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ORIGINAL ARTICLE

Prognostic significance of the lymphocyte-to-monocyte ratio and the tumor-infiltrating lymphocyte to tumor-associated macrophage ratio in patients with stage T3N0M0 esophageal squamous cell carcinoma

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Abstract
Purpose We assessed the prognostic significance of, and the relationship between, the pretreatment lymphocyte-to-monocyte ratio (LMR) and the TILs/tumor-associated macrophages (TAMs) ratio, in patients with esophageal squamous cell carcinoma (ESCC) of pathological stage T3N0M0 (pT3N0M0).
Methods A total of 220 newly diagnosed ESCC patients of stage pT3N0M0 who had not undergone neoadjuvant therapy were included. Densities of CD8+ TILs, CD4+ TILs, CD45RO+ TILs, and CD68+ TAMs were assessed by immunohistochemical staining of tissue microarray cores from all 220 pT3N0M0 ESCC patients (who underwent radical resection). Hematological biomarkers including lymphocyte and monocyte counts were obtained from routine preoperative blood test data, and the LMR and TILs/TAMs ratios calculated. Cutoff finder for survival prediction was plotted to find out the optimal cutoff point for each parameter.
Results The LMR and TILs/TAMs ratios were interrelated. On univariate analyses of data from the entire cohort, the LMR, CD45RO/CD68 ratio, and CD8/CD68 ratio were significantly associated with both OS and disease-free survival. Only the CD45RO/CD68 ratio was independently prognostic of survival on multivariate analysis.
Conclusions The prognostic significance of the CD45RO/CD68 ratio was higher than that of the LMR. The CD45RO/CD68 ratio is a useful independent prognostic marker in patients with pT3N0M0 ESCC who have undergone complete resection without neoadjuvant therapy.

Keywords Esophageal squamous cell cancer · Lymphocyte-to-monocyte ratio · TILs · Tumor-associated macrophages · Survival

Abbreviations

AJCC	American Joint Committee on Cancer
ALC	Absolute lymphocyte count
AMC	Absolute monocyte count
CI	Confidence interval
DFS	Disease-free survival
EAC	Esophageal adenocarcinoma
EC	Esophageal cancer
ESCC	Esophageal squamous cell cancer
HR	Hazard ratio
KPS	Karnofsky performance score
LMR	Lymphocyte-to-monocyte ratio
TAMs	Tumor-associated macrophages
TME	Tumor microenvironment

Introduction

Esophageal squamous cell cancer (ESCC) is one of the deadliest cancers, associated with high rates of recurrence and distant metastasis [1]. Despite recent

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progress in diagnostic procedures and multimodal treatment approaches, prognosis differs even among patients of the same TNM stage, especially those of middle stage [2]. The new staging system (7th edition) of the American Joint Committee on Cancer (AJCC) incorporated non-anatomical esophageal cancer (EC) characteristics including the extent of tumor differentiation and tumor location [2]. For example, patients of pathological stage T3N0M0 (pT3N0M0) were divided into stages IB, IIA, and IIB, according to histologic grade and cancer location, implying that survival may differ greatly among such patients [2]. Thus, identification of promising prognostic factors contributing to risk classification and clinical management could improve long-term survival.

Apart from work on various molecular signals and genetic mutations associated with ESCC progression, the relationship between local and systemic immune responses and ESCC prognosis has attracted much recent attention. It has become clear over the past two decades that, in terms of disease progression, the tumor microenvironment (TME) is as important as are genetic and epigenetic changes in cancer cells [3]. The TME has many components including complexes of local stromal cells, distantly recruited cells, and immune cells [3]. Of these TME cells, TILs and tumor-associated macrophages (TAMs) may reflect tumor biology and predict patient outcome. The densities of CD8+ and CD4+ TILs have been shown to be of prognostic utility, and tumor TAMs enhanced tumor cell invasion and metastasis [3, 4]. The circulating lymphocyte-to-monocyte ratio (LMR), a marker of systemic inflammation, has been shown to be independently prognostic of progression of a variety of solid tumors [5, 6]. It has been hypothesized that the LMR may reflect the TILs/TAM ratio, as the circulating levels of lymphocytes and monocytes may indicate the formation or the presence of TILs and TAMs, respectively [5–7]. However, to the best of our knowledge, the reason why the LMR is prognostic and the relationship between the LMR and the TILs/TAMs ratio remain poorly studied. Few reports have combined biomarkers to refine outcome predictions for ESCC patients. Our objective was to explore a possible correlation between the LMR and the TILs/TAMs ratio and to compare the prognostic utilities of these ratios in patients with ESCC.

Materials and methods

Patients

The study was approved by the Ethics Committee of our hospital. The inclusion criteria were: (1) ESCC that was histopathologically confirmed and pathologically staged as T3N0M0 after curative esophagectomy; (2) availability of blood test data obtained within 3 days prior to surgery; and (3) performance of a complete preoperative evaluation including endoscopic esophageal ultrasonography, computed tomography, and liver function testing. The exclusion criteria were prescription of any neoadjuvant therapy, immunosuppressive therapy (e.g., recent steroids), or immunotherapy, acute infection, any hematological disorders, and any prior history of a malignancy or an autoimmune disease. We finally included 220 newly diagnosed ESCC patients who were pathologically staged as T3N0M0 between June 2004 and December 2012. All patients provided written informed consent and agreed to their tumor tissue and clinical data being used for research purposes.

Clinical and laboratory parameters

The 7th edition of the AJCC TNM staging system was used to classify the tumor stage [2]. Tumor length (to the nearest 1 mm) was defined as the longest dimension measured on general postoperative pathological specimens. Tumor locations were categorized into the upper, middle, and lower esophagus. Tumor differentiation was graded as poor/not differentiated, moderately differentiated, or well differentiated.

All blood samples (collected from the forearm veins within 3 days prior to surgery) were placed in tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately sent for analysis. Peripheral lymphocytes and monocytes were counted by an automated hematology analyzer (XE-5000, Sysmex, Kobe, Japan). Each LMR was calculated by dividing the absolute lymphocyte count (ALC) by the absolute monocyte count (AMC). The optimal prognostic cutoffs for lymphocyte counts, monocyte counts, and the LMR were determined using the method established by Budczies et al. (http://molpath.charite.de/cutoff/) [8].

IHC

Postoperative specimens of all patients were subjected to immunohistochemical analysis performed using standard automated protocols. Sections (4 μm thick) were deparaffinized in xylene and rehydrated through baths with a graded alcohol series. The antigens were retrieved by microwaving under high pressure for 2 min. Sections were stained with the following primary antibodies: anti-CD68 (clone OTI4G1, Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China); anti-CD8 (clone SP16, Beijing Zhongshan Golden Bridge Biotechnology Company); anti-CD4 antibody (clone OTI6E10, Beijing Zhongshan Golden Bridge Biotechnology Company); and anti-CD45RO (clone OTI2E7, Beijing Zhongshan Golden Bridge Biotechnology Company) in a humidified chamber at 37 °C for 60 min

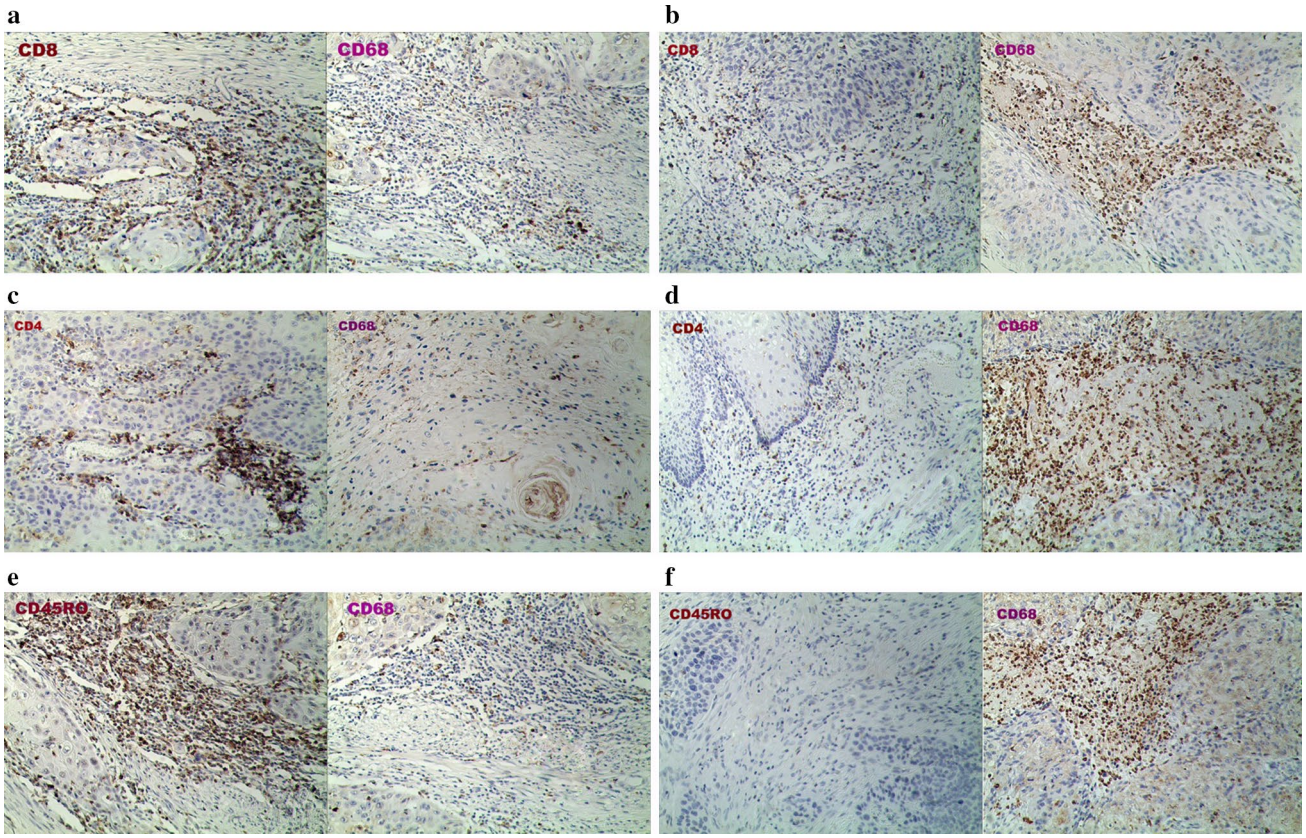

Fig. 1 Representative examples of high and low CD8/CD68 (**a, b**), CD4/CD68 (**c, d**), and CD45RO/CD68 ratios (**e, f**) expression (×200 magnification) in esophageal squamous cell carcinoma (ESCC) samples

and subsequently incubated with secondary goat antirabbit and goat antimouse antibodies (Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China) at 37 °C for 15 min. Reaction products were visualized by color reaction with 3,3′-diaminobenzidine and counterstained with hematoxylin.

Two independent pathologists blinded to clinical data reviewed all slides. The densities of CD68+ TAMs, CD8+ TILs, CD4+ TILs, or CD45RO+ TILs were evaluated in a medium-power field (200×) in a manner similar to recent recommendations in the literature [9]. In brief, TILs were scored as a percentage of stained lymphocytes in the stromal compartment on a semiquantitative scale (results in 5% steps beginning with 0%). The optimal prognostic cutoffs for TILs and TAMs were determined using the method established by Budczies et al. (http://molpath.charite.de/cutoff/) [8].

Statistical analysis

A Chi-square test was used to compare the differences in baseline and clinicopathological characteristics between the groups. Disease-free survival (DFS) was defined as time elapsed from date of surgery to that of local recurrence/distant metastasis, or to the date of last follow-up. OS was the time from the date of surgery to death from ESCC or the last follow-up. The Kaplan–Meier method was used to estimate the survival curves, and differences between subgroups were compared with the aid of the log-rank test. Cox's proportional hazards models were used to perform univariate and multivariate analysis defining hazard ratios (HRs) for variables relevant to DFS and OS. Two-sided p values and HRs with 95% confidence intervals (CIs) are reported. The optimal cutoff values for certain variables were determined using the method of Budczies et al. (http://molpath.charite.de/cutoff/) [8]. All statistical analyses were conducted with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). A two-sided p value <0.05 was considered to reflect statistical significance.

Results

Immune markers and clinical variables

Immunohistochemically, CD45RO+, CD4+, and CD8+ T cells, as well as CD68+ TAMs infiltrating ESCC tissue,

Table 1 Correlation between AMC and CD68+ TAM and clinicopathological characteristics

	No. of patients	%	AMC			CD68+ TAM		
			Low	High	*p*	Low	High	*p*
Age (years)					0.472			0.170
≤60	124	56.36	65	59		109	15	
>60	96	43.64	55	41		78	18	
Gender					0.554			0.558
Male	117	53.18	66	51		101	16	
Female	103	46.82	54	49		86	17	
KPS					0.322			0.833
<80	61	27.73	30	31		51	10	
≥80	159	72.27	90	69		136	23	
Tumor location					0.745			0.061
Upper	26	11.82	15	11		22	4	
Middle	88	40.00	50	38		69	19	
Lower	106	48.18	55	51		96	10	
Tumor differentiation					**0.001**			0.886
Well	88	40.00	52	36		76	12	
Moderate	84	38.18	53	31		71	13	
Poor	48	21.82	15	33		40	8	
Tumor length					**0.019**			**0.022**
≤4 cm	133	60.45	81	52		119	14	
>4 cm	87	39.54	39	48		68	19	
Perineural invasion					1.000			0.222
No	214	97.27	117	97		183	31	
Yes	6	2.73	3	3		4	2	
Vascular invasion					0.250			**0.011**
No	213	96.82	118	95		184	29	
Yes	7	3.18	2	5		3	4	
Treatment					0.550			0.304
Surgery only	102	46.36	51	51		91	11	
Adjuvant chemotherapy	46	20.91	25	21		39	7	
Adjuvant radiation	39	17.73	24	15		31	8	
Adjuvant chemoradiation	33	15.00	20	13		26	7	
Recurrence					1.000			0.466
No	38	17.27	21	17		34	4	
Yes	182	82.73	99	83		153	29	

Values in bold signify $p < 0.05$

AMC absolute monocyte count, *TAM* tumor-associated macrophages

were evident in the stroma (Fig. 1). Supplementary Figure S1 illustrates representative examples of CD8, CD4, CD45RO, and CD68 expression in ESCC samples. The immunohistochemical and hematological variables are shown in Supplementary Table S1. The optimal cutoffs for survival prediction were 36.80, 16.90, 5.00, and 15.10% for the CD68, CD8, CD4, and CD45RO counts, respectively. Similarly, an ALC of 1.431×10^9 /L and an AMC of 0.630×10^9 /L were the respective optimal cutoffs for prediction of survival. Ratios were defined as the proportions of CD45RO+ , CD4+ , and CD8+ T cells divided by the proportion of CD68+ TAMs. The LMR was calculated by dividing the lymphocyte count by the monocyte count. The optimal cutoffs were 3.364, 1.960, 1.671, and 1.980 for the LMR, and the CD4/CD68, CD8/CD68, and CD45RO/CD68 ratios, respectively. Representative examples of high and low CD8/CD68, CD45RO/CD68, and CD4/CD68 ratios are shown in Fig. 1.

The complete baseline characteristics of all patients (data on low- and high-level immune marker groups are shown separately) are presented in Table 1. High CD68 + TAM infiltrates correlated with longer tumor length and vascular

Table 2 Correlation between LMR and TILs/TAM ratio and clinicopathological characteristics

	LMR			CD45RO/CD68 ratio			CD8/CD68 ratio			CD4/CD68 ratio		
	Low	High	*p*	Low	High	*p*	Low	High	*p*	Low	High	*p*
Age (years)			0.787			0.250			0.683			0.455
≤60	72	52		87	37		98	26		97	27	
>60	54	42		74	22		78	18		79	17	
Gender			0.998			0.303			0.893			0.636
Male	67	50		89	28		94	23		95	22	
Female	59	44		72	31		82	21		81	22	
KPS			0.216			0.253			0.651			0.407
<80	39	22		48	13		50	11		51	10	
≥80	87	72		113	46		126	33		125	34	
Tumor location			0.896			0.382			0.054			0.464
Upper	16	10		21	5		21	5		23	3	
Middle	50	38		67	21		77	11		71	17	
Lower	60	46		73	33		78	28		82	24	
Tumor differentiation			**0.016**			**0.006**			0.248			**0.036**
Well	44	44		54	34		67	21		64	24	
Moderate	46	38		67	17		72	12		74	10	
Poor	36	12		40	8		37	11		38	10	
Tumor length			**0.023**			0.300			0.629			0.408
≤4 cm	68	65		94	39		105	28		104	29	
>4 cm	58	29		67	20		71	16		72	15	
Perineural invasion			0.702			0.195			0.602			1.000
No	123	91		155	59		170	44		171	43	
Yes	3	3		6	0		6	0		5	1	
Vascular invasion			0.701			0.194			0.349			0.349
No	121	92		154	59		169	44		169	44	
Yes	5	2		7	0		7	0		7	0	
Treatment			0.820			0.178			0.161			
Surgery only	61	41		77	25		85	17		84	18	0.330
Adjuvant chemotherapy	25	21		30	16		32	14		37	9	
Adjuvant radiation	23	16		26	13		30	9		27	12	
Adjuvant chemoradiation	17	16		28	5		29	4		28	5	
Recurrence			0.319			**<0.001**			0.130			**0.016**
No	19	19		19	19		27	11		25	13	
Yes	107	75		142	40		149	33		151	31	

Values in bold signify $p < 0.05$

LMR lymphocyte-to-monocyte ratio, *TILs* tumor-infiltrating lymphocytes, *TAM* tumor-associated macrophages

invasion (Table 1). No significant correlation was evident between CD68 status and gender, tumor location, or any other parameters, whereas a low AMC (<0.630 × 10^9 /L) and a high LMR (>3.364) were more frequent in patients with shorter tumors and tumors that were well or moderately differentiated (Table 1). Also, high CD45RO/CD68 and CD4/CD68 ratios were associated with both tumor differentiation ($p = 0.006$ and 0.036, respectively) and recurrence ($p < 0.001$ and $p = 0.006$, respectively) (Table 2). In contrast, we found no significant difference in age, tumor location, TNM stage, or any other variable between patients with high or low CD8, CD4, or CD45RO counts, and ALCs or CD8/CD68 ratios.

Correlation between tumor-infiltrating and hematological immune markers

A significant correlation was evident between the CD68 score and the AMC ($r = 0.315$, p <0.001) (Table 3). Also, the ALC exhibited a weak positive association with CD4

Table 3 Correlation of hematological and immunohistochemical variables with each other

	CD8	CD45RO	CD68	ALC	AMC
CD4					
r	0.050	*0.151*	0.048	*0.155*	−0.101
p	0.462	**0.025**	0.482	**0.021**	0.136
CD8					
r		0.128	−0.078	0.129	*−0.293*
p		0.058	0.250	0.056	**<0.001**
CD45RO					
r			0.024	0.011	−0.022
p			0.729	0.870	0.748
CD68					
r				*−0.149*	*0.315*
p				**0.027**	**<0.001**
ALC					
r					−0.118
p					0.082

Spearman's *r* coefficient test; values in bold signify $p < 0.05$

ALC absolute lymphocyte count, *AMC* absolute monocyte count

Table 4 Correlation of LMR and TILs/TAM ratio

LMR	CD45RO/CD68	CD8/CD68	CD4/CD68
r	0.235	0.402	0.309
p	**<0.001**	**<0.001**	**<0.001**

Spearman's *r* coefficient test; values in bold signify $p < 0.05$

LMR lymphocyte-to-monocyte ratio, *TILs* tumor-infiltrating lymphocytes, *TAM* tumor-associated macrophages

and a negative correlation with CD68 counts ($r = 0.115$ and -0.149; $p = 0.021$ and 0.027, respectively) (Table 3). The LMR was significantly correlated with the CD8/CD68, CD4/CD68, and CD45RO/CD68 ratios ($r = 0.402$, 0.309, and 0.235, all p values <0.001) (Table 4; Fig. 2a–c).

Survival analysis

Median follow-up time was 53.25 months. In total, 175 (79.09%) patients died from ESCC before the end of the follow-up. The median DFS and OS for the entire population were 40.0 months (95% CI 34.2–45.8 months) and 53.0 months (95% CI 48.0–58.0 months), respectively. Univariate analysis showed that conventional tumor histopathological features, including tumor length, tumor location, extent of differentiation, and vascular invasion, were prognostically significant (Table 5). No type of lymphocyte infiltrating the stroma and no hematological markers alone were of any prognostic significance. In contrast, except for the CD4/CD68 ratio (Table 5; Fig. 3g, h), high LMR and high CD8/CD68 and CD45RO/CD68 ratios were significantly associated with a favorable DFS ($p = 0.059$, 0.008, <0.001, respectively) and OS ($p = 0.050$, 0.002, <0.001, respectively) (Table 5; Fig. 3a–f). All of these factors ($p < 0.1$) were entered into multivariate analysis using the Cox's proportional hazards model. The CD45RO/CD68 ratio was strongly prognostic of DFS, with HRs of 0.913 (95% CI 0.841–0.990, $p = 0.028$) when used as a continuous variable (per point of increase) and 0.600 (95% CI 0.418–0.861, $p = 0.006$) when employed as a categorical variable (Table 5). In addition, the ratio was a good indicator of improved OS, with HRs of 0.899 (95% CI 0.825–0.979, $p = 0.014$) when used as a continuous variable (per point of increase) and 0.494 (95% CI 0.342–0.715, $p < 0.001$) when employed as a categorical variable (Table 5). Vascular invasion, extent of tumor differentiation, tumor location, and preoperative Karnofsky performance score (KPS) were independently associated with both DFS ($p = 0.027$, 0.003, 0.018 and 0.036, respectively) and OS ($p = 0.020$, <0.001, 0.009, and 0.011, respectively) (Supplementary Figures S2–S5).

Discussion

In the present study, we performed integrated analyses of local and systemic immune factors, including the LMR (based on the pretreatment peripheral blood cell counts), and tumor-infiltrating immune markers (evaluated using a tissue microarray method), including CD4+ , CD8+ , CD45RO+ TILs, and CD68+ TAMs, in 220 pT3N0M0 ESCC patients. We sought to evaluate the correlation between, and compare the prognostic abilities of, the LMR and the TILs/TAMs ratio. We found that LMR and TILs/TAM ratio are interrelated. Patients with low stromal CD45RO/CD68 ratios tended to exhibit reduced DFS and OS. Furthermore, a high CD45RO/CD68 ratio afforded better discrimination than did the LMR of subgroups with significantly poorer DFS and OS. Therefore, the stromal CD45RO/CD68 ratio may serve as a novel biomarker predicting the outcomes of patients with pT3N0M0 ESCC.

Tumor immune microenvironments can be analyzed effectively by performing IHC methods on resected tumors. Previous studies found that high or low levels of TILs and chemokines were of significant prognostic utility in ESCC patients [4, 10]. Moreover, two prior studies on EC patients evaluated the relationships among local and systemic inflammation, standard clinicopathological factors, and survival; most patients had esophageal adenocarcinoma (EAC); only a small proportion had ESCC [11, 12]. Although tumor inflammatory infiltration was irrelevant in terms of tumor necrosis, such infiltration played an important role in suppressing of tumor progression and

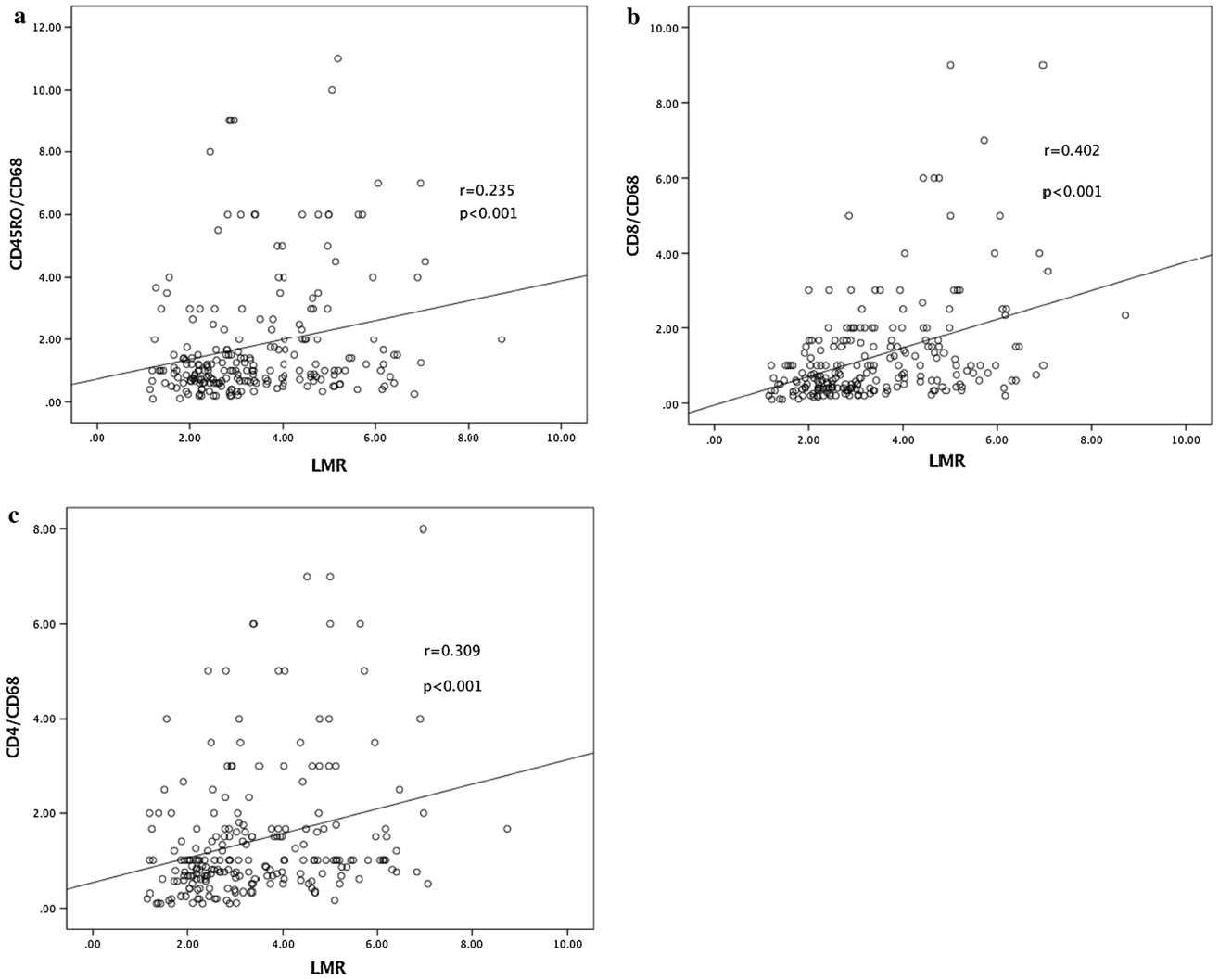

Fig. 2 Correlations between the CD45RO/CD68 ratio (**a**), the CD8/CD68 ratio (**b**), the CD4/CD68 ratio (**c**), and the lymphocyte-to-monocyte ratio (LMR)

metastasis in EAC, but not ESCC, patients [11, 12]. However, as the number of ESCC patients was small, and as the carcinogenesis of EAC and ESCC differs, any role played by inflammatory tumor infiltrates requires re-evaluation [11, 13]. In our present study, we found that the densities of single types of TILs or TAMs did not correlate with prognosis, but the combination of TAMs and TIL densities did show a correlation. This suggests that interactions among various immune cells in the TME may significantly affect ESCC progression.

CD45RO has been generally accepted as the optimal single marker for the entire memory T cell population, with the exception of T memory stem cells [14]. Memory T cells are acknowledged to respond faster upon re-stimulation with antigen compared with naive T cells [15]. Immunochemically, high-level expression of CD45RO+ T memory cells has been associated with better disease-related outcomes in various human cancers, including EAC and ESCC [16–18]. Chronic inflammation has long been regarded as a key risk factor for many human cancers [19]. Recently, a close association between chronic inflammation-associated genomic instability and esophageal carcinogenesis was reported in ESCC patients [20]. TAMs constitute prominent inflammatory cell populations in many types of tumor, playing pivotal roles in cancer-related inflammation [3, 19]. Although the roles played by TAMs in cancer development and progression are disputed, there is increasing evidence that a TAM-rich microenvironment increases the probability of metastasis and is associated with poor survival in patients with various cancers, including ESCC [11, 21–24]. VEGFA and EGF produced by TAMs foster angiogenic tissue programming and tumor growth, respectively, compatible with the positive relationship evident between CD68 expression, and both vascular invasion and tumor length [25–27].

Table 5 Univariate analysis of clinicopathological and immunohistochemical parameters associated with disease-free survival and overall survival

Variable	Disease-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age (≤60 vs. >60)	0.973 (0.726–1.304)	0.855			1.033 (0.765–1.393)	0.834		
Gender (male vs. female)	0.866 (0.646–1.161)	0.336			0.881 (0.653–1.188)	0.405		
Pre-op KPS (<80 vs. ≥80)	0.737 (0.534–1.017)	0.063	0.695 (0.495–0.976)	**0.036**	0.700 (0.505–0.971)	**0.032**	0.638 (0.451–0.902)	**0.011**
Tumor location								
Upper	Ref.	**0.044**	Ref.	**0.018**	Ref.	**0.036**	Ref.	**0.009**
Middle	0.979 (0.614–1.561)	0.928	0.952 (0.587–1.546)	0.843	0.943 (0.589–1.507)	0.805	0.879 (0.539–1.434)	0.607
Lower	0.678 (0.427–1.076)	0.099	0.623 (0.387–1.002)	**0.051**	0.646 (0.406–1.029)	0.066	0.560 (0.346–0.906)	**0.018**
Tumor length (≤4 cm vs.> 4 cm)	1.366 (1.017–1.834)	**0.038**	1.191 (0.870–1.630)	0.275	1.331 (0.984–1.799)	0.063	1.145 (0.828–1.582)	0.413
Differential grade								
Well	Ref.	**0.016**	Ref.	**0.003**	Ref.	**0.004**		**<0.001**
Moderate	1.272 (0.914–1.770)	0.153	1.360 (0.962–1.925)	0.082	1.296 (0.922–1.182)	0.135	1.389 (0.972–1.986)	0.071
Poor	1.763 (1.196–2.597)	**0.004**	1.997 (1.333–2.990)	**0.001**	1.964 (1.325–2.912)	**0.001**	2.294 (1.516–3.470)	**<0.001**
Perineural invasion (no vs. yes)	1.545 (0.683–3.492)	0.296			1.734 (0.766–3.925)	0.187		
Vascular invasion (no vs. yes)	3.175 (1.474–6.839)	**0.003**	2.451 (1.200–5.802)	**0.027**	3.457 (1.603–7.453)	**0.002**	2.577 (1.160–5.728)	**0.020**
Treatment regimens								
Surgery alone	Ref.	0.857			Ref.	0.915		
Adjuvant chemotherapy	1.087 (0.742–1.592)	0.669			1.042 (0.705–1.539)	0.836		
Adjuvant radiation	1.071 (0.721–1.591)	0.734			0.943 (0.623–1.427)	0.782		
Adjuvant chemoradiation	0.887 (0.571–1.380)	0.595			0.878 (0.560–1.377)	0.572		
Hematological markers								
ALC (low vs. high)	0.891 (0.613–1.294)	0.545			0.917 (0.624–1.347)	0.659		
AMC (low vs. high)	1.173 (0.876–1.570)	0.285			1.262 (0.937–1.700)	0.126		
LMR (low vs. high)	0.753 (0.560–1.011)	0.057	0.945 (0.686–1.303)	0.732	0.739 (0.546–1.000)	**0.050**	0.995 (0.716–1.384)	0.979
Immunohistochemical markers								
CD8+ TILs (low vs. high)	0.921 (0.687–1.235)	0.584			0.865 (0.641–1.169)	0.345		
CD4+ TILs (low vs. high)	0.877 (0.583–1.321)	0.531			0.851 (0.564–1.282)	0.440		
CD45RO+ TILs (low vs. high)	1.079 (0.787–1.479)	0.639			1.066 (0.774–1.469)	0.696		
TAM (CD68) (low vs. high)	1.318 (0.886–1.962)	0.173			1.352 (0.896–2.039)	0.151		
CD8/CD68 (low vs. high)	0.601 (0.411–0.877)	**0.007**	0.766 (0.473–1.240)	0.279	0.540 (0.364–0.802)	**0.002**	0.688 (0.421–1.126)	0.137
CD4/CD68 (low vs. high)	0.717 (0.486–1.057)	0.093	1.149 (0.714–1.851)	0.567	0.683 (0.455–1.025)	0.066	1.138 (0.694–1.866)	0.609
CD45RO/CD68 (low vs. high)	0.529 (0.371–0.754)	**<0.001**	0.600 (0.418–0.861)	**0.006**	0.494 (0.342–0.715)	**<0.001**	0.567 (0.389–0.827)	**0.003**

Table 5 continued

Variable	Disease-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
CD45RO/CD68 (as a continuous variable)	0.891 (0.820–0.968)	**0.006**	0.913 (0.841–0.990)	**0.028**	0.876 (0.803–0.956)	**0.003**	0.899 (0.825–0.979)	**0.014**

Values in bold signify $p < 0.05$

TILs tumor-infiltrating lymphocytes, *TAM* tumor-associated macrophages, *ALC* absolute lymphocyte count, *AMC* absolute monocyte count, *LMR* lymphocyte-to-monocyte ratio, *HR* hazard ratio, *CI* confidence interval, *Ref.* reference

Moreover, TAMs compromise effector cell functions in TMEs; TAMs express inhibitory receptors and secrete several cytokines, chemokines, and enzymes [25, 28]. The advantage of the CD45RO/CD68 index is that it combines the information on the status of both antitumor immunity and inflammation, thus comprehensively indicating the extent of the host immune response.

The preoperative, absolute peripheral monocyte count was suggested to be a useful prognostic marker in a large cohort of patients with resected ESCCs [29]. Moreover, recent studies have shown that a reduced LMR was associated with poor survival of cancer patients, including those with ESCC [30–32]. Our findings in a large cohort of ESCC patients are consistent with those of previous studies. The OS of patients with low LMRs was much poorer than that of those with high LMRs (median OS, 47.0 vs. 57 months). Also, the LMR was associated with both tumor differentiation and tumor length in our cohort. The LMR is a potentially valuable clinical biomarker, being easily calculated, reproducible, and low cost. Moreover, the LMR correlated with the TILs/TAMs ratio, suggesting that a systemic inflammatory response may reflect concurrent focal inflammation in the tumor, in agreement with data from previous studies on hepatocellular carcinoma [33]. However, LMR was not an independent prognostic indicator in our study. This warrants further investigation in a large prospective study of patients with ESCC.

The reason why lower level of LMR is associated with poor cancer outcomes remains unknown. Lymphopenia might weaken the efficacy of the immune system; cell-mediated cytotoxicity may be attenuated if the level of effector T cells is insufficient [6, 34]. Circulating monocytes may contribute to both tumor growth and reduced immunosurveillance. Serum monocytes are recruited locally and differentiate into macrophages only after infiltrating a tumor; the cells respond to the wide spectrum of chemokines and growth or differentiation factors, such as CCL-2 and CSF-1, produced by tumor and stromal cells in the TME. This may explain the moderate correlation evident between the CD68 and AMC scores, and the correlation between the LMR and the TILs/TAMs ratio [35, 36].

Previous studies evaluated the extent of T cell infiltration by using automated imaging software. However, such software distinguishes cells by color depths only, and cell shape, features, or position are not considered. Moreover, CD68 has recently been shown to not be a specific macrophage marker, but a lysosomal protein enriched not only in macrophages but also in non-myeloid cells including carcinomas [37]. In the present study, two independent pathologists blinded to clinical and pathological information evaluated the densities of TILs and TAMs. The data are superior to those obtained using automated imaging software; the pathologists considered cell morphology and position during the assessment. Moreover, our patients were more homogeneous than those of other cohorts; i.e., they were all of the same TNM stage (pT3N0M0). As the CD45RO/CD68 ratio was an independently favorable prognostic factor, it may be that this ratio will be a useful non-anatomical marker complementing TNM staging. However, studies on patients at other disease stages, and those who receive different initial treatments (including neoadjuvant therapy and definitive therapies), are required.

There were certain limitations to our study. First, a previous study on other tumor types has specifically identified infiltrating inflammatory cells as "invasive margin" or "cancer cell nests" by location [38]. We could not analyze ESCC specimens in this manner because the primary infiltration pattern of ESCC is perivascular stromal infiltration; the diffuse pattern is very rare. Therefore, most lymphocytes are located in the stroma, and few infiltrate into cancer cell nests (which are thus difficult to evaluate). Moreover, we found that patients with high CD4/CD68 status had a propensity toward reduced recurrence and longer survival. The associations did not reach statistical significance (Table 5; Fig. 3g, h). This is probably because different subpopulations of CD4+ TILs exert distinct functions [15]. More detailed classification is required in the future. In addition, in our study, we did not classify circulating lymphocytes in detail; this may cause us to

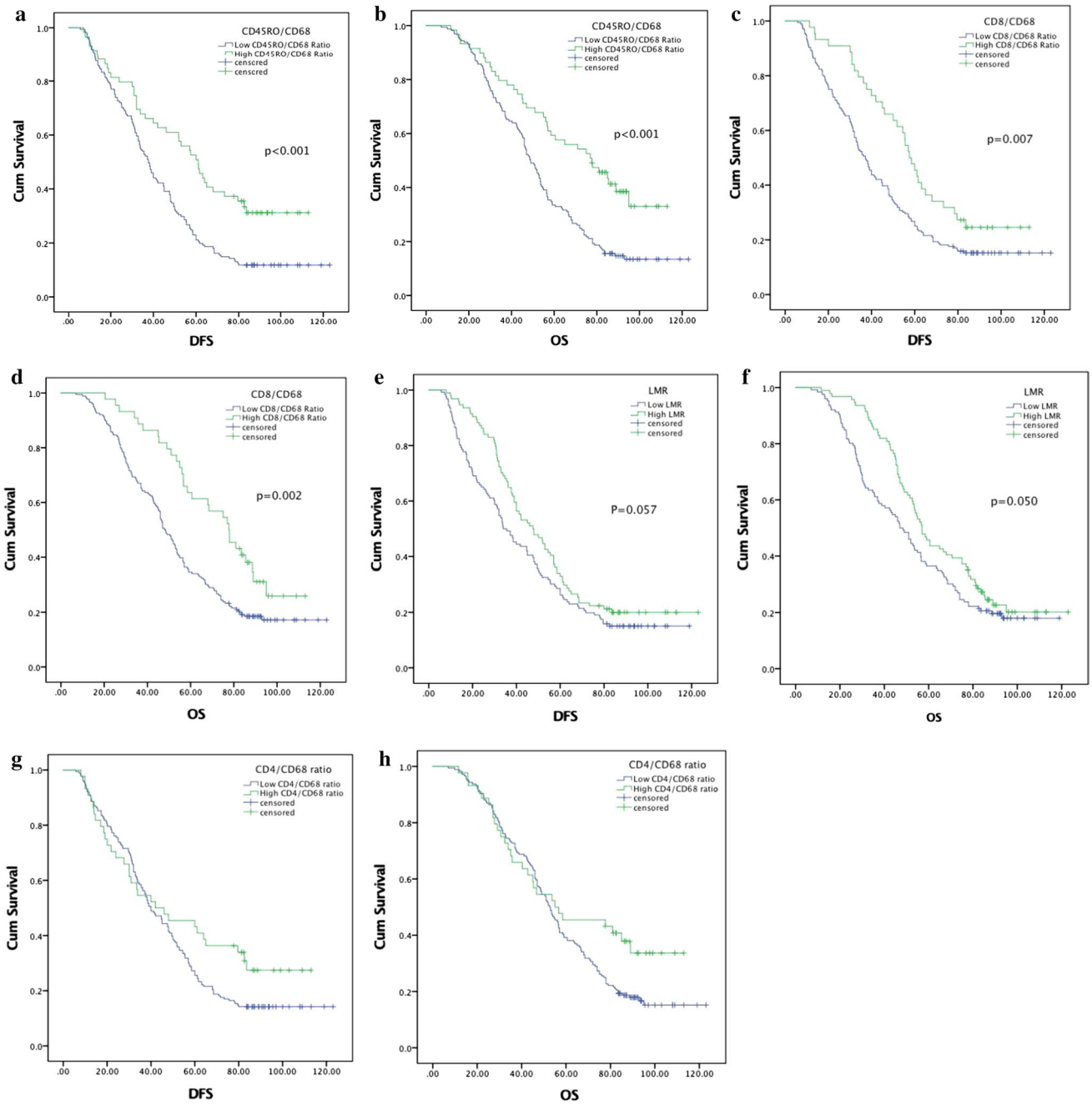

Fig. 3 Kaplan–Meier analysis of disease-free survival and overall survival in terms of the CD45RO/CD68 ratio (**a, b**), the CD8/CD68 ratio (**c, d**), the LMR (**e, f**), and the CD4/CD68 ratio (**g, h**)

underestimate (to some extent) the correlation between the levels of circulating lymphocytes and TILs, and the impact of circulating lymphocyte levels on survival. However, the levels of circulating lymphocyte subpopulations, such as CD4+ , CD8+ ,and CD45RO+ lymphocytes, supposedly correlate with survival in patients with several types of solid tumors embracing nasopharyngeal carcinoma, cervical cancer, and hepatocellular carcinoma (most of which are virus-associated tumors) [39–41]. Virus-specific immunity may increase the numbers of T cell subpopulations, thus improving survival [42]. However, the etiology and pathogenesis of ESCC remain unclear. Recently, the InterSCOPE study, the largest sero-epidemiological study on HPV in ESCC, revealed the absence of viral biological activity for 51 mucosotropic HPV types in ESCC from high-incidence regions [43]. However, it remains true that the impact of circulating lymphocytes on survival of ESCC patients requires further study.

In summary, we show that the tumor-infiltrating CD45RO/CD68 ratio may more comprehensively indicate host immune response status than do the LMR and other single markers. The CD45RO/CD68 ratio may be a useful prognostic biomarker in patients with pT3N0M0 ESCC and is a promising candidate marker of the immunoscore. Further research on the local and systemic immune responses of ESCC patients treated in different centers is warranted.

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Compliance with ethical standards

Conflict of interest No author has any conflict of interest.

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