Tumor-Infiltrating CD4+ Lymphocytes Predict a Favorable Survival in Patients with Operable Esophageal Squamous Cell Carcinoma

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Background:
The immune status within the tumor microenvironment has not been well determined in esophageal squamous cell carcinoma (ESCC). The aim of this study was to investigate the distributions of tumor-infiltrating T lymphocytes (TILs), and analyze their associations with clinical characteristics and prognosis; as well as investigate the expression of programmed death-ligand 1 (PD-L1) which has been identified as a favorable indicator of prognosis in our previous study on ESCC.

Material/Methods:
Five hundred and thirty-six patients who underwent radical surgery for ESCC between January 2008 and April 2012 in Department of Thoracic Surgery at Zhejiang Cancer Hospital were included in the study. Immunohistochemistry was used to investigate the infiltration of various TILs (CD3+, CD4+, CD8+ T lymphocytes) in ESCC tissues. Chi-square test and Cox proportional hazards regression were used to explore the correlations between TILs abundance and clinicopathological variables and survival.

Results:
The infiltration of intraepithelial CD4+ (iCD4+) lymphocytes was markedly higher than it in the stromal region (44.2% for intraepithelial versus 28.9% for stromal, p<0.001). Moreover, increased iCD4+ lymphocytes were significantly associated with longer overall survival (OS, p=0.001) in univariate analysis and were identified as an independent predictor for improved OS in multivariate analysis (hazard ratio [HR]=0.67, 95% confidence interval [CI]: 0.51–0.88, p=0.040). Unexpectedly, combined with our previous study results, the TILs infiltration in ESCC showed an inverse association with the expression of PD-L1 (p=0.027).

Conclusions:
Our results suggested that iCD4+ lymphocytes infiltration could be a favorable indicator for prognosis in ESCC.

MeSH Keywords:
Antigens, CD4 • Esophageal Neoplasms • Immunochemistry • Survival Analysis • T-Lymphocytes, Helper-Inducer

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Background

Esophageal cancer is the sixth most common cause of cancer-related deaths worldwide [1]. Approximately, 70% of global esophageal cancer cases occur in China, where it is the fourth cause of cancer death [2]. Esophageal squamous cell carcinoma (ESCC) accounts for more than 90% of esophageal cancer cases in Chinese patients [3]. Despite recent improvements in therapy, the outcome of ESCC remains dismal [4,5].

Recent advances in cancer immunological therapeutic strategies have revealed the importance of signaling between programmed death-1 (PD-1), mainly expressed on antigen-experienced T cells, and its ligand PD-L1, expressed on tumor cells and antigen presenting cells [6]. The combination of PD-1 and PD-L1 is the key immune checkpoint receptor to inhibit T-cell activation [6], inducing impaired immune response and worse prognosis in various cancers [7,8]. Clinical trials have demonstrated that patients with malignant melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma benefit from anti-PD-1 and anti-PD-L1 therapy [9–11]. However, recent literature reported that patients with high expression of PD-1 and PD-L1 had better prognosis in breast cancer, metastatic melanoma, colorectal cancer, pulmonary squamous cell carcinoma, and ovarian cancer [12–16]. Moreover, only a fraction of patients respond to PD-1 or PD-L1 blockage [17,18]. Therefore, the role of PD-L1 as a predictive biomarker for immune treatment response in various solid tumors remains controversial [8,19,20].

We previously identified PD-L1 expression of 41.4% (222/536) in our ESCC cohort [21], and found that PD-L1 expression differed significantly by tumor location, grade, lymph node metastases, and disease stage [21]. Moreover, patients with positive PD-L1 expression had reduced risk for disease relapse compared to those without PD-L1 expression [21]. Our finding of an inverse association between the expression of this favorable predictor PD-L1 and lymph node metastasis prompted us to speculate that PD-L1 might be induced by an active immune response speculation that T-cell proliferation.

High numbers of tumor-infiltrating lymphocytes (TILs) have been repeatedly shown to provide a significant survival advantage in ESCC. Particularly the presence of T cells (CD3+) and various T cell subpopulations (e.g., CD4+, CD8+, CD103+) have been shown to be indicators of a better prognosis [22,23]. Considering that PD-L1 expression could be induced by activated T cells [24,25], we next explored the potential association between PD-L1 expression and TIL abundance in ESCC.

In the present study, we systematically investigated the clinical significance of tumor-infiltrating (intraepithelial and stromal) lymphocytes in patients using 536 ESCC tissue samples. The survival impacts of various immunological factors were also assessed.

Material and Methods

Study population

Five hundred and thirty-six patients who underwent radical surgery for ESCC between January 2008 and April 2012 in Department of Thoracic Surgery at Zhejiang Cancer Hospital were included in the study. No patients received neoadjuvant treatment or immunotherapy. The demographic and clinical data of patients including age, gender, tumor site, histological grade, lymph node status, pTNM stage, smoking experience, alcohol drinking, and family history were abstracted from clinical records. The extent of the disease was determined by pTNM staging based on the 7th IUC/AJCC recommendations. All tissue specimens used in our study were obtained from the tissue bank of Zhejiang Cancer Hospital and all patients provided informed consent before surgery. The protocol of the study was approved by the Institutional Review Board of Zhejiang Cancer Hospital.

Follow-up visits were performed every three months for the first two years after surgery, and every six months in the third year and yearly thereafter. At each visit, a clinical history was taken and a physical examination was performed. Routine diagnostic imaging methods included gastroscopy and computer tomography (CT). The median follow-up time was 32.7 months with a range from 1.0 to 88.7 months. The overall survival (OS) data were available for 451 patients (84.1%); among whom 261 patients (57.9%) died during follow-up. Disease free survival (DFS) data were available for 403 patients (75.2%), and 224 patients (41.8%) underwent disease relapse, of which 190 patients (84.8%) died.

Tissue microarray

Formalin-fixed paraffin-embedded (FFPE) surgical specimens were hematoxylin and eosin (H&E) stained, and ESCC was confirmed by two senior pathologists independently. The paraffin tissue blocks of 536 cases of esophageal cancer were used in the construction of the tissue microarray. In brief, the H&E-stained standard slides were reviewed from each section of esophageal cancer tissue, and one representative tumor area of each tumor (2 mm diameter) was removed from the FFPE tissue blocks. Tissue microarray was sectioned at 3 μm and mounted on glass slides for the immunohistochemistry analysis.

Immunohistochemistry analysis

Standard immunohistochemical analysis was performed against primary antibody directed CD3 (clone SP7, diluted at 1: 100, Abcam, Cambridge, UK), CD4 (clone B468A1, diluted at 1: 200, Santa Cruz, Texas, USA) and CD8 (clone 144B, diluted at 1: 100, Abcam, Cambridge, UK). Antigen retrieval was achieved using EDTA buffer (pH 9.0) in a pressure cooker for 20 minutes. After neutralization of endogenous peroxidase, tissue
microarray slides were pre-incubated with blocking serum and then were incubated with primary antibody for 45 minutes at room temperature. After three five-minute washes with PBS, the slides were treated with the horseradish peroxidase (HRP)-labeled secondary antibody (Dako, Glostrup, Denmark) for 20 minutes at room temperature, then washed in PBS. Finally, the reaction products were visualized with 3,3’-diaminobenzidine (DAB, Dako, Glostrup, Denmark) and the slides were counterstained with hematoxylin. After being dehydrated, slides were mounted in resin.

Evaluation of immunohistochemistry

The tumor sections were screened at low power magnification (100×), and high power fields (400×) were selected to evaluated the immunoreactivity. All immunostained slides were examined independently by two senior pathologists in a blind manner. The average of the values obtained by the two observers was recorded and used in the statistical analysis. In accordance with previously published approaches [26], intraepithelial TILs (iTILs) were defined as lymphocytes located within tumor cell nests or in direct contact with the ESCC epithelial cells, whereas stromal TILs (sTILs) were defined as lymphocytes in the adjacent peritumoral stroma without direct contact with the carcinoma cells. Areas of necrosis and artifacts, and cells within the vessels were omitted. All samples were scored according to the frequency of positive cells in all nucleated cells (as percentage) on the stained TMA core. The median leukocyte count was used as cutoff to categorize each case into either a high or low group. The tissues having no TILs were excluded from our study; hence data of 514 patients were used for statistical analysis.

Statistical analysis

Differences between groups were compared using the chi-square test or the Mann-Whitney U test for variables. Spearman’s coefficient rank test was used to evaluate the correlation between tumor-infiltrating T lymphocyte, and PD-L1 expression. Associations with DFS and OS were analyzed using the Kaplan-Meier method, log-rank test and the Cox proportional hazards model. All the statistical analyses were performed with SPSS 13.0 for Windows (Chicago, IL, USA), and p value ≤0.05 in a two-tailed test was considered statistically significant.

Results

Expression of TIL

The infiltrating T lymphocytes were categorized by location in the tumor intraepithelium (i) or stroma (s), and the representative images of CD3+, CD4+, CD8+ lymphocyte infiltration are shown in Figure 1. The expressions of CD3, CD4, and CD8 were found to be located on both the membrane and in the cytoplasm of TILs. A total of 536 patients were enrolled in this study. Among them, 40.9% (210 patients) had intraepithelial CD3 (iCD3) expression, and 39.1% (199 patients) had stromal CD3 (sCD3) expression, but no significant difference between the infiltration of iCD3+ and sCD3+ lymphocytes was found (p>0.05). Regarding CD8 expression in the 527 patients, 16.7% (88 patients) were intraepithelial expression, and 15.4% (81 patients) were stromal expression. Also, the accumulation of iCD8+ lymphocytes was not related to the sCD8+ lymphocytes (p>0.05). Importantly, the infiltration of iCD4+ lymphocytes was 44.2% (234/530), which was significantly higher than that in stromal regions (28.9%, 153/530, p<0.001).

Clinicopathological associations with TIL expression

The demographics of 536 patients are summarized in Table 1. The median age of the patients at diagnosis was 60 (range 37–77) years. Among these patients, 73.5% (394/536) were younger than 65 years, 86.0% (464/536) were male, 25.7% (138/536) had family history, 71.3% (382/536) were alcohol drinkers, and 75.2% (403/536) were smokers. Within the cohort, 35 patients (6.5%) had well differentiated tumors, 377 (70.3%) moderately differentiated tumors, and 124 (23.1%) poorly differentiated tumors. In accordance with the 7th IUCC/AICC staging system, 61 (11.4%) patients were stage I, 195 (36.4%) patients were stage II, 273 (50.9%) patients were stage III, and seven (1.3%) patients were stage IV. In the present study, we analyzed the correlations between the clinicopathological features and the infiltration of CD3+, CD4+, and CD8+ lymphocytes in ESCC tissues. As shown in Table 1–6, increased iCD3+, sCD4+, and iCD8+ lymphocytes were significantly associated with advanced tumor differentiation (p<0.05). Furthermore, high infiltrations of sCD4+ and sCD8+ lymphocytes were more frequently observed in patients with alcohol drinking and smoking experience (p<0.05). However, sCD3+ and iCD4+ lymphocytes had no significant correlation with any of the clinicopathological parameters (p>0.05).

Correlations between PD-L1 and TIL expression

Based on the fact that PD-L1 expression had close interactions with TILs in the tumor microenvironment [20], combining our previous study of an inverse association between PD-L1 expression and lymph node metastasis [21], we hypothesized that PD-L1 expression could be induced by increased T-cell infiltration. Then the relevance of PD-L1 expression and various TIL markers was investigated. Unexpectedly, results showed an inverse trend of PD-L1 expression and lymphocytes infiltration, especially in sCD3+ lymphocytes (Table 7, p=0.027). Correlation analysis revealed the similar results...
Though infiltrated T cells have been reported to induce better outcome in ESCC [23], the patients in our study with PD-L1 expression were more likely to have less T cells infiltration.

Prognostic impacts of the immune microenvironment

To identify variables of potential prognostic significance in the patients with ESCC, the impacts of TILs subgroup and other clinicopathological parameters on the prognosis were explored. Table 7 shows that increased iCD4+ lymphocytes were significantly associated with prolonged overall survival (OS) in ESCC ($r=-0.098, p=0.027$). Though infiltrated T cells have been reported to induce better outcome in ESCC [23], the patients in our study with PD-L1 expression were more likely to have less T cells infiltration.

### Discussion

In our previous study, we identified that PD-L1 could be a favorable indicator of prognosis in ESCC and patients with its expression were more likely to have less lymph node metastasis [21]. We thus hypothesized the positive prognostic effect of PD-L1 might be induced by increased T-cell infiltration. However, in this study, we detected an inverse association between immune T-cell abundance and PD-L1 expression in ESCC, indicating the interaction of PD-L1 and PD-1 might inhibit the functions of TILs.

Our findings in this study are in accordance with earlier reports about a significantly inverse correlation between PD-L1 expression and infiltrated T lymphocytes [27–29]. In addition, a study of NSCLC elucidated that the PD-L1 protein expression pattern was related to changeable T-cell density within the tumor microenvironment [30]. Therefore, the favorable prognostic effect of PD-L1 might be based on regulatory of the complicated immune network, like the activation of different immune cell subpopulations, like γδ T cells [31],
Table 1. Clinicopathological associations with iCD3 expression in ESCC.

| Category           | All cases | iCD3       | p Value |
|--------------------|-----------|------------|---------|
|                    | <65       | ≥65        |         |
| Age                | 139 (27.0%) | 75 (54.0%) | 64 (46.0%) | 0.145 |
| Gender             |           |            |         |
| Male               | 444 (86.4%) | 265 (59.7%) | 179 (40.3%) | 0.530 |
| Female             | 70 (13.6%)  | 39 (55.7%)  | 31 (44.3%)  |         |
| Tumor site         |           |            |         |
| Upper              | 15 (2.9%)   | 8 (53.3%)   | 7 (46.7%)   | 0.784 |
| Middle             | 146 (28.4%) | 84 (57.5%)  | 62 (42.5%)  |         |
| Lower              | 353 (68.7%) | 212 (60.1%) | 141 (39.9%) |         |
| Tumor grade        |           |            |         |
| Well               | 32 (6.2%)   | 23 (71.9%)  | 9 (28.1%)   | 0.020 |
| Moderate           | 362 (70.4%) | 222 (61.3%) | 140 (38.7%) |         |
| Poor               | 120 (23.3%) | 59 (49.2%)  | 61 (50.8%)  |         |
| Tumor stage        |           |            |         |
| I                  | 58 (11.3%)  | 33 (56.9%)  | 25 (43.1%)  | 0.544 |
| II                 | 188 (36.6%) | 115 (61.2%) | 73 (38.8%)  |         |
| III                | 262 (51.0%) | 151 (57.6%) | 111 (42.4%) |         |
| IV                 | 6 (1.2%)    | 5 (83.3%)   | 1 (16.7%)   |         |
| Family history     |           |            |         |
| Yes                | 132 (25.7%) | 73 (55.3%)  | 59 (44.7%)  | 0.298 |
| No                 | 382 (74.3%) | 231 (60.5%) | 151 (39.5%) |         |
| Alcohol history    |           |            |         |
| Yes                | 363 (70.6%) | 208 (57.3%) | 155 (42.7%) | 0.187 |
| No                 | 151 (29.4%) | 96 (63.6%)  | 55 (36.4%)  |         |
| Smoking history    |           |            |         |
| Yes                | 386 (75.1%) | 234 (60.6%) | 152 (39.4%) | 0.237 |
| No                 | 128 (24.9%) | 70 (54.7%)  | 58 (45.3%)  |         |
| BMI                |           |            |         |
| <18                | 70 (13.6%)  | 38 (54.3%)  | 32 (45.7%)  | 0.274 |
| 18–25              | 395 (76.8%) | 241 (61.0%) | 154 (39.0%) |         |
| >25                | 49 (9.5%)   | 25 (51.0%)  | 24 (49.0%)  |         |
| Total              | 514         | 304 (59.1%) | 210 (40.9%) |         |

Bold-italic value was statistically significant (p≤0.05).
Table 2. Clinicopathological associations with sCD3 expression in ESCC.

| Category                 | All cases | sCD3 | p Value |
|--------------------------|-----------|------|---------|
|                          |           | –    | +       |
| Age                      |           |      |         |
| <65                      | 139 (27.3%) | 82 (59.0%) | 57 (41.0%) | 0.588 |
| ≥65                      | 370 (72.7%) | 228 (61.6%) | 142 (38.4%) |         |
| Gender                   |           |      |         |
| Male                     | 439 (86.2%) | 268 (61.0%) | 171 (39.0%) | 0.867 |
| Female                   | 70 (13.8%) | 42 (60.0%) | 28 (40.0%) |         |
| Tumor site               |           |      |         |
| Upper                    | 15 (2.9%) | 7 (46.7%) | 8 (53.3%) | 0.276 |
| Middle                   | 146 (28.7%) | 84 (57.5%) | 62 (42.5%) |         |
| Lower                    | 348 (68.4%) | 219 (62.9%) | 129 (37.1%) |         |
| Tumor grade              |           |      |         |
| Well                     | 31 (6.1%) | 19 (61.3%) | 12 (38.7%) | 0.653 |
| Moderate                 | 361 (70.9%) | 224 (62.0%) | 137 (38.0%) |         |
| Poor                     | 117 (23.0%) | 67 (57.3%) | 50 (42.7%) |         |
| Tumor stage              |           |      |         |
| I                        | 58 (11.4%) | 35 (60.3%) | 23 (39.7%) | 0.212 |
| II                       | 187 (36.7%) | 117 (62.6%) | 70 (37.4%) |         |
| III                      | 358 (70.3%) | 218 (60.9%) | 140 (39.1%) |         |
| IV                       | 6 (1.2%) | 6 (100.0%) | 0 (0.0%) |         |
| Family history           |           |      |         |
| Yes                      | 129 (25.3%) | 70 (54.3%) | 59 (45.7%) | 0.074 |
| No                       | 380 (74.7%) | 240 (63.2%) | 140 (36.8%) |         |
| Smoking history          |           |      |         |
| Yes                      | 358 (70.3%) | 218 (60.9%) | 140 (39.1%) | 0.247 |
| No                       | 151 (29.7%) | 92 (60.9%) | 55 (39.1%) |         |
| BMI                      |           |      |         |
| <18                      | 70 (13.8%) | 40 (57.1%) | 30 (42.9%) | 0.176 |
| 18–25                    | 391 (76.8%) | 246 (62.9%) | 145 (37.1) |         |
| >25                      | 48 (9.4%) | 24 (50.0%) | 24 (50.0%) |         |
| Total                    | 509 (100%) | 310 (60.9%) | 199 (39.1%) |         |
| Category          | All cases | iCD4 | p Value |
|-------------------|-----------|------|---------|
|                   |           | –    | +       |
| Age               |           |      |         |
| <65               | 141       | 73   | 68      | 0.255   |
| ≥65               | 389       | 223  | 166     |         |
| Gender            |           |      |         |
| Male              | 459       | 260  | 199     | 0.348   |
| Female            | 71        | 36   | 35      |         |
| Tumor site        |           |      |         |
| Upper             | 16        | 7    | 9       | 0.586   |
| Middle            | 152       | 87   | 65      |         |
| Lower             | 362       | 202  | 160     |         |
| Tumor grade       |           |      |         |
| Well              | 35        | 18   | 17      | 0.784   |
| Moderate          | 372       | 211  | 161     |         |
| Poor              | 123       | 67   | 56      |         |
| Tumor stage       |           |      |         |
| I                 | 59        | 31   | 28      | 0.264   |
| II                | 193       | 102  | 91      |         |
| III               | 271       | 157  | 114     |         |
| IV                | 7         | 6    | 1       |         |
| Family history    |           |      |         |
| Yes               | 134       | 76   | 58      | 0.815   |
| No                | 396       | 220  | 176     |         |
| Alcohol history   |           |      |         |
| Yes               | 377       | 212  | 165     | 0.780   |
| No                | 153       | 84   | 69      |         |
| Smoking history   |           |      |         |
| Yes               | 399       | 225  | 174     | 0.661   |
| No                | 131       | 71   | 60      |         |
| BMI               |           |      |         |
| <18               | 71        | 37   | 34      | 0.733   |
| 18–25             | 407       | 231  | 176     |         |
| >25               | 52        | 28   | 24      |         |
| Total             | 530       | 296  | 234     | 0.442   |

Table 3. Clinicopathological associations with iCD4 expression in ESCC.
Table 4. Clinicopathological associations with sCD4 expression in ESCC.

| Category          | All cases | sCD4 | p Value |
|-------------------|-----------|------|---------|
|                   |           | –    | +       |
| **Age**           |           |      |         |
| <65               | 141 (26.6%) | 96 (68.1%) | 45 (31.9%) | 0.351 |
| ≥65               | 389 (73.4%) | 281 (72.2%) | 108 (27.8%) |
| **Gender**        |           |      |         |
| Male              | 459 (86.6%) | 323 (70.4%) | 136 (29.6%) | 0.325 |
| Female            | 71 (13.4%) | 54 (76.1%) | 17 (23.9%) |
| **Tumor site**    |           |      |         |
| Upper             | 16 (3.0%) | 13 (81.3%) | 3 (18.7%) | 0.264 |
| Middle            | 152 (28.7%) | 114 (75.0%) | 38 (25.0%) |
| Lower             | 362 (68.3%) | 250 (69.1%) | 112 (30.9%) |
| **Tumor grade**   |           |      |         |
| Well              | 35 (6.6%) | 27 (77.1%) | 8 (22.9%) | **0.017** |
| Moderate          | 372 (70.2%) | 275 (73.9%) | 97 (26.1%) |
| Poor              | 123 (23.2%) | 75 (61.0%) | 48 (39.0%) |
| **Tumor stage**   |           |      |         |
| I                 | 59 (11.1%) | 40 (67.8%) | 19 (32.2%) | 0.323 |
| II                | 193 (36.4%) | 140 (72.5%) | 53 (27.5%) |
| III               | 271 (51.1%) | 190 (70.1%) | 81 (29.9%) |
| IV                | 7 (1.3%) | 7 (100.0%) | 0 (0.0%) |
| **Family history** |           |      |         |
| Yes               | 134 (25.3%) | 95 (70.9%) | 39 (29.1%) | 0.944 |
| No                | 396 (74.7%) | 282 (71.2%) | 114 (28.8%) |
| **Alcohol history** |           |      |         |
| Yes               | 377 (71.1%) | 257 (68.2%) | 120 (31.8%) | **0.018** |
| No                | 153 (28.9%) | 120 (78.4%) | 33 (21.6%) |
| **Smoking history** |           |      |         |
| Yes               | 399 (75.3%) | 275 (68.9%) | 124 (31.1%) | **0.050** |
| No                | 131 (24.7%) | 102 (77.9%) | 29 (22.1%) |
| **BMI**           |           |      |         |
| <18               | 71 (13.4%) | 48 (67.6%) | 23 (32.4%) | 0.527 |
| 18–25             | 407 (76.8%) | 289 (71.0%) | 118 (29.0%) |
| >25               | 52 (9.8%) | 40 (76.9%) | 12 (423.1%) |
| **Total**         | 530 | 377 (71.1%) | 153 (28.9%) |

Bold-italic value was statistically significant (p≤0.05).
### Table 5. Clinicopathological associations with iCD8 expression in ESCC.

| Category               | All cases | iCD8 | p Value |
|------------------------|-----------|------|---------|
|                        |           | –    | +       |         |
| **Age**                |           |      |         |         |
| <65                    | 140 (26.6%) | 117 (83.6%) | 23 (16.4%) | 0.920   |
| ≥65                    | 387 (73.4%) | 322 (83.2%) | 65 (16.8%)  |         |
| **Gender**             |           |      |         |         |
| Male                   | 458 (86.9%) | 385 (84.1%) | 73 (15.9%)  | 0.228   |
| Female                 | 69 (13.1%)  | 54 (78.3%)  | 15 (21.7%)  |         |
| **Tumor site**         |           |      |         |         |
| Upper                  | 16 (3.0%)   | 14 (87.5%)  | 2 (12.5%)   | 0.608   |
| Middle                 | 151 (28.7%) | 129 (85.4%) | 22 (14.6%)  |         |
| Lower                  | 360 (68.3%) | 296 (82.2%) | 64 (17.8%)  |         |
| **Tumor grade**        |           |      |         |         |
| Well                   | 34 (6.5%)    | 34 (100.0%) | 0 (0.0%)    | 0.004   |
| Moderate               | 370 (70.2%) | 311 (84.1%) | 59 (15.9%)  |         |
| Poor                   | 123 (23.3%) | 94 (76.4%)  | 29 (23.6%)  |         |
| **Tumor stage**        |           |      |         |         |
| I                      | 59 (11.2%)  | 50 (84.7%)  | 9 (15.3%)   | 0.958   |
| II                     | 193 (36.6%) | 162 (83.9%) | 31 (36.1%)  |         |
| III                    | 268 (50.9%) | 221 (82.5%) | 47 (17.5%)  |         |
| IV                     | 7 (1.3%)    | 6 (85.7%)   | 1 (14.3%)   |         |
| **Family history**     |           |      |         |         |
| Yes                    | 133 (25.2%) | 112 (84.2%) | 21 (15.8%)  | 0.745   |
| No                     | 394 (74.8%) | 327 (83.0%) | 67 (17.0%)  |         |
| **Alcohol history**    |           |      |         |         |
| Yes                    | 376 (71.3%) | 310 (82.4%) | 66 (17.6%)  | 0.406   |
| No                     | 151 (28.7%) | 129 (85.4%) | 22 (14.6%)  |         |
| **Smoking history**    |           |      |         |         |
| Yes                    | 397 (75.3%) | 332 (83.6%) | 65 (16.4%)  | 0.726   |
| No                     | 130 (24.7%) | 107 (82.3%) | 23 (17.7%)  |         |
| **BMI**                |           |      |         |         |
| <18                    | 71 (13.5%)  | 55 (54.3%)  | 16 (45.7%)  | 0.365   |
| 18–25                  | 404 (76.7%) | 340 (84.1%) | 64 (15.9%)  |         |
| >25                    | 52 (9.9%)   | 44 (51.0%)  | 8 (49.0%)   |         |
| **Total**              | 527        | 439 (83.3%) | 88 (16.7%)  |         |

Bold-italic value was statistically significant (p≤0.05).
**Table 6.** Clinicopathological associations with sCD8 expression in ESCC.

| Category          | All cases | sCD8 | p Value |
|-------------------|-----------|------|---------|
|                   | –        | +    |         |
| **Age**           |          |      |         |
| <65               | 140 (26.6%) | 120 (85.7%) | 20 (14.3%) | 0.678 |
| ≥65               | 387 (73.4%) | 326 (84.2%) | 61 (15.8%) | |
| **Gender**        |          |      |         |
| Male              | 458 (86.9%) | 384 (83.8%) | 74 (16.2%) | 0.197 |
| Female            | 69 (13.1%) | 62 (89.9%) | 7 (10.1%) | |
| **Tumor site**    |          |      |         |
| Upper             | 16 (3.0%) | 13 (81.3%) | 3 (18.8%) | 0.665 |
| Middle            | 151 (28.7%) | 131 (86.8%) | 20 (13.2%) | |
| Lower             | 360 (68.3%) | 302 (83.9%) | 58 (16.1%) | |
| **Tumor grade**   |          |      |         |
| Well              | 123 (23.3%) | 104 (84.6%) | 19 (15.4%) | 0.832 |
| Moderate          | 370 (70.2%) | 312 (84.3%) | 58 (15.7%) | |
| Poor              | 34 (6.5%) | 30 (88.2%) | 4 (11.8%) | |
| **Tumor stage**   |          |      |         |
| I                 | 59 (11.2%) | 49 (83.1%) | 10 (16.9%) | 0.701 |
| II                | 193 (36.6%) | 164 (85.0%) | 29 (15.0%) | |
| III               | 268 (50.9%) | 226 (84.3%) | 42 (15.7%) | |
| IV                | 7 (1.3%) | 7 (1.6%) | 0 (0.0%) | |
| **Family history**|          |      |         |
| Yes               | 133 (25.2%) | 113 (85.0%) | 20 (15.0%) | 0.902 |
| No                | 394 (74.8%) | 333 (84.5%) | 61 (15.5%) | |
| **Alcohol history**|        |      |         |
| Yes               | 376 (71.3%) | 309 (82.3%) | 67 (17.8%) | **0.014** |
| No                | 151 (28.7%) | 137 (90.7%) | 14 (9.3%) | |
| **Smoking history**|         |      |         |
| Yes               | 397 (75.3%) | 328 (82.6%) | 69 (17.4%) | **0.025** |
| No                | 130 (24.7%) | 118 (90.8%) | 12 (9.2%) | |
| **BMI**           |          |      |         |
| <18               | 71 (13.5%) | 61 (85.9%) | 10 (14.1%) | 0.658 |
| 18–25             | 404 (76.7%) | 339 (83.9%) | 65 (16.1%) | |
| >25               | 52 (9.9%) | 46 (88.5%) | 6 (11.5%) | |
| **Total**         | 527       | 446 (84.6%) | 81 (15.4%) | |

Bold-italic value was statistically significant (p≤0.05).
and the secretion of certain cytokines such as IL-10 and interferon γ [32–34]. Meanwhile, the prognostic significance of PD-L1 has been proven to be relevant to the mutational burden [35], inducing increased presentation of neo-antigens by tumor cells [17]. However, other studies about the presence of TILs in human tumor systems including ESCC have showed a positive or no association with PD-L1 expression [33,36–38]. Taken together, the regulatory effect of the complicated immune network in the tumor microenvironment has not been fully determined until now.

In the present study, we also explored the impact of tumor-infiltrating lymphocytes on the clinical significance in ESCC. The cooperation of CD4+ and CD8+ T cells has been well-documented in host immune responses against various tumors including ESCC [22,23]. In our study, the total number of CD4+ TILs in intraepithelium was markedly higher than those in stromal areas. Consistent with our study, Hatogai et al. also found intraepithelial TILs had significantly increased levels compared to stromal compartments, conferring more clinical prognostic value in ESCC [38]. In line with previous reports in ovarian carcinoma [39], we observed that patients with increased infiltration of iCD4+ lymphocytes had longer OS compared to those with decreased infiltration, while sCD4+ lymphocytes was not correlated with the outcomes in ESCC.

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### Table 7. The associations of T cells infiltration with programmed death-ligand 1 (PD-L1) expression and the survival of esophageal squamous cell carcinoma patients.

| Variables | NO. of patients (%) | PD-L1 expression | | | | |
|-----------|---------------------|------------------|---|---|---|---|
|           | Positive | Negative | Positive | Negative | Positive | Negative |
|           |          |          |       |          |          |          |
| Intraepithelium | CD3+ T cell | Positive | 210 (40.9) | 81 (38.6) | 129 (61.4) | 129 (61.4) | 0.143 | 1.008 | 0.978 | 0.954 | 0.847 |
|           | Negative | 304 (59.1) | 137 (45.1) | 167 (54.9) | (0.596–1.702) | (0.589–1.544) |
|           | CD4+ T cell | Positive | 234 (44.2) | 98 (41.9) | 136 (58.1) | 0.940 | 0.664 | 0.130 | 0.517 | 0.007 |
|           | Negative | 296 (55.8) | 123 (41.6) | 173 (58.4) | (0.392–1.127) | (0.319–0.838) |
|           | CD8+ T cell | Positive | 88 (16.7) | 29 (33.0) | 59 (67.0) | 0.079 | 0.731 | 0.325 | 0.943 | 0.841 |
|           | Negative | 439 (83.3) | 189 (43.1) | 250 (56.9) | (0.391–1.365) | (0.533–1.670) |
| Stroma | CD3+ T cell | Positive | 199 (39.1) | 72 (36.2) | 127 (63.8) | (0.027) | 0.935 | 0.771 | 1.061 | 0.802 |
|           | Negative | 310 (60.9) | 143 (46.1) | 167 (53.9) | (0.594–1.472) | (0.670–1.680) |
|           | CD4+ T cell | Positive | 153 (28.9) | 60 (39.2) | 93 (60.8) | 0.460 | 0.431 | 0.418 | 0.398 | 0.373 |
|           | Negative | 377 (71.1) | 161 (42.7) | 216 (57.3) | (0.056–3.307) | (0.052–3.029) |
|           | CD8+ T cell | Positive | 81 (15.4) | 31 (38.3) | 50 (61.7) | 0.539 | 0.873 | 0.597 | 0.651 | 0.071 |
|           | Negative | 446 (84.6) | 187 (41.9) | 259 (58.1) | (0.528–1.444) | (0.408–1.037) |

Bold-italic values were statistically significant (p<0.05); HR – hazard ratio; CI – confidence interval.
Th1 subset is active in secreting pro-inflammatory cytokines, whereas a pro-tumorigenic effect can be predominant when Th2 cells secrete anti-inflammatory cytokines [40]. However, in our study, no correlations between the infiltration of iCD3+ lymphocytes and sCD3+ lymphocytes and their impacts on the prognosis were found. And iCD8+ and sCD8+ lymphocytes infiltration was also not associated with clinical outcome in ESCC. Notably, one study found increased CD8+ TILs infiltration were independent favorable prognostic factors in esophageal adenocarcinoma [41], while another study indicated that the TILs were not the prognostic markers for long-term survival in patients with esophageal adenocarcinoma [42]. These controversial results were likely caused by factors in the tissue microenvironment which may alter T-cell phenotype and function early during esophageal disease progression and may represent targets for immune intervention [43]. Further investigations regarding the impact on clinical outcomes of the balance of immune infiltrating cells are needed.

Additionally, the presence of stromal TILs infiltration more than intraepithelial infiltration was correlated with alcohol drinking and smoking experience. As previously described, smoking history of a patient was associated with a greater risk of cancer development than patients without those characteristics, and were characterized by abundant and deregulated inflammation [44], suggesting a linkage between inflammatory processes affecting the smokers or alcohol drinkers and immune response status of tumors [30]. The selective recruitment of T cells to the stromal compartment of these esophageal cancers suggests that these stromal TILs are less affected by tumor-derived inhibitory factors than in the intraepithelial regions, which are located in direct contact with malignant cells [30]. Unlike an earlier study [22], we observed patients with increased TILs were more likely to harbor poor tumor differentiation; meanwhile, some other authors reported no association of TILs accumulation with tumor grade of ESCC [23,38]. Potential explanations for this finding might be induced from a misbalance of the effect of tumor-derived factors and immune responses.

Based on a large cohort of operable ESCC patients, our study systematically described the immune status in the tumor microenvironment. To our knowledge, this is the first study that has indicated that increased iCD4+ lymphocytes infiltration was...
Table 8. Prognostic factors for disease free survival and overall survival of operable ESCC patients estimated by multivariate Cox regression analyses.

| Parameters                  | DFS                  | OS                  |
|-----------------------------|----------------------|---------------------|
|                             | HR (95% CI) | p Value  | HR (95% CI) | p Value  |
| Intraepithelial CD3+ T cell | Negative | 1.00     | 1.00       |          |
|                             | Positive  | 1.01 (0.60–1.70)  | 0.978  | 1.26 (0.76–2.11)  | 0.371  |
| Stromal CD3+ T cell         | Negative | 1.00     | 1.00       |          |
|                             | Positive  | 0.94 (0.60–1.47)  | 0.771  | 1.17 (0.78–1.75)  | 0.442  |
| Intraepithelial CD4+ T cell | Negative | 1.00     | 1.00       |          |
|                             | Positive  | 0.66 (0.40–1.13)  | 0.130  | 0.67 (0.51–0.88)  | 0.004  |
| Stromal CD4+ T cell         | Negative | 1.00     | 1.00       |          |
|                             | Positive  | 0.43 (0.06–3.31)  | 0.418  | 0.41 (0.05–3.31)  | 0.386  |
| Intraepithelial CD8+ T cell | Negative | 1.00     | 1.00       |          |
|                             | Positive  | 0.73 (0.39–1.37)  | 0.325  | 0.89 (0.50–1.58)  | 0.325  |
| Stromal CD8+ T cell         | Negative | 1.00     | 1.00       |          |
|                             | Positive  | 0.87 (0.53–1.44)  | 0.597  | 0.65 (0.40–1.04)  | 0.075  |

Bold-italic value was statistically significant (p ≤ 0.05); HR – hazard ratio; CI – confidence interval; Adjusting for age, sex, tumor site, stage, grade, smoking experience, PD-L1 expression, alcohol drinking and family history.

Conclusions

The iCD4+ lymphocytes infiltration is an independent favorable factor for prognosis in ESCC. The TILs infiltration in ESCC tissues was inversely associated with the expression of PD-L1. Therefore, the profiles of immune cells may provide additional information for patient outcomes and help to stratify patients who will benefit more from immunotherapy.

References:

1. Chen W, Zheng R, Baade PD et al: Cancer statistics in China, 2015. Cancer J Clin, 2016; 66(2): 115–32
2. Song Y, Li L, Ou Y et al: Identification of genomic alterations in oesophageal squamous cell cancer. Nature, 2014; 509(7498): 91–95
3. Xu Y, Yu X, Chen Q, Mao W: Neoadjuvant versus adjuvant treatment: which one is better for resectable esophageal squamous cell carcinoma? World J Surg Oncol, 2012; 10: 173
4. Kleinberg L, Forastiere AA: Chemoradiation in the management of esophageal cancer. J Clin Oncol, 2007; 25(26): 4110–17
5. Han X, Lu N, Pan Y, Xu J: Nimotuzumab combined with chemotherapy is a promising treatment for locally advanced and metastatic esophageal cancer. Med Sci Monit, 2017; 23: 412–18
6. Pardoll DM: The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer, 2012; 12(4): 252–64
7. Reiss KA, Forde PM, Brahmer JR: Harnessing the power of the immune system via blockade of PD-1 and PD-L1: A promising new anticancer strategy. Immunotherapy, 2014; 6(4): 459–75

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25. Kondo A, Yamashita T, Tamura H et al: Interferon-gamma and tumor necrosis factor-alpha induce an immunoinhibitory molecule, B7-H1, via nuclear factor-kappaB activation in blasts in myelodysplastic syndromes. Blood, 2010; 116(7): 1124–31

26. Wang J, Jia Y, Wang N et al: The clinical significance of tumor-infiltrating neutrophils and neutrophil-lymphocyte ratio in non-small cell lung cancer patients. Cancer Res, 2016; 76(22): 6739–49

27. Hamaishi I, Mandai M, Iwasaki M et al: Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci USA, 2007; 104(9): 3360–65

28. Karunarathne DS, Horne-Debets JM, Huang JX et al: Programmed death-1 ligand 2-mediated regulation of the PD-L1 to PD-1 axis is essential for establishing CD4+ T cell immunity. Immunol, 2016; 45(2): 333–45

29. Dong H, Strome SE, Salomao DR et al: Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med, 2002; 8(6): 793–800

30. Parra ER, Beherens C, Rodriguez-Canales J et al: Image analysis-based assessment of PD-L1 and tumor-associated immune cell density supports distinct intratumoral microenvironment groups in non-small cell lung cancer patients. Clin Cancer Res, 2016; 22(24): 6278–89

31. Iwasaki M, Tanaka Y, Kobayashi H et al: Expression and function of PD-1 in human gammadelta T cells that recognize phosphoantigens. J Immunol, 2011; 181(2): 345–55

32. Kinter AL, Godbout EJ, McNally JP et al: The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. J Immunol, 2008; 181(10): 6738–46

33. Ohigashi Y, Sho M, Yamada Y et al: Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. Clin Cancer Res, 2005; 11(8): 2947–53

34. Wang S, Bajorath J, Files DB et al: Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. J Exp Med, 2003; 197(9): 1083–91

35. Le DT, Uram JN, Wang H et al: PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med, 2015; 372(6): 2509–20

36. Lipson EL, Vincent IG, Loyo M et al: PD-1 expression in the Merkel cell carcinoma microenvironment: association with inflammation, Merkel cell polyomavirus and overall survival. Cancer Immunol Res, 2013; 1(1): S4–63

37. Kim MV, Koh I, Kim S et al: Clinopathological analysis of PD-L1 and PD-L2 expression in pulmonary squamous carcinoma: Comparison with tumor-infiltrating T cells and the status of oncogenic drivers. Lung Cancer, 2015; 89: 126–32

38. Hatogai K, Kitano S, Fuji S et al: Comprehensive immunohistochimical analysis of tumor microenvironment immune status in esophageal squamous cell carcinoma. Oncotarget, 2015; 6(10): 4752–64

39. Clarke B, Tinker AV, Lee CH et al: Intraepithelial T cells and prognosis in ovarian carcinoma: novel associations with stage, tumor type, and BRCA1 loss. Mod Pathol, 2009; 22(3): 393–402

40. Crotty S: Follicular helper CD4 T cells (TFH). Annu Rev Immunol, 2011; 29: 333–45

41. Noble F, Mellows T, McCormath Matthews LH et al: Tumor infiltrating lymphocytes correlate with improved survival in patients with oesophageal adenocarcinoma. Cancer Immunol Immunother, 2016; 65(6): 651–62

42. Zingg U, Montani M, Frey DM et al: Tumour-infiltrating lymphocytes and survival in patients with adenocarcinoma of the oesophagus. Eur J Surg Oncol, 2010; 36(7): 670–77

43. Kavanagh ME, Conroy MJ, Clarke NE et al: Impact of the inflammatory microenvironment on T-cell phenotype in the progression from reflux oesophagitis to Barrett oesophagus and oesophageal adenocarcinoma. Cancer Lett, 2016; 370(1): 117–24

44. Chung KF, Adcock IM: Multifaceted mechanisms in COPD: Inflammation, immunity, and tissue repair and destruction. Eur Respir J, 2008; 31(6): 1334–56