Prognostic Impact of CD204-Positive Macrophages in Lung Squamous Cell Carcinoma

Possible Contribution of Cd204-Positive Macrophages to the Tumor-Promoting Microenvironment

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Introduction: Tumor-associated macrophages (TAMs) are recruited into cancer-induced stroma and produce a specific microenvironment for cancer progression. CD204 (+) TAMs are reportedly related to tumor progression and clinical outcome in some tumors. The aim of this study was to clarify the correlation between CD204 (+) TAMs and the clinicopathological features of lung squamous cell carcinoma.

Methods: We investigated the relationships between the numbers of CD204 (+) TAMs and clinicopathological factors, microvessel density, and the numbers of Foxp3 (+) lymphocytes in 208 consecutively resected cases. We also examined the relationships between the numbers of CD204 (+) TAMs and the expression levels of cytokines involved in the migration and differentiation of CD204 (+) TAMs.

Results: A high number of CD204 (+) TAMs in the stroma was significantly correlated with an advanced p-stage, T factor, N factor, and the presence of vascular and pleural invasion. A high number of CD204 (+) TAMs in the stroma was also a significant prognostic factor for all p-stages and p-stage I. Moreover, the numbers of CD204 (+) TAMs were correlated with the microvessel density and the numbers of Foxp3 (+) lymphocytes. A high number of CD204 (+) TAMs was strongly correlated with the tissue expression level of monocyte chemoattractant protein-1. CD204 (+) TAMs were shown to be significant independent prognostic factors in a multivariate analysis.

Conclusions: CD204 (+) TAMs were an independent prognostic factor in lung squamous cell carcinoma. CD204 (+) TAMs, along with other tumor-promoting stromal cells such as regulatory T cells and endothelial cells, may create tumor-promoting microenvironments.

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The tumor microenvironment is composed of not only tumor cells, but also stromal cells including macrophages, lymphocytes, fibroblasts, and endothelial cells. Stromal cells are known to interact with cancer cells and to produce a specific microenvironment capable of influencing tumor progression.[1] In the host immune system in cancer tissue, tumor-associated macrophages (TAMs) as well as cancer-associated fibroblasts are important components of the tumor microenvironment, and some kinds of macrophages are known to act on tumor progression.[2] Recent immunological studies have identified two different functions of polarized macrophage activation, as exhibited by classically activated (M1) and alternatively activated (M2) macrophage phenotypes.[3,4] These subpopulations of macrophages have different receptor expression patterns and different cytokine and chemokine productions. M1-polarized macrophages have an interleukin (IL)-12^{high}, IL-23^{high}, IL-10^{low} phenotype and defend the body against pathogens and tumor cells by inducing interferon-gamma and producing tumor necrosis factor α and nitric oxide. M1-polarized macrophages reportedly play a role in tumor suppression. However, M2-polarized macrophages have an IL-12^{low}, IL-23^{low}, IL-10 ^{high} phenotype and have high expression levels of class A scavenger receptor (CD204) and mannose receptor (CD163).[5–7] M2-polarized macrophages play a role in tumor-supportive functions, such as tumorigenesis, angiogenesis, matrix remodeling, and immune-suppression.[8] Recent studies have reported that the number of CD204-positive TAMs within a primary tumor is related to tumor progression and outcome in glioma, ovarian epithelial tumors, pancreatic cancer, and lung adenocarcinoma.[6,9–11]

Although squamous cell carcinoma of the lung is second only to adenocarcinoma of the lung, its treatmentt has not yet been sufficiently effective. Recently, the development of

cancer therapy targeting cancer stromal cells has been proposed.[12] In the current study, we examined whether the numbers of CD204 (+) TAMs recruited into the cancer tissue are related to clinicopathological factors of lung squamous cell carcinoma. Furthermore, we examined the matching correlations between CD204 (+) TAMs and other types of cancer stromal cells, including regulatory T cells and endothelial cells.

MATERIALS AND METHODS

Patients

Between January 2000 and December 2006, a total of 255 patients with lung squamous cell carcinoma underwent surgery with curative intent at our hospital. We excluded 47 cases with poor-quality surgical specimens, and the remaining 208 cases were included in this study. The median follow-up period was 5.7 years.

Histopathology Studies

Surgical specimens were fixed in 10% formalin or methanol and embedded in paraffin. Sections that are 4-μm–thick were stained using the hematoxylin and eosin method. Vascular invasion and pleural invasion were evaluated using the Verhoeff-van-Gieson method. The histologic diagnoses were based on the third revised World Health Organization histologic classification. Disease stages were based on the 7th edition of TNM classification.

Evaluation of Clinicopathological Factors

The clinical characteristics were retrieved from the available clinical records. The following clinicopathological factors were investigated retrospectively to assess their impact on survival: age, sex, smoking history, pathologic stage, pathologic T status, pathologic nodal involvement, lymphatic permeation, vascular invasion, and pleural invasion.

Antibodies and Immunohistochemistry

The slides were deparaffinized with xylene, rehydrated, and antigen-retrieved in a microwave oven for 20 minutes. After the inhibition of endogenous peroxidase activity, individual slides were then incubated overnight at 4°C with mouse antihuman CD204 antibody (Scavenger Receptor class A-E5; Transgenic, Japan) at a final dilution of 1:400, mouse antihuman CD34 antibody (QBEND/10; Acris Antibodies, Herford, Germany) at a final dilution of 1:400, and mouse monoclonal antihuman Foxp3 antibody (236A/E7; Abcam, Japan) at a final dilution of 1:150. The slides were then incubated with EnVision (Dako, Denmark), and the color reaction was developed in 2% 3, 3-diaminobenzidine in 50 mM Tris-buffer (pH7.6) containing 0.3% hydrogen peroxidase. Finally, the sections were counterstained with Meyer hematoxylin.

Evaluation of Immunohistochemistry

Two pathologists (S.H. and G.I.) selected a hot spot within a section and counted the CD204 (+) TAMs in the cancer stroma and nest in five high-power microscopic fields (×400; 0.0625 mm^2). The average counts were recorded and cases were divided into two groups with low and high numbers of CD204 (+) TAMs according to the median value. The absolute number of Foxp3-positive lymphocytes in the stroma, was counted in five randomly selected high-power microscopic fields (×400; 0.0625 mm^2), and the average counts were recorded. As for the microvessel density (MVD), the five most vascular areas (hot spots) in the invasive foci within a section were selected, and vessels labeled with anti-CD34 monoclonal antibody were counted in five high-power microscopic fields (×400; 0.0625 mm^2). The average counts were recorded as the MVD. In these studies, we selected areas in the central area within a cancer tissue, and necrotic areas were excluded.

Tissue Samples, RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA was extracted from 13 lung squamous cell carcinoma cases.. Samples of both cancer tissue and noncancerous tissue were collected and immediately homogenized in QIAzol Lysis reagent (QIAGEN, CA) with Tissue Lyser II (QIAGEN) and stored at −80°C until use. The total RNA was isolated from the tissues using a QIAshredderTM (250) (QIAGEN) and an RNeasy Mini Kit (250) (QIAGEN) according to the manufacturer's instructions. The RNA was reverse transcribed to synthesize cDNA using a primerscript RT reagent kit (Takara Biochemicals, Osaka, Japan). To quantitatively compare the mRNA levels of each cytokine, we performed a real-time polymerase chain reaction using SYBR Premix Ex TaqII (Takara) with the Smart Cycler II (Takara). The sense and antisense primers used for quantitative amplification of the cytokine mRNAs and for amplification of glyceraldehyde-3-phosphate dehydrogenase as an internal control are shown in (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A347). The amount of template cDNA was expressed by the threshold cycles (G), determined from the amplification curve (exponential curve), and the threshold level for the detection of the polymerase chain reaction product. The expression level of each gene was reported as the ratio of its expression to the level of glyceraldehyde-3-phosphate dehydrogenase gene expression in the same sample. The ratio between the level of cytokine expression in the cancer tissue to the level of expression in the noncancerous tissue was calculated for each case. The median number of CD204 (+) TAMs was used to divide the cases into high and low CD204 (+) TAM groups.

Statistical Analysis

The distributions of CD204 (+) TAMs in the stroma and MVD and Foxp3-positive lymphocytes were tested for correlations by calculating the Spearman rank correlation coefficients. Overall survival (OS) was measured from the date of surgery until the date of death from any cause or to the date on which the patient was last known to be alive. Recurrence-free survival (RFS) was measured from the date of surgery until the date of recurrence or until the date the patient was last known to be disease free. Survival curves were estimated using the Kaplan–Meier method, and differences in survival

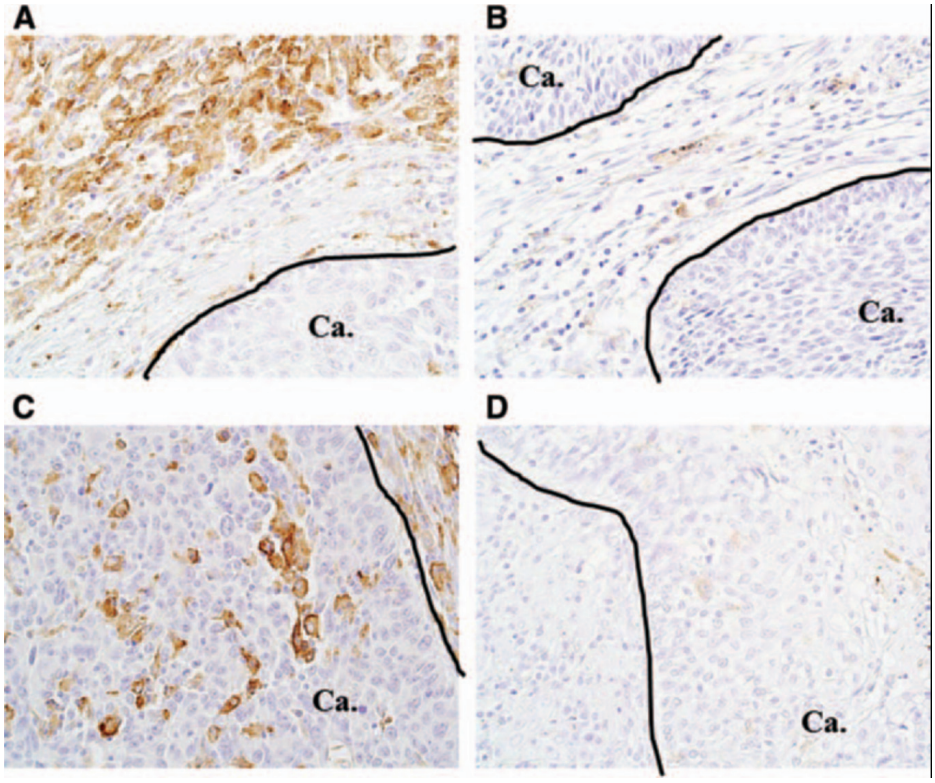

FIGURE 1. *A,* Immunohistochemical staining of squamous cell carcinoma with antihuman CD204 antibody for tumor-associated macrophages in the stroma and nest. Cases with a high number of CD204 (+) TAMs in the stroma, and *B,* a low number of CD204 (+) TAMs in the stroma. *C,* Cases with a high number of CD204 (+) TAMs in the nest, and *D,* a low number of CD204 (+) TAMs in the nest. TAMs, tumor-associated macrophages.

were compared using the log-rank test. A p value less than 0.05 was considered significant. Statistical analysis software (SPSS, version II) was used to perform the analyses.

RESULTS

Correlations between the numbers of CD204 (+) TAMs in the cancer stroma and nest and clinicopathological factors.

A representative case of immunohistochemical staining results for CD204 is shown in Fig. 1. All the patients were classified into two groups according to the median values: 30 for the stroma, and nine for the nest. A high CD204 (+) TAM count in the stroma was significantly correlated with the pathologic stage, pathologic T status, pathologic nodal involvement, and vascular and pleural invasion (Table 1). However, lymphatic permeation was significantly less frequent in the group with a high CD204 (+) TAM count in the nest, compared with those with a low CD204 (+) TAM count in the nest (Supplemental Table 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A348).

Evaluation of CD204 (+) TAMs in Stroma and Nest as Prognostic Factors

The OS and RFS were significantly shorter in the group with a high CD204 (+) TAM count in the stroma compared with the group with a low CD204 (+) TAM count in the stroma, for all stages (p = 0.0005 and p = 0.0002, respectively) (Fig. 2A). In the p-stage I patients, the OS and RFS were also significantly shorter in the group with a high CD204 (+) TAM count in the stroma, compared with the group with a low CD204 (+) TAM count in the stroma (p = 0.0154 and p = 0.0071) (Fig. 2*B*). A high CD204 (+) TAM count in the stroma was marginally related to the OS and RFS among the p-stage II patients (Fig. 2*C*), but no significant relation with prognosis was seen among the p-stage III patients (Fig. 2*D*). In contrast, no relationship was seen between a high CD204 (+) TAM count in the nest and the prognosis (data not shown).

Correlations among the numbers of CD204 (+) TAMs in the stroma, Foxp3-positive lymphocytes, and the microvessel density Recent studies showed that CD204 (+) TAMs contribute to the development of neovascularization and immunesuppression. We examined the numbers of Foxp3 (+) lymphocytes (regulatory T cells) and the MVD in all the cases (Fig. 3A–D). The numbers of CD204 (+) TAMs in the stroma were strongly correlated with the MVD ($p < 0.001$; $r_s = 0.471$) and were moderately correlated with the numbers of Foxp3-positive lymphocytes ($p = 0.034$; $r_s = 0.147$) (Fig. 3*E* and *F*).

Correlations between the numbers of CD204 (+) TAMs in the stroma and cytokine expression in the cancer tissues. We examined the expressions of factors involved in the recruitment of TAMs, regulatory T cells, and endothelial cells. The correlations between MVD monocyte chemoattractant protein-1 (MCP-1), IL-10, transforming growth factor β, and vascular endothelial growth factor (VEGF) expression and the numbers of CD204 (+) TAMs in the tumor tissue specimens (n = 13) were analyzed. The ratios of MCP-1 expression in the cancer tissues to their levels of expression in noncancerous tissues were significantly higher in the CD204 high group (p = 0.032) (Fig. 4A). The ratios of IL-10 and transforming growth factor β expressions in the cancer tissues to their levels of expression in noncancerous tissue were marginal higher in the CD204 high group (p = 0.063 and p = 0.086,

TABLE 1. Relationship between CD204 (+) Tumor-Associated Macrophages in Stroma and Clinical Features

Variables	CD204 Low (n = 93)	CD204 High (n = 115)	p^a
Sex			
Male	85	103	
Female	8	12	0.6556
Age (yr)			
<70	49	63	
70≤	44	52	0.7632
Smoking history			
Never smoker	3	10	
Smoker	90	105	0.1052
Surgical procedures			
Lobectomy + segmentectomy	82	94	
Pneumonectomy	11	21	0.2475
Pathological stage			
Stage I	56	48	
Stage II–IIIA	37	67	0.0081[b]
T states			
T1	34	24	
T2–4	59	91	0.0121[b]
Lymph node metastasis			
N(−)	65	60	
N(+)	28	55	0.0095[b]
Vascular invasion			
V(−)	37	28	
V(+)	56	87	0.0169[b]
Lymphatic permeation			
Ly(−)	71	87	
Ly(+)	22	28	0.9076
Pleural invasion			
P(−)	66	68	
P(+)	27	47	0.018[b]

[a]Pearson 2 test.
[b]p <0.05.

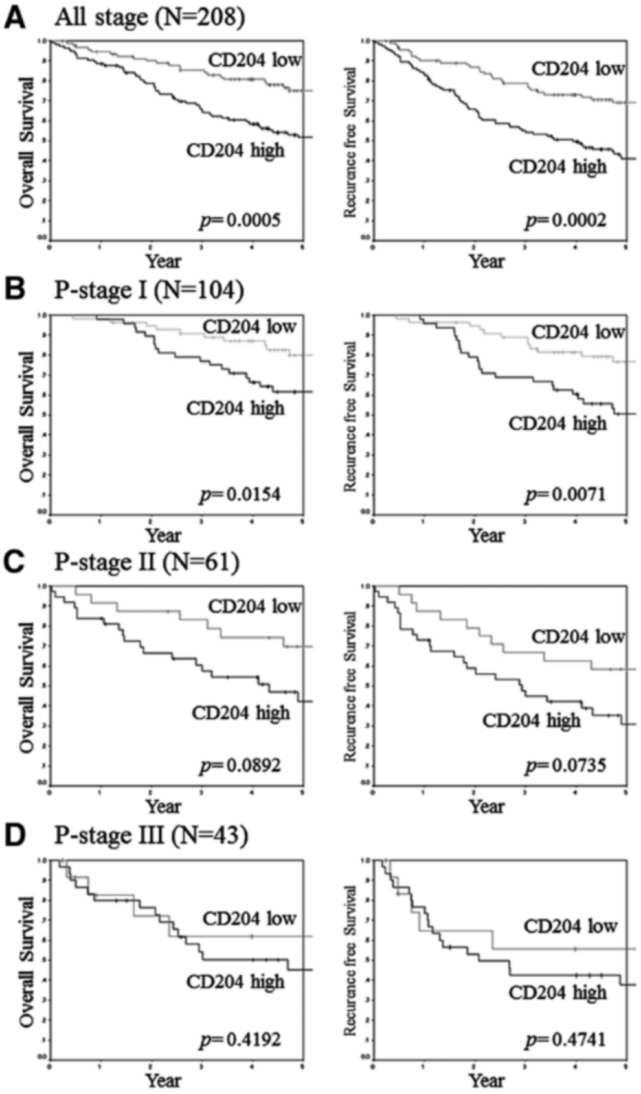

FIGURE 2. Kaplan–Meier analysis stratified according to a high or low number of CD204 (+) TAMs in the stroma. The Kaplan–Meier analysis for overall survival and recurrence-free survival according to the numbers of CD204 (+) TAMs in the stroma are shown for all stages *A*, for p-stage I, *B*, for p-stage II, *C*, and for p-stage III, *D*,. TAMs, tumor-associated macrophages.

respectively) (Fig. 4*B*, *C*). The difference in the expression of VEGF between the two groups was not significant (Fig. 4*D*).

Univariate and Multivariate Analyses of Factors Associated with Prognosis

A univariate analysis identified four significant risk factors for OS: CD204 (+) TAMs in the stroma, p-T status, vessel invasion, and pleural invasion (Table 2). In a multivariate analysis, the presence of CD204 (+) TAMs and the p-T status were shown to be statistically significant independent predictors of the OS (Table 3).

DISCUSSION

In this study, we first showed that the numbers of CD204 (+) TAMs in the tumor stroma, but not in the tumor nest, were correlated with several conventional prognostic factors in squamous cell carcinoma of the lung. Furthermore, we showed that the cases with higher numbers of CD204 (+) TAMs in the stroma had a poor clinical outcome, particularly in the early stages. These results were consisted with previous reports in other types of malignancies, such as glioma, ovarian epithelial tumors, pancreatic cancer, and lung adenocarcinoma.[6,9–11] Moreover, the numbers of CD204 (+) TAMs were related to the numbers of Foxp3 (+) lymphocytes and the MVD. These data suggested that in squamous cell carcinoma tissue, CD204 (+) TAMs, along with other tumor-promoting stromal cells such as regulatory T cells and endothelial cells, create a specific microenvironment that supports tumor progression.

TAMs are known to induce the proliferation, survival, and invasion of tumor cells by producing wide range

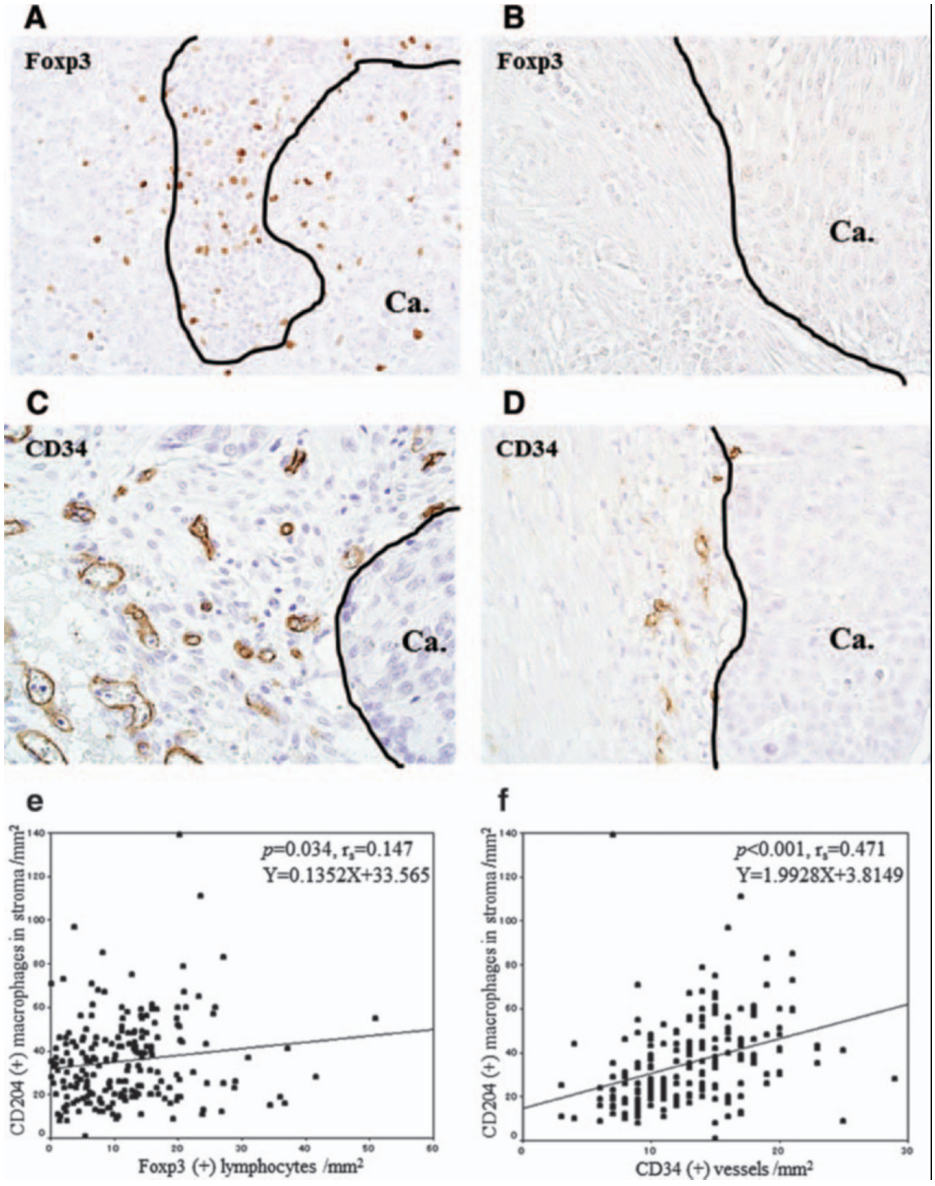

FIGURE 3. Immunohistochemical staining of squamous cell carcinoma tissue with antihuman Foxp3 antibody and antihuman CD34 antibody. *A*, Cases with a high number of Foxp3 positive lymphocytes, and *B*, a low number of Foxp3 positive lymphocytes. *C*, Cases with a high MVD, and *D*, a low MVD. *E*, Correlations between the numbers of CD204 (+) TAMs in the stroma and the MVD, *F*, the number of Foxp3 (+) lymphocytes. TAMs, tumor-associated macrophages; MVD, microvessel density.

of factors, such as matrix metalloproteinases (MMP).[1,13–16] Hagemann et al.[17] reported that TAMs change to M2 phenotype macrophages, CD204 (+) TAMs, after cocultivation with ovarian cancer cells. CD204 (+) TAMs showed a significant up-regulation of mRNA for the genes MMP−1, −2, −7, −9, and −14. Another article reported that the co-cultivation of breast cancer cells with macrophages led to the enhanced invasiveness of the cancer cells as a result of tumor necrosis factor α-dependent MMP-9 secretion from the TAMs.[18] These observations may explain the enhanced vascular and pleural invasion of squamous cell carcinoma cells in the high CD204 (+) TAMs groups in the current study.

We found that the numbers of CD204 (+) TAMs were strongly correlated with the MVD. Kawahara et al.[19] showed similar results that M2 macrophages were correlated with the MVD in gastric cancer. Given the previous report that CD204 (+) TAMs secrete proangiogenic factors, including VEGF,[17] the positive relation observed between CD204 (+) TAMs and the MVD in the present study is understandable. However, the numbers of CD204 (+) TAMs were not associated with the expression of VEGF in the tumor tissue. This discrepancy might be caused by the fact that angiogenesis also depends on angiogenic factors other than VEGF.

The number of CD204 (+) TAMs in the stroma was marginally correlated with the number of Foxp3 (+) lymphocytes, which was partly consistent with the results for intrahepatic cholangiocarcinoma.[20] Foxp3 (+) T cells down-regulate the immune response by attenuating the host's antitumor T cells, potentially permitting unrestricted growth, subsequent metastasis, and recurrence.[21,22] Taking these into consideration, CD204 (+) TAMs may not only enhance tumor cell invasiveness directly, but may also create a more tumor-promoting microenvironment by recruiting endothelial cells and regulatory T cells.

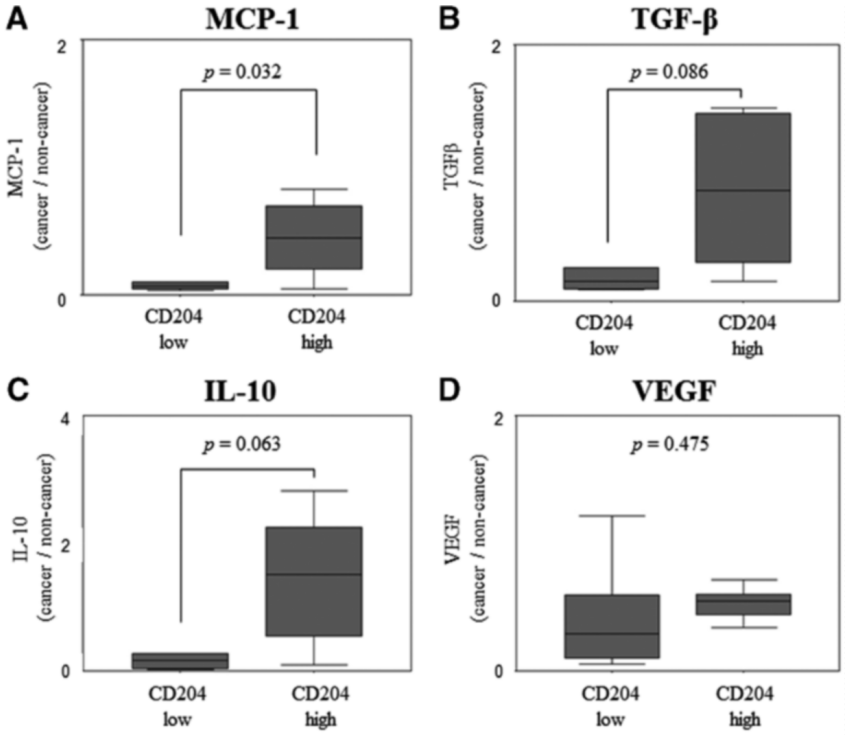

FIGURE 4. Relative mRNA expression in CD204-low and CD204-high cases. The levels of mRNA expression shown are the ratios of expression in cancer tissues relative to the expression in noncancerous tissues, as determined using a quantitative real-time polymerase chain reaction. MCP-1, monocyte chemoattractant protein-1; TGF β, transforming growth factor β; VEGF, vascular endothelial growth factor; IL-10, interleukins-10.

TABLE 2. Univariate Analysis for Overall Survival (N =208)

Variables	Category	All Cases	5-Year Survival (%)	p^a
Age				
Median, yrs (range)	69 (46–88)			0.1436
	<70	112	66	
	≥70	96	58	
Sex	Female	20	48	0.3689
	Male	188	63	
Smoking	Never	13	35	0.1894
	Ever	195	64	
Surgical procedures	Lobectomy segmentectomy	176	63	0.2908
	Pneumonectomy	32	56	
T factor	≤T1	58	84	0.0006[b]
	>T1	150	54	
N factor	pN0	125	66	0.1891
	pN1/pN2	83	56	
Lymphatic permeation	Absent	158	61	0.4973
	Present	50	67	
Vascular invasion	Absent	65	73	0.0381[b]
	Present	143	57	
Pleural invasion	Absent	134	70	0.0013[b]
	Present	74	47	
CD204 (+) TAMs in stroma	Low	93	75	0.0005[b]
	High	115	52	
CD204 (+) TAMs in nest	Low	91	63	0.5894
	High	117	61	

[a]Log-rank test.
[b]p 0.05.
TAMs, tumor-associated macrophages.

TABLE 3. Multivariate Analysis for Overall survival

Variables	Favorable	Unfavorable	Overall Survival		
			Hazard Ratio	95% Confidence Interval	*p*
T factor	≤T1	>T1	0.486	0.246–0.957	0.037
Vascular invasion	Absent	Present	1.124	0.650–1.942	0.676
Pleural invasion	Absent	Present	1.447	0.895–2.340	0.132
CD204 (+) TAMs in stroma	Low	High	2.053	1.273–3.311	0.003

TAMs, tumor-associated macrophages.

In the present study, we showed that the tissue expression of MCP-1 was significantly correlated with the numbers of CD204 (+) TAMs. MCP-1 has been reported as a key cytokine that induces the migration, accumulation, and differentiation of the M2 phenotype and contributes to the recruitment of CD204 (+) TAMs into the tumor tissue.[11,23] Moreover, MCP-1 can act directly on endothelial cells to promote angiogenesis.[24] Although no significant association was seen between the number of CD204 (+) TAMs and the VEGF mRNA level, MCP-1 might contribute to an increase in the MVD.

In this study, there 10 patients received postoperative adjuvant chemotherapy and 21 patients received chemotherapy after recurrence. However, there are no differences in the prognosis with or without postoperative adjuvant chemotherapy (RFS; $p = 0.2329$, OS; $p = 0.2548$) and chemotherapy after recurrence (OS; $p = 0.1318$). Among 198 patients who did not receive adjuvant chemotherapy, a high number of CD204 (+) TAMs in the stroma was also a significant prognostic factor for all *p*-stages and *p*-stage I (All p-stages: RFS $p = 0.0002$, OS $p = 0.0012$, *p*-stageI: RFS $p = 0.0169$, OS $p = 0.0369$). Therefore CD204-positive TAM was a strongly independent prognostic factor, even subtracting the effect of treatment.

In a recent report, the actions of bisphosphonates on macrophages not only impaired TAMs recruitment, but also inhibited the release of proangiogenic factors capable of affecting TAMs by reversing their polarization from the M2 to the M1 phenotype.[25] Moreover, the depletion of TAMs by clodrolip, which consists of a liposome encapsulating clodronate or zoledronic acid in combination with sorafenib, significantly inhibited tumor progression in hepatocellular carcinoma in vitro.[26] Our current results suggest that the targeting of CD204 (+) TAMs may be useful as a supplemental therapy for conventional cancer-treatment regimens for lung squamous cell carcinoma.

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