ANTICANCER RESEARCH *41*: 4515-4522 (2021)
doi:10.21873/anticanres.15262

Prognostic Value of CD45Ro+ T-Cell Expression in Patients With Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: The role of tumour-infiltrating CD45Ro+ T-cells in oral squamous cell carcinoma (OSCC) is unclear. This study aimed to evaluate prognostic biomarkers for OSCC. Patients and Methods: We determined the density of tumour-infiltrating CD45Ro+ T cells in the parenchyma and stroma at the tumour centre (TCe) and invasive front (IF) and examined the association between the density of these cells and histopathological status in 142 patients. Results: Five-year overall survival (OS) and recurrence-free survival were favourable in patients with high CD45Ro+ T-cell density in the TCe stroma. OS was favourable in patients with high CD45Ro+ T-cell density in the IF stroma. Stepwise Cox regression model analysis indicated that CD45Ro+ T-cells in the stroma of the IF and TCe were an independent prognostic factor for OS. Conclusion: CD45Ro+ T-cells in the stroma of the IF and TCe play a role in cancer immune surveillance and may be a useful prognostic factor.*

Oral cancer represents 1-3% of all cancers, with histopathologically classified oral squamous cell carcinoma (OSCC) accounting for approximately 90% of oral cancer cases (1). In recent years, immunotherapy, such as immune checkpoint blockade and chimeric antigen receptor T-cell therapy, has attracted attention as a new option for the treatment of cancer. However, the prognosis of patients with OSCC remains poor and has not improved in the past three decades (2). One of the reasons for this is that tumour responses to immunotherapy are not exactly the same. Solid tumours are thought to have more complicated microenvironments that may play complex roles in the host immune response (3). The key to resolve this situation may be the use of tumour-infiltrating lymphocytes (TILs), which are a major component of the tumour microenvironment (4). TILs are a manifestation of the immune response between tumour cells and immune effector cells (5). Many studies have reported a significant association between the presence of TILs and patient survival (6-9). However, results remain controversial because there are many different types of T-lymphocytes that likely play different roles in the tumour microenvironment. Previously, we examined the relationship between TILs and survival in patients with OSCC (10, 11). To further investigate this relationship, we focused on the role of CD45Ro+ T-cells in the tumour microenvironment.

CD45 is a constitutively active cell-surface tyrosine phosphatase involved in leukocyte signalling (12). The expression of different CD45 isoforms occurs in a cell type-specific manner and depends on the activation state and differentiation stage of cells. CD45Ro is one of the most suitable single markers for human memory T-cells, which accurately represent the activation status of T-cells (13). CD45Ro+ T-cells are often increased in solid tumours (13). Although memory T-cells protect against microbes, whether they can prevent cancer recurrence and metastasis and contribute to the survival rate after tumour resection is unclear. Some studies have suggested an association between the density of CD45Ro+ T-cells and cancer prognosis, but their results remain unclear (14, 15). In fact, only a few reports have examined the role of CD45Ro+ T-cells in OSCC (16-18), and there is currently a lack of evidence supporting their use as a biomarker.

If memory T-cells can prevent recurrence and metastasis, the presence of CD45Ro+ T-cells in TILs might induce the

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Key Words: Tumour-infiltrating lymphocytes, oral cancer, biomarker, CD45Ro, prognosis.

immune response to prevent recurrence and metastasis after tumour resection. The purpose of this study was to clarify the association between CD45Ro$^+$ T-cell infiltration and clinical outcome in OSCC patients. In addition, we aimed to provide further evidence on the clinical value of tumour-infiltrating CD45Ro$^+$ T-cells as a prognostic biomarker for OSCC.

Patients and Methods

Patients. Our retrospective study followed the guidelines of the Declaration of Helsinki and its subsequent versions. Approval for this study was obtained from the Institutional Review Board of Sapporo Medical University. Written informed consent was obtained from all study participants. We used tissue samples collected from patients who were diagnosed with OSCC and who underwent definitive surgery between January 2004 and December 2015 at Sapporo Medical University Hospital. Tissue samples were processed routinely, embedded in paraffin, and cut into 4-μm sections. None of the patients received any form of neoadjuvant or adjuvant chemo- and/or radiotherapy before or after surgery.

Immunohistochemical staining. The presence of CD45Ro$^+$ T-cells in surgical specimens was evaluated via immunohistochemical staining as described previously (10). The slides were stained with primary monoclonal antibodies targeting CD45Ro$^+$ T-cells [1:200; Mouse Anti-CD45RO Antibody (UCH-L1) (sc-1183); Santa Cruz Biotechnology, Dallas, TX]. To more accurately identify tumour cells, tissue sections were incubated with a primary monoclonal antibody against pan-cytokeratin (1:200; clone AE1/AE3; Abcam, Cambridge, UK). Haematoxylin and eosin staining was used for pathological assessment of tumour characteristics. Negative controls were processed in the same manner but were not incubated with the primary antibodies.

Histopathological and immunohistopathological evaluation. The evaluation method was based on our previous report (10). Haematoxylin and eosin-stained slides were evaluated for lymphovascular invasion, perineural invasion, and histopathological grading. CD45Ro$^+$ T-cells were evaluated in four different areas: the parenchyma and stroma at the tumour centre (TCe) and invasive front (IF). First, CD45Ro$^+$ T-cells were identified under low power magnification (×40); then, CD45Ro$^+$ T-cells in the four regions of the tumour were counted. For counting, we chose the areas with the most intense CD45Ro$^+$ T-cell staining in the four tumour regions and performed counting under high-power magnification (×400). Tumour areas with artefacts and necrotic or apoptotic features were excluded. CD45Ro$^+$ T-cells in the IF were counted in areas containing small clusters or nests at the deepest invading margins. Location identification was simplified by AE1/AE3 staining. At least three random fields were examined to determine the density of the tumour-infiltrating CD45Ro$^+$ T-cells in each tumour compartment; in cases of heterogeneity, we used the cell count that was most representative of the entire section. CD45Ro$^+$ T-cell density was assessed by three authors (KK, SS, and KN) on a personal computer equipped with DP2-BSW software for a microscope with a digital camera (Olympus, Tokyo, Japan; Figure 1). Labels bearing the patient's pathological number were covered. We analysed the prognostic role of CD45Ro$^+$ T-cell density in the four different tumour areas. Stratified cell density was also estimated in relation to clinicopathological findings, including sex, age, anatomical sites, primary tumour, regional lymph nodes, and stage grouping. Clinical staging was assessed according to the eighth edition of the American Joint Committee on Cancer Staging Manual (19). Tumour histological grade was classified according to the World Health Organization classification (20).

Statistical analyses. Overall survival (OS), recurrence-free survival (RFS), and metastasis-free survival (MFS) were used as indicators of the survival rate. MFS was examined in 111 N0 cases. The patients were divided into high or low groups using the cut-off point of the median density of CD45Ro$^+$ T-cells. OS, RFS, and MFS were analysed using the Kaplan–Meier method and compared using the log-rank test for each group. Variables that had prognostic potential were evaluated using a stepwise method in Cox regression models. We used the following variables: clinical characteristics (sex, age, anatomical site, and stage grouping), pathological characteristics (histopathological grading, lymphovascular invasion, and perineural invasion), and immunohistochemical findings (infiltrating CD45Ro$^+$ T-cells in the tumour parenchyma and stroma at the TCe and IF). Two-tailed *p*-values of <0.05 were considered statistically significant. SPSS statistical software (version 23.0 for Windows; IBM, Armonk, NY, USA) was used for statistical analyses.

Results

Tumour characteristics. A total of 240 primary OSCC patients were treated by curative surgery from January 2004 to December 2015. Of these, 142 were treated without neoadjuvant or adjuvant chemo- and/or radiotherapy. In addition, sufficient tissue samples from these patients were available for further analyses. The clinicopathological characteristics of patients are summarized in Table I. The 142 OSCC patients comprised 80 men (56.3%) and 62 women (43.7%), with a median age of 68 years (range=33-93 years) at first presentation. Anatomical sites included the tongue/floor of the mouth (94 patients, 66.2%) and other regions of the oral cavity (48 patients, 33.8%). Forty-five patients were stage I (31.7%), 61 were stage II (42.9%), and 36 were stage III/IV (25.4%) according to the American Joint Committee on Cancer staging classification. Seventy-five patients were grade 1 (52.8%), 62 were grade 2 (43.7%), and 5 were grade 3 (3.5%) according to histopathological grading. Lymphovascular invasion was present in 26 patients (18.3%) and absent in 116 (81.7%); perineural invasion was present in 13 patients (9.2%) and absent in 129 (90.8%).

Relationship between clinicopathological data and survival rates. The median follow-up period for all patients was 89 months (range=4-192 months). Primary tumours recurred in 16 patients (11.2%), and regional lymph node relapse was found in 26 patients (18.3%). Five-year OS and RFS were 83.8% and 63.4%, respectively. Detailed results are shown in Table I. Age, lymphovascular invasion, and perineural invasion were correlated with both OS and RFS.

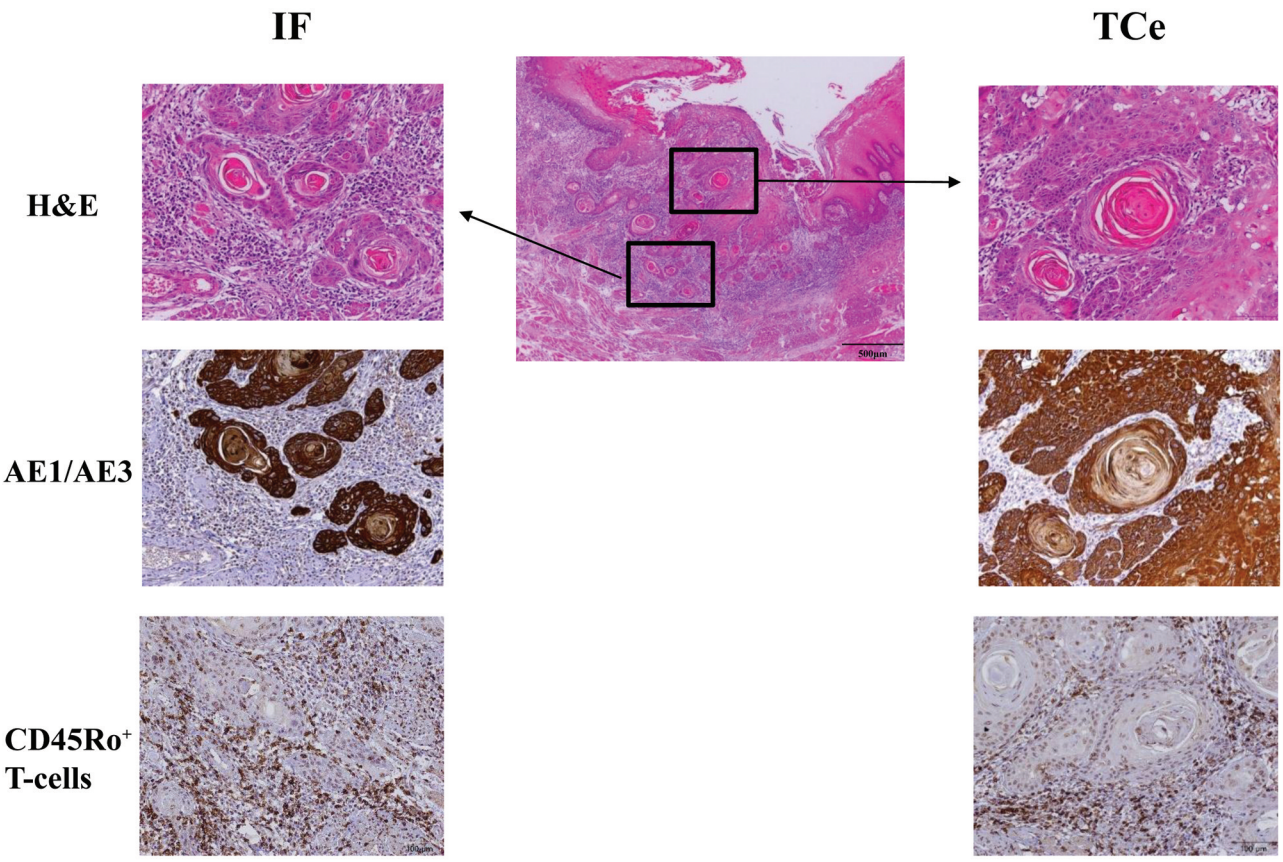

Figure 1. *Histopathological panel of oral squamous cell carcinoma tissues. We examined four distinct compartments: tumour parenchyma and stroma at the tumour centre (TCe) and invasive front (IF). To clarify the infiltration of the IF, we used a primary monoclonal antibody against pan-cytokeratin (AE1/AE3). CD45Ro+ T-cells were first identified using haematoxylin and eosin (H&E) staining under low magnification (×40); then, these cells were counted visually in areas with the greatest density under high magnification (×400) in the four compartments of the tumour.*

Density of tumour-infiltrating CD45Ro+ T-cells. The median number and range of tumour-infiltrating CD45Ro+ T-cells in each compartment were as follows: TCe parenchyma, 1 (range=0-8); TCe stroma, 22 (range=0-91); IF parenchyma, 1 (range=0-18); and IF stroma, 33 (range=0-124). A higher number of CD45Ro+ T-cells infiltrated the tumour stroma compared with the tumour parenchyma. Detailed results are shown in Table II.

Relationship between CD45Ro+ T-cell density and survival. Patients with high CD45Ro+ T-cell density in the tumour stroma of the IF and TCe showed significantly better OS compared with those with low CD45Ro+ T-cell density (IF: 94.4% vs. 72.9%, respectively, $p<0.001$; TCe: 94.4% vs. 72.9%, respectively, $p<0.001$). In contrast, there was no significant difference between high/low CD45Ro+ T-cell density in the parenchyma of the TCe and IF (upper panel in Figure 2). Patients with high CD45Ro+ T-cell density in the stroma of the TCe showed significantly better RFS than patients with low CD45Ro+ T-cell density (lower panel in Figure 2). In contrast, there was no significant difference in MFS between patients with high/low CD45Ro+ T-cell density (Figure 3). Stepwise Cox regression model analysis indicated that CD45Ro+ T-cell density in the stroma of the IF was an independent prognostic factor for OS (hazard ratio, 3.97; 95% confidence interval, 1.21-13.04; $p=0.02$). This result suggests that it is necessary to focus on CD45Ro+ T-cell density in the tumour stroma rather than in the parenchyma. Detailed results are shown in Table III.

Discussion

CD45Ro is a surface marker expressed on memory T-cells, which are considered to be preserved for months after being generated and are responsible for the rapid and amplified response to secondary antigen exposure (14). CD45Ro+ T-cells

Table I. *Patient and tumour characteristics. Five-year overall survival (OS) and recurrence-free survival (RFS) according to clinicopathological variables.*

Characteristic	No. of patients	Percentage	OS		RFS	
			Survival rate	Log-rank test (*p*-Value)	Survival rate	Log-rank test (*p*-Value)
Gender						
Male	80	56.3	83.8%	0.97	66.3%	0.31
Female	62	43.7	83.9%		59.7%	
Age (years)						
<68	73	51.4	94.5%	<0.001	72.6%	0.01
≥68	69	48.6	72.5%		53.6%	
Anatomical site						
Tongue/Floor of the mouth	94	66.2	92.1%	0.002	69.7%	0.07
Other	48	33.8	74.2%		56.1%	
Primary tumour						
T1	49	34.5	95.9%	0.001	69.4%	0.11
T2	79	55.6	81.0%		63.3%	
T3/4	14	9.9	57.1%		42.9%	
Regional lymph nodes						
N (–)	111	78.2	88.3%	0.002	65.8%	0.22
N (+)	31	21.8	67.7%		54.8%	
Stage grouping						
Stage I	45	31.7	97.8%	<0.001	71.1%	0.18
Stage II	61	42.9	83.6%		63.9%	
Stage III/IV	36	25.4	66.7%		52.8%	
Histopathological grading						
Grade 1	75	52.8	88.0%	0.32	70.7%	0.003
Grade 2	62	43.7	79.0%		58.1%	
Grade 3	5	3.5	80.0%		20.0%	
Lymphovascular invasion						
Absent	116	81.7	86.7%	0.04	67.5%	0.01
Present	26	18.3	68.2%		40.9%	
Perineural invasion						
Absent	129	90.8	86.8%	0.001	67.4%	<0.001
Present	13	9.2	53.8%		23.1%	

OS, Overall survival; RFS, recurrence-free survival.

Table II. *Patient distribution according to the location and density of tumour-infiltrating CD45Ro$^+$ T-cells.*

CD45Ro$^+$ T-cell infiltration location			No. of patients	Percentage
TCe	Stroma	Cut-off point=22		
		High: ≥22	72	50.7
		Low: <22	70	49.3
	Parenchyma	Cut-off point=1		
		High: ≥1	50	35.2
		Low: <1	92	64.8
IF	Stroma	Cut-off point=33		
		High: ≥33	72	50.7
		Low: <33	70	49.3
	Parenchyma	Cut-off point=1		
		High: ≥1	43	30.3
		Low: <1	99	69.7

IF, Invasive front; TCe, tumour centre.

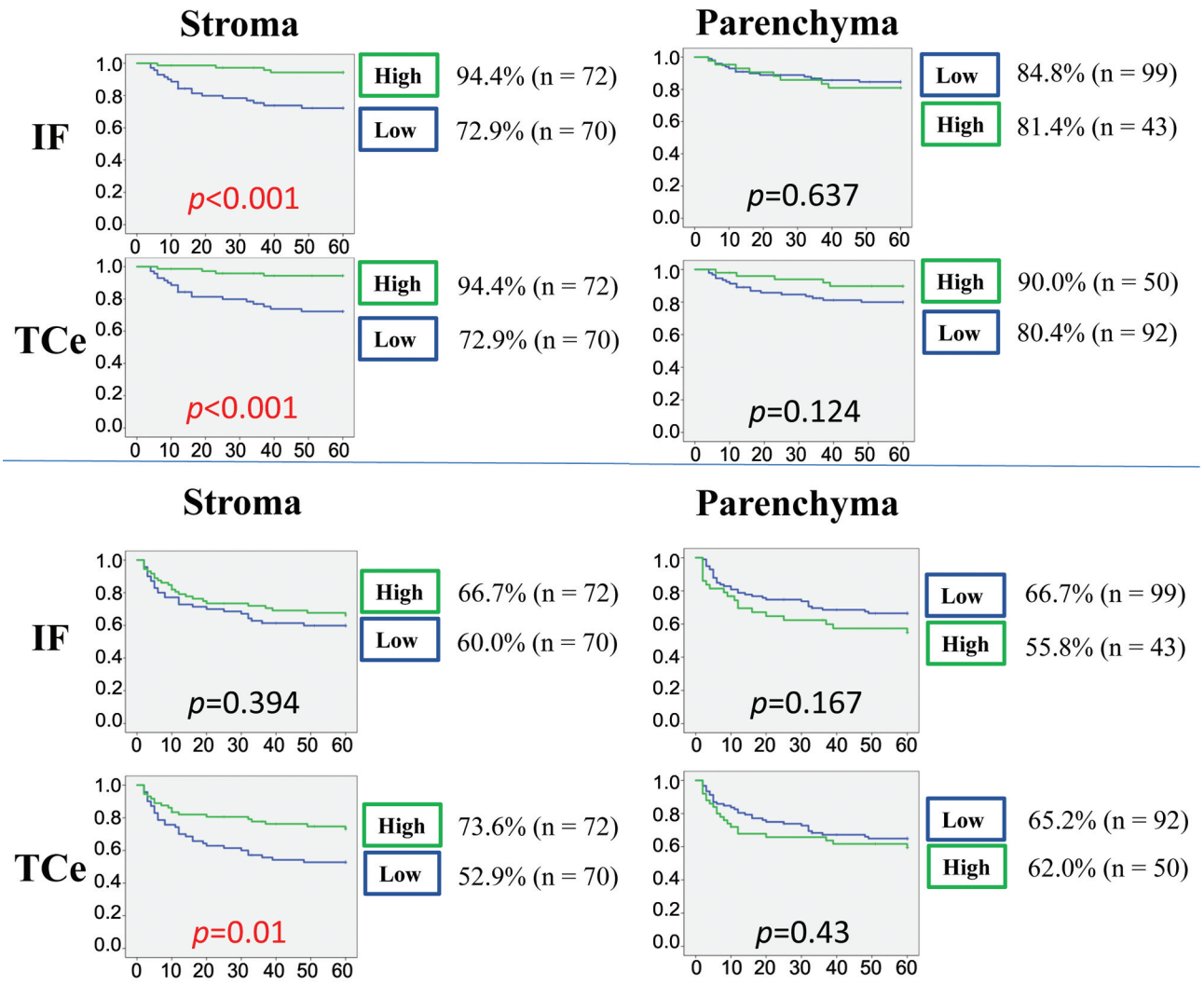

Figure 2. *Relationships between 5-year overall survival (upper panel) and recurrence-free survival (lower panel) with tumour-infiltrating CD45Ro+ T-cells. Blue and green lines indicate the number of CD45Ro+ T-cells below and above the median, respectively. Horizontal axes indicate time in months and vertical axes indicate the cumulative survival rate. Patients with high CD45Ro+ T-cell density in the stroma of the invasive front (IF) and tumour centre (TCe) showed significantly better overall survival than patients with low CD45Ro+ T-cell density. Patients with high CD45Ro+ T-cell density in the stroma of the TCe showed significantly better recurrence-free survival than patients with low CD45Ro+ T-cell density.*

are reported to be associated with better OS and disease-free survival in solid tumours such as nasopharyngeal carcinoma (21) and colorectal cancer (22). However, Hotta et al. revealed paradoxical OS in renal cell carcinoma (23). In OSCC, only a few reports have indicated that high infiltration of CD45Ro+ T-cells correlates with better survival (16-18), which is consistent with our results. Therefore, our report further enhances the prognostic usefulness of CD45Ro+ T-cells.

However, the exact mechanisms underlying the CD45Ro+ T-cell-mediated improvement of survival remain unclear. This improvement may partially be related to the following features of CD45Ro+ T-cells: 1) they are the one of the hallmarks of adaptive immunity; 2) they display a low activation threshold; 3) they proliferate vigorously, despite minimal co-stimulation; and 4) they persist throughout an individual's life with stem cell-like multipotency and self-renewal characteristics (24). From a molecular-biological aspect, splicing of CD45mRNA shows that CD45Ro contains exons 3 and 7 but not exons 4-6 and this isoform is expressed on effector and memory T cells, some B subsets, activated monocytes/macrophages, and granulocytes (25). CD45Ro enhances activation mediated by both T cell

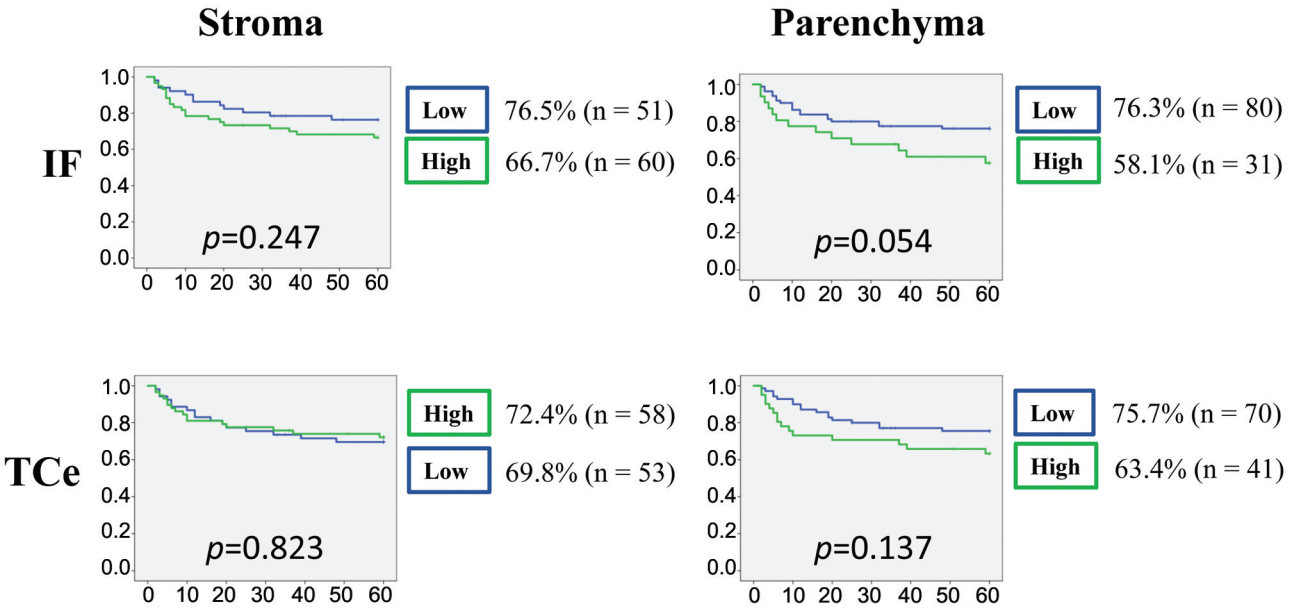

Figure 3. *Relationships between 5-year metastasis-free survival and tumour-infiltrating CD45Ro+ T-cells. There was no significant difference in metastasis-free survival between patients with high/low CD45Ro+ T-cell density. IF, invasive front; TCe, tumour centre.*

receptor and B cell receptor signalling (26, 27). Also, cytosolic calcium levels and protein kinase C activity are higher in CD45Ro$^+$ T-cells and are sufficient for inducing T cell activation (28, 29). More importantly, tumour-infiltrating CD45Ro$^+$ T-cells that have experienced tumour antigens are probably effector memory CD8$^+$ T-cells, which can secrete interferon-γ and granzyme to induce potent anti-tumour immune responses. In situ immune reactions can reflect and influence systematic anti-tumour capability. After resection of a primary tumour, central memory T-cells, as another subset of CD45Ro$^+$ memory T-cells, increase in number, home to the secondary lymphoid organs, and exhibit a persistent anti-tumour effect through various mechanisms such as interferon-γ production. Interferon-γ mRNA expression is significantly higher in tumours with high CD45Ro$^+$ T-cell density than in tumours with low CD45Ro$^+$ T-cell density in advanced gastric cancer tissue (30). Thus, it is reasonable to assume that CD45Ro$^+$ T-cells can respond to and eliminate residue tumour cells, thereby improving the survival rate.

In this study, we found that a high number of CD45Ro$^+$ T-cells infiltrated the stroma of the TCe and IF compared to the parenchyma, suggesting that the above molecular mechanism and its possible involvement in the secretion of interferon-γ are predominantly in the stroma. Therefore, a high level of CD45Ro$^+$ T-cell infiltration into the stroma of the TCe and IF was associated with a significantly higher survival rate compared with low-level infiltration, indicating that CD45Ro$^+$ memory T-cells may serve as an independent prognostic factor in OSCC. However, this study has several limitations. First, it used retrospective methods for data collection. Second, the number of patients was relatively

Table III. *Multivariate analysis with Cox proportional hazards model for overall survival and recurrence-free survival.*

Immunohistochemical, clinical, and pathological findings	OS		
	HR	95% CI	*p*-Value
IF-Stroma	3.97	1.21-13.04	0.02
TCe-Stroma	3.38	1.06-10.78	0.03
Age	4.46	1.46-13.56	0.008
Stage grouping	3.13	1.54-6.37	0.002
Lymphovascular invasion	2.66	1.04-6.78	0.04
	RFS		
	HR	95% CI	*p*-Value
Perineural invasion	2.91	1.43-5.90	0.003
Age	1.94	1.10-3.41	0.02

Clinical, pathological, and immunohistochemical findings were used. CI, Confidence interval; HR, hazard ratio; IF, invasive front; OS, overall survival; RFS, recurrence-free survival; TCe, tumour centre.

small. Other limitations include different immunostaining methods, scoring systems, and cut-off points used in each report. It is necessary to consider a unified large-scale study to obtain more reliable data.

In conclusion, CD45Ro+ T-cells are associated with a favourable clinical outcome in patients with OSCC, suggesting that these cells might be a prognostic biomarker. However, it is necessary to gather more functional data for CD45Ro+ T-cells. To develop a treatment strategy targeting CD45Ro+ T-cells, future studies should include prospective designs with appropriate multivariate analyses of large cohorts.

Conflicts of Interest

The Authors declare that they have no competing financial interest.

Authors' Contributions

Conceptualization: Kazushige Koike, Koyo Nishiyama. Data curation: Kazushige Koike, Koyo Nishiyama, Shota Shimizu. Formal analysis: Kazushige Koike, Koyo Nishiyama, Shota Shimizu. Funding acquisition: Kazushige Koike, Akihiro Miyazaki. Investigation: Kazushige Koike, Koyo Nishiyama, Shota Shimizu. Methodology: Kazushige Koike, Koyo Nishiyama, Shota Shimizu. Project administration: Kazushige Koike, Akihiro Miyazaki. Resources: Kazushige Koike, Koyo Nishiyama, Hironari Dehari, Kazuhiro Ogi, Takanori Sasaki, Shota Shimizu, Takashi Sasaya, Kei Tsuchihashi, Hiroyoshi Hiratsuka, Akihiro Miyazaki. Software: Kazushige Koike, Tomoko Sonoda. Supervision: Tadashi Hasegawa, Hiroyoshi Hiratsuka, Akihiro Miyazaki. Validation: Kazushige Koike, Koyo Nishiyama, Shota Shimizu. Visualization: Kazushige Koike, Koyo Nishiyama, Shota Shimizu. Writing-original draft: Kazushige Koike. Writing-review&editing: Koyo Nishiyama, Hironari Dehari, Kazuhiro Ogi, Takanori Sasaki, Shota Shimizu, Takashi Sasaya, Kei Tsuchihashi, Tomoko Sonoda, Tadashi Hasegawa, Hiroyoshi Hiratsuka, Akihiro Miyazaki.

Acknowledgements

We thank ThinkSCIENCE, Inc. (Tokyo, Japan) for English language editing of this manuscript. This work was supported by JSPS KAKENHI Grant Numbers JP19K10292 and JP20K18675.

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Received May 31, 2021
Revised June 29, 2021
Accepted July 8, 2021