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Prognostic Implications of Infiltrating T Cells Within Tumors for Patients With Esophageal Squamous Cell Carcinoma: A Retrospective Cohort Study

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Research article

Keywords: Tumor-infiltrating lymphocytes, T cells, Esophageal cancer, Prognosis

Posted Date: July 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-46066/v1

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Abstract

Background: Infiltrating T lymphocytes within tumors have been recognized to be associated with patient survival in various cancer types. For esophageal squamous cell carcinoma (ESCC), the prognostic implications of infiltrating CD3+ and CD8+ T cells have yet to be validated in more extensive patient groups.

Methods: We evaluated the numbers of infiltrating CD3+ and CD8+ T cells within tumors from 461 patients with ESCC using immunohistochemistry and analyzed its impacts on overall survival.

Results: The median number of infiltrating CD3+ and CD8+ T cell per field was 16.0 (*P*25-*P*75: 9.2-28.6) and 8.0 (3.8-20.2), respectively, with obvious correlation between them (r_s = 0.65, $p < 0.0001$). Age and tumor grade were associated with the numbers of infiltrating T cells (all $p < 0.05$). A high number of infiltrating CD3+ T cells was associated with prolonged overall survival (unadjusted HR for high *vs* low: 0.73, 95% CI: 0.55-0.98), whereas CD8+ T cell number was not (0.90, 0.68-1.20). Multivariate Cox analysis identified that T stage, lymph node metastasis, and infiltrating CD3+ T cell number were independent prognostic factors (all $p < 0.05$).

Conclusions: Our results indicate that CD3+ T cell infiltration within tumors is an independent prognostic factor for ESCC patients, whereas CD8+ T cell infiltration has no prognostic implications.

Background

Esophageal cancer (EC) includes two main histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). Globally, ESCC accounts for the vast majority of EC cases, especially in Central and South-Eastern Asia including China, while EAC is the most common histological subtype in many western countries.[1] EC is estimated to be the seventh most commonly diagnosed cancer and sixth leading cause of cancer death worldwide, with a projected 572,000 new cases and 509,000 ten thousand deaths in 2018.[2] In China, there were an estimated 258,000 new EC cases and 193,000 deaths in 2014.[3] Although survival of EC patients has significantly improved due to advances in early screening and therapies, the prognosis is still poor. According to the results of CONCORD-3 programme released in 2018, the age-standardized 5-year net survival of EC patients in most countries was in the range 10–30% in 2010-14, compared to 29.7% in 2010-14 in China.[4]

The immune system plays an important role in the genesis, development, and prognosis of cancer. Indeed, “evading immune destruction” has been proposed as one of the hallmarks defining cancer.[5] Moreover, immune contexture, defined as the nature, density, functional state and distribution of infiltrating immune cells within the tumor,[6] is recognized to impact the prognosis and treatment response of cancer patients.[7, 8] For instance, in melanoma, patients with higher numbers of infiltrating CD8 + T cells within pre-treatment tumor samples were more likely responded to pembrolizumab (PD-1 inhibitor).[9] A favorable impact of infiltrating CD8 + T cells on patient survival has also been observed in many cancer

types, including breast,[10] colorectal,[11] and ovarian cancer.[12] Especially for colorectal cancer, the concept of “immunoscore”, a scoring methodology considering the CD3+/CD8+/CD45RO + T cell counts both in the tumor center and invasive margin, has been proposed as a complement of the classical TNM classification system,[13] forming a new combined TNM-immune classification system of cancer.[11, 14, 15]

Specific to ESCC, one study[16] has suggested that intratumoral CD8 + T cell infiltration was an independent protective prognostic factor, while another study[17] indicated that CD4 + and CD8 + T cells can cooperatively improve prognosis of ESCC patients. A recent study[18] also verified the favorable prognostic implication of CD8 + T cell infiltration. However, another study[19] indicated that CD8 + T cells infiltration does not provide prognostic information for ESCC patients. Nevertheless, these results need to be validated in more extensive ESCC patients.

In the present study, we evaluated the number of infiltrating overall (CD3+) and cytotoxic (CD8+) T cells within tumors of ESCC patients. The associations between the number of infiltrating T cells, clinicopathological characteristics and overall survival (OS) were analyzed.

Methods

Patients

A total of 461 ESCC patients (334 males and 127 females; age range: 34–83 years) who received radical esophagectomy in the Cancer Hospital of Shantou University Medical College from March 2009 to December 2013 were included. None of them had received any pre-surgery anticancer treatment. Follow-up and clinicopathological information, including age, gender, smoking and drinking behavior before operation, tumor position, tumor grade, TNM stage, and resection margin infiltration, were collected from archived medical records. Patients died during the in-hospital stay were excluded. Overall survival (OS) was defined as the period from the date of operation to the date of death or loss to follow-up.

Immunohistochemistry (ihc) Staining For Cd3 And Cd8

Formaldehyde-fixed and paraffin-embedded surgical specimens of tumor tissues were subjected to IHC staining for CD3 and CD8. First, tumor tissues were sectioned at 4 μm thickness followed by deparaffinage in xylene and rehydration in a graded series of ethanol. Then, heat-mediated antigen retrieval was performed with Tris-EDTA in a pressure cooker at 125 ℃ for 2 minutes. After natural cooling, sections were immersed in 3% hydrogen peroxide to quench endogenous peroxidase activity. Subsequently, sections were incubated with rabbit monoclonal primary antibodies for CD3 (Clone: SP7, Maixin Biotechnologies, Fuzhou, China) or CD8 (Clone: SP16, Maixin Biotechnologies, Fuzhou, China) overnight at 4 ℃. The next day, sections were incubated with secondary antibody at 37 ℃ for 30 minutes before visualization with the DAB system. Finally, sections were counterstained with hematoxylin, then dehydrated and mounted with coverslips.

Quantification Of Infiltrating Cd3 + and Cd8 + t Cells Number

IHC images of five random high power (400X) fields in the tumor cell nest or stroma were captured for each section using an Olympus microphotographic system. Number of CD3 and CD8 positive (membrane) cells (Fig. 1A) in each image was manually counted with Image-Pro Plus 6.0 software. Then, for each marker, the number of T cells was defined as the mean value of positive cell counts in five images.

Statistical analysis

All statistical analyses were performed using R 3.5.2 software. The correlation between infiltrating CD3 + and CD8 + T cell numbers was evaluated by Spearman rank correlation coefficient (r_s). Differences in infiltrating T cell numbers between patients with different clinicopathological characteristics were compared using the χ^2 test or Fisher's exact probability test. For overall survival, survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate Cox proportional hazards regression models were performed to identify prognostic factors of OS. The significance level was set at $\alpha = 0.05$.

Results

Infiltrating CD3+/CD8 + T cells within tumors

The number of infiltrating CD3 + and CD8 + T cells within tumors were evaluated in 461 ESCC patients. The median value for CD3 + and CD8 + T cell infiltration was 16.0 (P_{25}-P_{75}: 9.2–28.6) and 8.0 (3.8–20.2), respectively. There was an obvious correlation between the number of infiltrating CD3 + and CD8 + T cells ($r_s = 0.65$, $p < 0.0001$). Among 461 ESCC patients, using the medians as cut-off levels, 230 patients were classified into a high CD3 density group, while 227 patients were classified into a high CD8 density group. Figure 1A shows representative immunohistochemical staining images for CD3 and CD8.

Association Between Clinicopathological Parameters And Infiltrating T Cells

Table 1 summarizes the association between clinicopathological parameters and infiltrating T cells. There was a trend for CD3 + T cell infiltration to increase with age ($p = 0.0163$), but the trend was not statistically significant for CD8 + T cells ($p = 0.0907$). Smoking and drinking seemed to slightly decrease the CD3 + and CD8 + T cell numbers, although the differences were not statistically significant. Patients with a positive resection margin had a higher CD8 + T cell infiltration ($p = 0.0187$), but not for CD3 + T cells ($p = 1$). Tumor grade was associated with both CD3+ ($p = 0.0097$) and CD8+ ($p = 0.0111$) T cell infiltration, and well-differentiated tumors had lower levels of T cell numbers. Association of other clinicopathological features including TNM stages with CD3 + or CD8 + T cell numbers was not observed.

Table 1
Clinicopathological parameters and CD3+/CD8+ T cells infiltration.

		$CD3^+$ T cells			$CD8^+$ T cells		
Parameters	**No. p**	**Low**	**High**	**P-value**	**Low**	**High**	**P-value**
Total, n (%)	461	231 (50.1)	230 (49.9)		234 (50.8)	227 (49.2)	
Gender, n (%)							
Male	334	168 (50.3)	166 (49.7)	0.8942	168 (50.3)	166 (49.7)	0.7488
Female	127	63 (49.6)	64 (50.4)		66 (52.0)	61 (48.0)	
Age (years), n (%)							
<55	139	80 (57.6)	59 (42.4)	0.0163	78 (56.1)	61 (43.9)	0.0907
55–64	226	114 (50.4)	112 (49.6)		116 (51.3)	110 (48.7)	
>64	96	37 (38.5)	59 (61.5)		40 (41.7)	56 (58.3)	
Smoking, n (%)							
No	147	71 (48.3)	76 (51.7)	0.5950	72 (49.0)	75 (51.0)	0.6010
Yes	314	160 (51.0)	154 (49.0)		162 (51.6)	152 (48.4)	
Drinking, n (%)							
No	287	141 (49.1)	146 (50.9)	0.5890	144 (50.2)	143 (49.8)	0.7469
Yes	174	90 (51.7)	84 (48.3)		90 (51.7)	84 (48.3)	
Location[a], n (%)							
Top	48	25 (52.1)	23 (47.9)	0.9571	21 (43.8)	27 (56.2)	0.3483
Middle	322	162 (50.3)	160 (49.7)		171 (53.1)	151 (46.9)	
Bottom	85	42 (49.4)	43 (50.6)		40 (47.1)	45 (52.9)	

		CD3+ T cells			CD8+ T cells		
Resection margin, n (%)							
Negative	452	226 (50.0)	226 (50.0)	1[b]	233 (51.5)	219 (48.5)	0.0187[b]
Positive	9	5 (55.6)	4 (44.4)		1 (11.1)	8 (88.9)	
Grade, n (%)							
Well	113	68 (60.2)	45 (39.8)	0.0097	71 (62.8)	42 (37.2)	0.0111
Moderate	267	118 (44.2)	149 (55.8)		123 (46.1)	144 (53.9)	
Poor	81	45 (55.6)	36 (44.4)		40 (49.4)	41 (50.6)	
T stage[a], n (%)							
T1	10	4 (40.0)	6 (60.0)	0.2187	3 (30.0)	7 (70.0)	0.5512
T2	59	36 (61.0)	23 (39.0)		32 (54.2)	27 (45.8)	
T3	226	106 (46.9)	120 (53.1)		115 (50.9)	111 (49.1)	
T4	147	77 (52.4)	70 (47.6)		77 (52.4)	70 (47.6)	
Lymph node metastasis[a], n (%)							
N0	206	104 (50.5)	102 (49.5)	0.9897	113 (54.9)	93 (45.1)	0.1694
N1	236	119 (50.4)	117 (49.6)		114 (48.3)	122 (51.7)	
Distant metastasis[a], n (%)							
M0	434	220 (50.7)	214 (49.3)	0.2824[b]	222 (51.2)	212 (48.8)	1[b]
M1	7	2 (28.6)	5 (71.4)		4 (57.1)	3 (42.9)	

[a] Parameter with missing information; [b] *P* value was calculated by Fisher's exact test.

Association Between Infiltrating T Cells And Overall Survival

A total of 434 ESCC patients with complete information of clinicopathological characteristics and overall survival was included in the survival analysis. The median follow-up time for all patients was 44.2 months. Median overall survival time for all patients was 81.8 months, with a 3-year overall survival of 65.3% and a 5-year overall survival of 54.6%.

For patients with a high tumor density of CD3 + T cells, the 3-year overall survival and 5-year overall survival was 71.7% and 58.3%, respectively, while 58.9% and 50.6% for patients with a low tumor density of CD3 + T cells (unadjusted HR for high *vs* low: 0.73, 95% CI: 0.55–0.98, $p = 0.0336$; Fig. 1B). For patients in the high CD8 density group, 3-year and 5-year overall survivals were 68.7% and 55.8%, respectively, but 62.0% and 53.3% for patients in the low CD8 density group (unadjusted HR for high *vs* low: 0.90, 95% CI: 0.68–1.20, $p = 0.4758$; Fig. 1B).

The univariate Cox proportional hazards regression model identified drinking, resection margin, tumor grade, T stage, lymph node metastasis, distant metastasis, and CD3 + T cell number as significant prognostic factors (all $p < 0.05$; Table 2). These factors were subsequently included in a multivariate Cox regression mode, indicating that only T stage, lymph node metastasis, and infiltrating CD3 + T cell number were independent prognostic factors (all $p < 0.05$; Table 2).

Table 2
Univariate and multivariate Cox proportional hazards regression model for overall survival.

Parameters	No. p	Univariate		Multivariate	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Gender					
Female *vs* male	122/312	0.74 (0.53–1.03)	0.0749		
Age (years)					
55–64 *vs* < 55	213/131	1.12 (0.80–1.57)	0.5010		
>64 *vs* < 55	90/131	1.20 (0.80–1.80)	0.3838		
Smoking					
Yes *vs* no	293/141	1.33 (0.97–1.82)	0.0751		
Drinking					
Yes *vs* no	162/272	1.35 (1.01–1.79)	0.0414	1.12 (0.83–1.51)	0.4479
Location					
Middle *vs* top	308/45	1.33 (0.78–2.26)	0.2983		
Bottom *vs* top	81/45	1.69 (0.94–3.01)	0.0816		
Resection margin					
Positive *vs* negative	8/426	2.50 (1.17–5.32)	0.0177	1.18 (0.52–2.66)	0.6989
Grade					
Moderate *vs* well	248/111	1.19 (0.83–1.68)	0.3427	1.08 (0.74–1.57)	0.6903
Poor *vs* well	75/111	1.73 (1.13–2.65)	0.0114	1.35 (0.87–2.11)	0.1818
T stage					
T1 *vs* T4	10/146	0.10 (0.01–0.69)	0.0198	0.14 (0.02–1.01)	0.0512
T2 *vs* T4	58/146	0.42 (0.25–0.69)	0.0006	0.46 (0.28–0.77)	0.0028

Parameters	No. p	Univariate		Multivariate	
		HR (95% CI)	P-value	HR (95% CI)	P-value
T3 *vs* T4	220/146	0.53 (0.39–0.72)	< 0.0001	0.59 (0.43–0.80)	0.0007
Lymph node metastasis					
N1 *vs* N0	230/204	2.31 (1.71–3.13)	< 0.0001	1.98 (1.44–2.72)	< 0.0001
Distant metastasis					
M1 *vs* M0	7/427	3.30 (1.35–8.06)	0.0090	2.18 (0.82–5.74)	0.1162
$CD3^+$ T cells					
High *vs* low	213/221	0.73 (0.55–0.98)	0.0336	0.71 (0.53–0.96)	0.0246
$CD8^+$ T cells					
High *vs* low	211/223	0.90 (0.68–1.20)	0.4758		

Discussion

Due to the ability of generating cytotoxic T lymphocytes (CTLs), CD8 + T cells have been considered the major anti-cancer effector cells.[20] Thus, it is biologically sound that a higher number of infiltrating CD8 + T cells correlates with favorable prognosis in many cancer types.[10–12] However, although there is an obvious correlation between infiltrating CD3 + and CD8 + cell numbers, our results indicate that infiltrating CD8 + T cells within tumors do not provide prognostic information for ESCC patients, whereas CD3 + T cell number is an independent prognostic factor.

For infiltrating CD8 + T cells, our results are consistent with a previous study,[19] but inconsistent with two other studies.[16,17] This inconformity suggests that infiltrating CD8 + T cells in esophageal tumors include different functional subgroups that are unable to be solely identified by IHC. Indeed, it has been reported that infiltrating bystander CD8 + T cells, which do not recognize tumor antigens and thus have no ability to kill neoplastic cells, exist in human lung and colorectal tumors.[21] For infiltrating CD3 + T cells, our results are consistent with the only available study[19] for ESCC. Both studies demonstrate that CD3 + T cell infiltration is an independent favourable prognostic factor. As discussed in the previous study,[19] CD3 + T cells represent the overall T cells infiltration within tumors and can be used to identify whether a specific tumor is in a T-cell-inflamed tumor microenvironment or not, which might be able to predict the response to immune checkpoint inhibitors.[22] Thus, for ESCC patients, measuring the number of

infiltrating CD3 + T cells is a more appropriate method than measuring CD8 + T cells for prognostic implications, indicating its potential in clinical application for cancer classification.

Our results shows both infiltrating CD3 + and CD8 + T cell numbers increase with age. This phenomenon has never been described in ESCC, and the potential mechanism remains elusive. Moreover, our results show well-differentiated tumors are associated with lower numbers of infiltrating CD3 + and CD8 + T cells, while moderately-differentiated tumors have highest numbers of infiltrating T cells. An early study[23] reported analogous associations between tumor grades and lymphocytic infiltration. A possible reason is that well-differentiated tumors are characterized by complete cancer cell nests constituting a physic barrier to hinder the infiltration of T cells.

There are some limitations in our study. First, although the sample size is bigger than several previous studies, the participants of our study were from a single hospital, which limits the representativeness of the present study. Second, only overall survival of patients was followed, without more specific information on relapse and disease-free survival.

Conclusions

Our study demonstrates that infiltrating CD3 + T cells number within tumors is an independent prognostic factor for ESCC patients, while CD8 + T cells has no prognostic implication. The promising TNM-immune classification system of cancer would also be appropriate for ESCC, but needs some modifications and more validation studies.

Abbreviations

EC
Esophageal cancer; ESCC:Esophageal squamous cell carcinoma; EAC:Esophageal adenocarcinoma; OS:Overall survival; IHC:Immunohistochemistry;

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shantou University Medical College (SUMC-2015-15). All patients or their families signed the informed consent.

Consent for publication

Not applicable.

Availability of data and materials

All data of this study are available from the corresponding author by reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by National Natural Science Foundation of China (81572684, 81772997) and Natural Science Foundation of Guangdong Province (2016A030313070).

Authors' contribution

PY and MS designed this study; PY, PX, YG, QG, and HY performed related experiments. HH and SC provided the clinical and prognostic information of patients. PY wrote the manuscript. DT and MS supervised the study. All authors approved the manuscript.

Acknowledgments

Thanks to professor Stanley Lin for his work on language editing.

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Figures

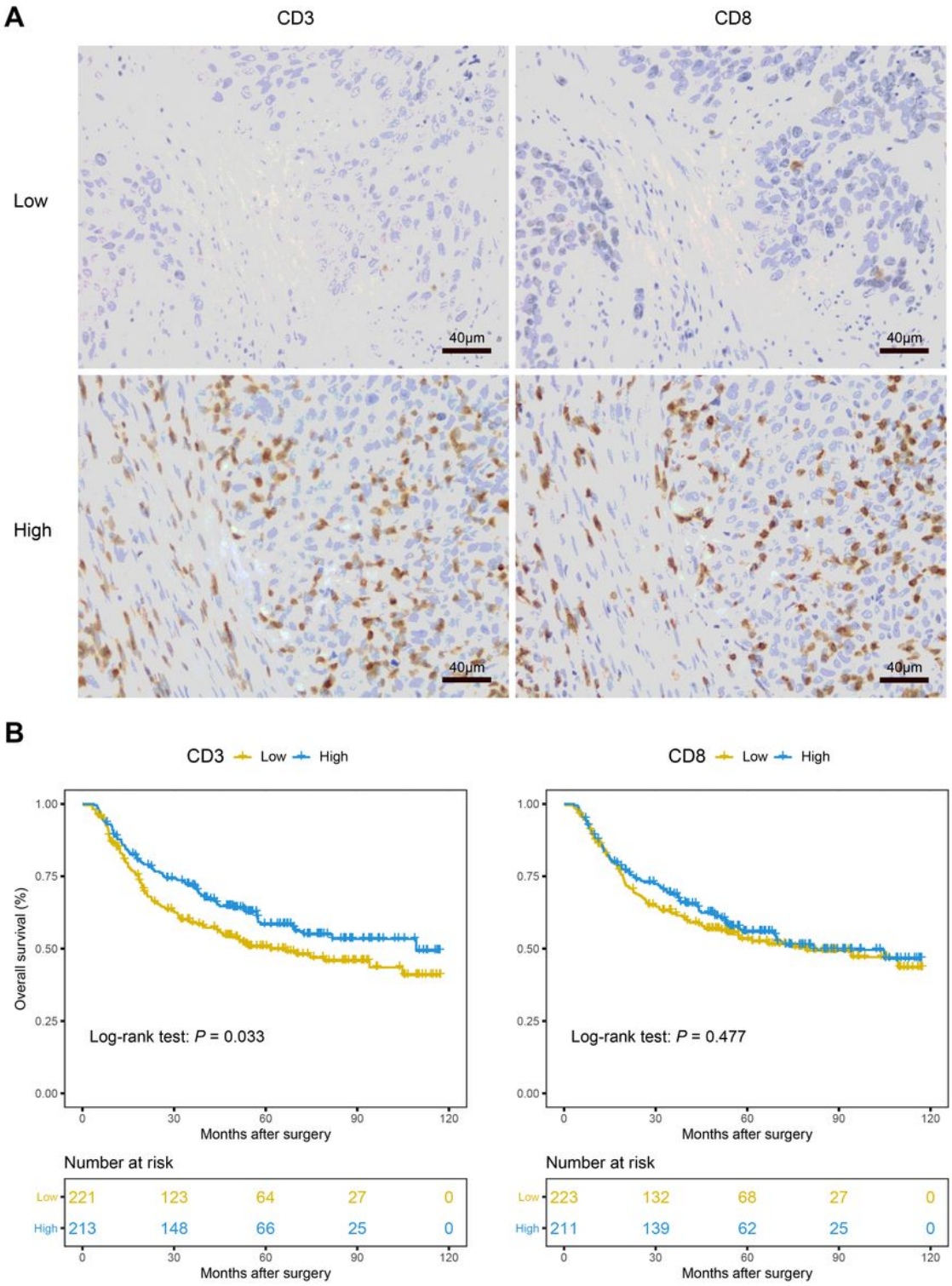


Figure 1

(A) Representative IHC images for CD3 (left) and CD8 (right) staining; (B) Overall survival for ESCC patients with different numbers of infiltrating CD3+ (left) and CD8+ T cells (right).