib *via* downregulation of PD-L1 in endothelium. Additionally, downregulation of PD-L1 in endothelial cells was achieved by a relatively low concentration of 0.1 μM anlotinib (48.036 ng/mL) but did not make an impact on tumor cells[9] and was approximately equal to the stable blood concentration of anlotinib (10 mg) administered in the ALTER0303 trial pharmacokinetic study (data not published). The average daily exposed dose of anlotinib was only 10.2 mg among two therapeutic arms[17], which provides the possibility of accelerating the efficacy of anti-PD-L1 by decreasing the baffling infiltration of $CD8^+$ cells by $PD\text{-}L1^+$BECs and inactivating $CD8^+$ T cells proximal to the lymphatic vasculature by $PD\text{-}L1^+$LECs. The PD-L1 in tumor cells, as the target of antibody, were still retained and a dose-dependent systemic adverse effect was assuaged. The direct anti-angiogenic and anti-tumoral functions of anlotinib may help obtain efficacy. However, acceleration of immunotherapy likely accounted for the robust effect

because 3.1-month mPFS was gained by anlotinib alone (daily dose = 12 mg) in advanced NSCLC[37].

Nevertheless, cases with high PD-L1 expression in TCs but not BECs indicate a weaker immune barrier and a longer mPFS was not achieved in the combined therapy group compared to the TQB2450 alone group (**Table 3**).

In summary, this study demonstrated that PD-L1 in LECs and BECs inside tumors can screen the population for the benefits of immunotherapy plus anlotinib and we believe that PD-L1 in LECs and BECs could be another ideal marker in addition to PD-L1 in TCs that was proven to predict efficacy in our study[17]. However, further studies are warranted to explore these effects.

This study had some limitations. First, owing to ethical reasons and consent rejection, it was difficult to obtain tissues dynamically and monitor changes in PD-L1 protein expression in microvessel endothelial cells during treatment. Second, because many specimens were obtained by puncture biopsy, the amount of available tissue was insufficient to create an animal model of patient-derived tumor xenograft (PDX) to further explore the mechanism of action underlying PD-L1 protein in microvessel endothelial cells induced by tumors and to prepare a special strain of BECs and LECs. Third, because only 75 Chinese participants were enrolled, we could not extend our conclusion to other populations. Thus, further long-term and large-scale observations are required to confirm this hypothesis.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conceptualization: Kai Li and Baohui Han.
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Technical support for immunofluorescence staining: provided by Ruili Wang.
All the authors have reviewed and approved the submitted manuscript.

Data availability statement

The authors confirm that data supporting the findings of this study are available in the article.

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