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Original Article

Immunoscore based on CD3$^+$ and cytotoxic CD8$^+$ T-lymphocyte densities predicts prognosis and chemotherapy efficacy in esophageal squamous cell carcinoma

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Abstract: The current American Joint Committee on Cancer/Union for International Cancer Control tumor-node-metastasis (AJCC/UICC-TNM) staging system for esophageal squamous cell carcinoma (ESCC) does not incorporate an immune-based classification. This study aimed to evaluate the prognostic value of the Immunoscore (IS), based on the densities of CD3$^+$ and cytotoxic CD8$^+$ T lymphocytes, and its ability to predict the efficacy of chemotherapy in patients with ESCC. The IS was determined by immunohistochemical staining for CD3 and CD8 and was calculated according to the densities of CD3$^+$ and CD8$^+$ cells in the tumor center (CT) and invasive margin (IM). A total of 255 patients with previously untreated ESCC who underwent curative resection were randomly assigned to training and validation sets. Among them, 169 received postoperative chemotherapy. Recurrence-free survival (RFS) and overall survival (OS) were the endpoints of the prognostic analyses. In the training set, patients with a high IS had the lowest risk of recurrence. The 5-year RFS rates were 9.7%, 44.7%, and 90.1% among patients with low, intermediate, and high IS values, respectively (hazard ratio [HR] for high vs. low IS, 0.29; P<0.001). A high IS was also significantly associated with prolonged OS (P<0.001). Multivariable Cox regression analysis showed that the associations of IS with RFS and OS were independent of clinicopathological variables (HR for high vs. low IS: 0.29 for RFS and 0.53 for OS; both P<0.001). The IS accounted for the largest proportion of the total χ^2 statistic in predicting both RFS and OS (P<0.001). The IS was also significantly associated with RFS and OS among patients who received postoperative chemotherapy (both P<0.001). These findings were further confirmed in the validation set. The IS provides more accurate prognostic stratification and prediction of chemotherapy efficacy. It may complement the AJCC/UICC-TNM staging system in patients with operable ESCC.

Keywords: Immunoscore, esophageal squamous cell carcinoma, chemotherapy, prognosis

Introduction

Esophageal cancer (EC) is the eleventh most common malignancy and the seventh leading cause of cancer-related death worldwide [1]. Esophageal squamous cell carcinoma (ESCC) is one of the major histological subtypes of EC, accounting for approximately 90% of all cases [2]. The management of ESCC remains challenging, primarily because of its aggressive biological behavior [3]. Therefore, innovative approaches are urgently needed to improve prognostic assessment and treatment decision-making for patients with ESCC [4].

The current staging system for ESCC is the AJCC/UICC-TNM staging system [5]. However, patients with the same TNM stage may experience markedly different clinical outcomes despite receiving standardized treatment, highlighting an important limitation of this system. Moreover, the current TNM classification does not account for the antitumor immune response of patients with ESCC. Incorporating an

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immune-based prognostic scoring system, such as the Immunoscore (IS), may help overcome this limitation.

Cancer cells interact with and modulate the immune system, disrupting the balance between tumor progression and host immune surveillance [6-8]. In recent years, tumor-infiltrating lymphocytes (TILs) have been increasingly recognized as favorable prognostic indicators in various cancers, reflecting the essential role of the tumor microenvironment (TME) in cancer progression [9]. Extensive evidence has demonstrated that infiltration by T cells, including $CD3^+$ T cells and cytotoxic $CD8^+$ T lymphocytes, is associated with prolonged disease-free survival (DFS) and/or overall survival (OS) across cancers with diverse histological characteristics and anatomical origins, including squamous cell carcinoma, melanoma, large-cell lung cancer, urothelial carcinoma [10], and adenocarcinoma [11].

In ESCC, the TME has been recognized as a key determinant of antitumor immunity and therapeutic response [12]. Cytotoxic $CD8^+$ T lymphocytes have been identified as an independent prognostic factor in patients with EC [13]. High densities of $CD3^+$ and $CD8^+$ T cells have also been observed in esophageal adenocarcinoma [14]. In addition, cytotoxic $CD8^+$ T-cell infiltration is involved in the regulatory mechanisms underlying ESCC metastasis [15]. Chemotherapy-resistant tumor cells may evade immune recognition by restricting cytotoxic $CD8^+$ T-cell infiltration [16]. Furthermore, $CD3^+$ and cytotoxic $CD8^+$ T cells have demonstrated prognostic value in patients with ESCC treated with immune checkpoint inhibitors [17].

However, the prognostic and chemotherapy-predictive value of an IS based on the densities of $CD3^+$ and cytotoxic $CD8^+$ T cells in ESCC has rarely been investigated and remains unclear. Therefore, this study aimed to evaluate the clinical utility of the IS for stratifying patients with ESCC and to determine its value in predicting prognosis and chemotherapy efficacy.

Methods

Patient population

Data from patients with ESCC who underwent surgical resection at the 989th Hospital of the Joint Logistic Support Force of the Chinese People's Liberation Army (Luoyang, China) between August 2016 and September 2022 were retrieved from the records of the Departments of Cardiothoracic Surgery and Pathology. The inclusion criteria were as follows: histologically confirmed ESCC, sporadic disease, and the availability of complete clinical information and tissue specimens. The exclusion criteria were recurrent cancer, metastatic cancer, malignancies other than ESCC or systemic malignant disease, and receipt of preoperative neoadjuvant therapy.

The tissue-banking procedures and informed consent forms were approved by the Medical Ethics Committee of the 989th Hospital of the Joint Logistic Support Force of the Chinese People's Liberation Army (approval no. 989LLSC2024-1218). The study was conducted in accordance with the 1964 Declaration of Helsinki and its subsequent amendments. Participants were randomly assigned to training and validation sets with balanced clinicopathological characteristics. The training set was used to establish the IS and evaluate its ability to predict prognosis and chemotherapy efficacy, whereas the validation set was used to further assess its predictive performance.

All included patients were followed for more than 3 years, with the final follow-up conducted in December 2025. Follow-up was performed through telephone interviews and outpatient imaging examinations to document distant metastasis, recurrence, recurrence-free survival (RFS), and overall survival (OS). Clinicopathological data were extracted from the pathological diagnostic system, including age, gender, postoperative TNM stage [5], tumor differentiation, perineural invasion, and venous tumor emboli.

Pathological specimen processing

Immediately after surgical excision, ESCC specimens were fixed in 4% neutral-buffered formaldehyde for 12-24 h to preserve tissue morphology and structure. The fixed specimens were subsequently dehydrated by sequential immersion in graded ethanol solutions of 70%, 80%, 95%, and 100%, with each step lasting 3 min. After dehydration, the specimens were cleared in xylene and embedded in molten paraffin to produce paraffin blocks. The blocks were sec-

tioned at a thickness of 3-4 μm using an RM2255 microtome (The Lycra Company, Wilmington, DE, USA), and the sections were mounted on glass slides. Finally, the sections were stained with hematoxylin and eosin (BA4027, Zhuhai Beisuo Biotechnology Co., Ltd., Zhuhai, Guangdong, China).

Immunohistochemistry (IHC)

Two 3-μm sections were prepared from each formalin-fixed, paraffin-embedded tumor specimen and routinely deparaffinized and rehydrated. The sections were washed twice with phosphate-buffered saline (PBS-0061, Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, Fujian, China) for 3 min per wash. Antigen retrieval was performed in sodium citrate buffer (pH 7.3; MVS-0101, Fuzhou Maixin Biotechnology Development Co., Ltd.) using an autoclave for 5 min. Endogenous peroxidase activity was blocked with Hydrogen Peroxide Block (SP KIT-A2, Fuzhou Maixin Biotechnology Development Co., Ltd.) for 15 min.

The sections were incubated separately with antibodies against CD3 (1:50; clone SP7; Kit-0003, Fuzhou Maixin Biotechnology Development Co., Ltd.) and CD8 (1:100; clone SP16; RMA-0514, Fuzhou Maixin Biotechnology Development Co., Ltd.) at 37°C for 1.5 h. After two 5-min washes with buffer, the sections were processed using a standard two-step indirect streptavidin-biotin method with the Strept AB Complex/HRP Duet Mouse/Rabbit Amplification Kit (Kit-5020; Fuzhou Maixin Biotechnology Development Co., Ltd.), with each incubation lasting 30 min.

For chromogenic detection, 1-2 drops of DAB Plus Chromogen were added to 1 mL of DAB Plus Substrate (DAB1031; Fuzhou Maixin Biotechnology Development Co., Ltd.). The prepared solution was applied to the sections, which were incubated for 3 min. The sections were then thoroughly rinsed with tap water, counterstained with Mayer's hematoxylin (BA4021; Zhuhai Beisuo Biotechnology Co., Ltd.), dehydrated, cleared, and mounted. Negative controls were prepared by omitting the primary antibody, and gastric cardia lymph node tissue was used as the positive control.

Determination of the IS

$CD3^+$ and cytotoxic $CD8^+$ T cells were evaluated using a cell-counting method. Based on the boundary between tumor and adjacent normal tissue, the intratumoral region was defined as the tumor center (CT), whereas the tumor-normal tissue interface was defined as the invasive margin (IM). The entire section was initially examined at low magnification (×40) to identify TIL hotspots with high densities of positively stained lymphocytes in the CT and IM regions. The three hotspots containing the largest numbers of TILs in each region were selected for counting at ×400 magnification using an Olympus CX41 microscope, with a field area of 0.196 mm^2 (**Figure 1**).

Two senior pathologists who were blinded to the patients' clinicopathological characteristics, including age and pathological diagnosis, independently evaluated all sections. Inter-observer reproducibility was assessed using the Wilcoxon signed-rank test. The mean value obtained from the two observers was used as the final cell density.

Receiver operating characteristic (ROC) curve analysis based on 5-year OS was used to determine the optimal cutoff value for each cell-density variable according to the maximum Youden index. The densities of $CD3^+$ and $CD8^+$ cells in the CT and IM regions were separately assigned a score of 0 for low infiltration (Lo; below the cutoff value) or 1 for high infiltration (Hi; equal to or above the cutoff value). The four individual scores were then summed to generate the IS.

The resulting five-category IS ranged from 0 to 4 and was designated I0, I1, I2, I3, or I4, respectively. For the three-category classification, a low IS (ISLo) was defined as a score of 0 or 1, an intermediate IS (ISInt) as a score of 2 or 3, and a high IS (ISHi) as a score of 4. For the two-category classification, ISLo was defined as a score of 0 or 1, whereas intermediate-to-high IS (ISInt+Hi) was defined as a score of 2, 3, or 4 (**Figure 2**).

Statistical analysis

Statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Age was normally or approximately nor-

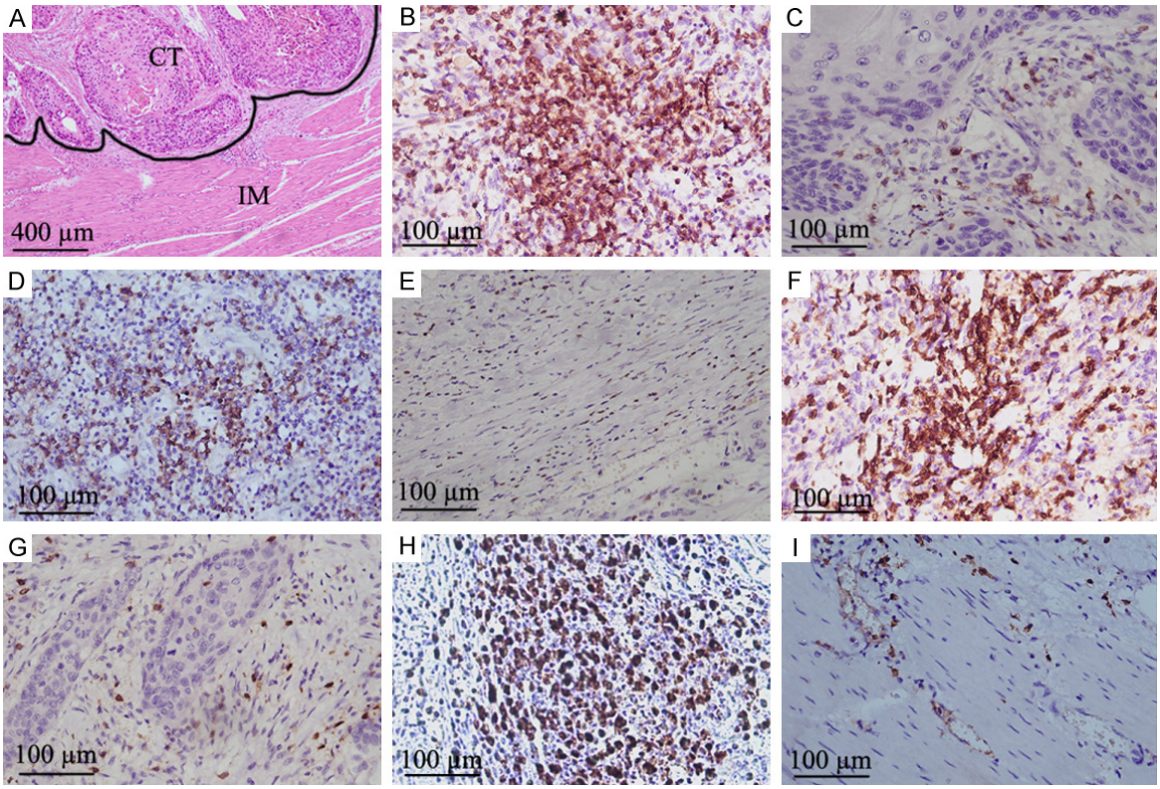

Figure 1. Immunostaining (IHC) of $CD3^+$ and cytotoxic $CD8^+$ T lymphocytes for Immunoscore evaluation. (A) Representative CD3 and CD8 IHC section in an Esophageal squamous cell carcinoma (ESCC) resected specimen indicating the central of tumor (CT) and invasive margin (IM) (100×); (B) High density of $CD3^+$ T cell in CT; (C) Low density of $CD3^+$ T cell in CT; (D) High density of $CD3^+$ T cell in IM; (E) Low density of $CD3^+$ T cell in IM; (F) High density of $CD8^+$ T cell in CT; (G) Low density of $CD8^+$ T cell in CT; (H) High density of $CD8^+$ T cell in IM; (I) Low density of $CD8^+$ T cell in IM (B-I, 400×).

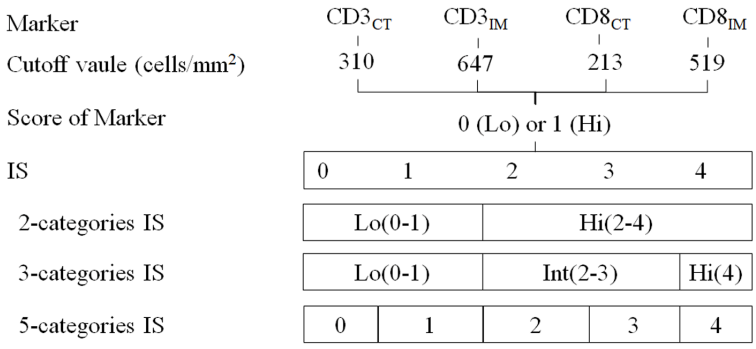

Figure 2. Immunoscore (IS) calculation method. The cutoff value of $CD3^+$ and cytotoxic $CD8^+$ T lymphocytes density was calculated by Receiver Operating Characteristic (ROC) curve analysis based on 5-year OS. The cell density was converted into score as 0 (Low (Lo), < cutoff value) or 1 (High (Hi), ≥ cutoff value). The scores of CD3 CT, CD3 IM, CD8 CT and CD8 IM were then translated into an IS. The IS can be categorized into 2-, 3- or 5-categories.

mally distributed and is presented as the mean ± standard deviation. An independent-samples t test was used to compare age between two groups, and one-way analysis of variance was used for comparisons among three groups.

Categorical variables, including TNM stage, T stage, tumor differentiation, and N stage, were compared using the chi-square test or Fisher's exact test, as appropriate. Gender was categorized as male or female. Tumor differentiation was classified as poor, moderate, or well differentiated, and tumor location was classified as upper, middle, or lower. Perineural invasion, venous tumor emboli, and recurrence were categorized as absent (No) or present (Yes). T stage was classified as T1a, T1b, T2, or T3; N stage as N0, N1, N2, or N3; and TNM stage as IA, IB, IIA, IIB, IIIA, IIIB, or IVA. The two-category IS was classified as low (Lo) or intermediate/high

(Int+Hi). The three-category IS was classified as low (Lo), intermediate (Int), or high (Hi), and the five-category IS was classified as I0, I1, I2, I3, or I4. RFS and OS were analyzed using the Kaplan-Meier method and Cox proportional hazards regression models to evaluate the prognostic value of the IS. A two-sided *P* value <0.05 was considered statistically significant. Categorical variables are expressed as number of cases (percentage).

Results

Patient characteristics

According to the inclusion and exclusion criteria, 255 patients were included, comprising 165 men and 90 women. The mean age at diagnosis was 65.66 ± 7.75 years. TNM stage was IA in 8.2% of patients (21/255), IB in 16.1% (41/255), IIA in 12.2% (31/255), IIB in 28.6% (73/255), IIIA in 8.2% (21/255), IIIB in 22.7% (58/255), and IVA in 3.9% (10/255). The median follow-up duration was 50 months (range, 3-121 months). Lymph node metastasis was present in 99 patients and absent in 156 patients. A total of 169 patients received postoperative chemotherapy. By the end of follow-up, 58.8% of patients (150/255) had experienced recurrence or distant metastasis. Participants were randomly assigned to training and validation sets with balanced clinicopathological characteristics (**Table 1**; all P>0.05).

Distribution and expression of CD3⁺ and cytotoxic CD8⁺T lymphocytes and the IS

$CD3^+$ and cytotoxic $CD8^+$ T cells were quantified by IHC. CD3 and CD8 staining was predominantly localized to the cytoplasm and cell membrane of lymphocytes in the CT and IM regions (**Figure 1**). No significant differences were observed between the two observers in the counted densities of $CD3^+$ and $CD8^+$ cells in either the CT or IM regions (Wilcoxon signed-rank test; all *P*>0.05).

In the training set, the median $CD3^+$ cell density in the CT (CD3 CT) was 305 cells/mm^2 (range, 18-1,778 cells/mm^2), and the median $CD8^+$ cell density in the CT (CD8 CT) was 255 cells/mm^2 (range, 18-1,935 cells/mm^2). The median $CD3^+$ cell density in the IM (CD3 IM) was 870 cells/mm^2 (range, 40-2,425 cells/mm^2), and the median $CD8^+$ cell density in the IM (CD8 IM) was 558 cells/mm^2 (range, 35-2,015 cells/mm^2). ROC curve analysis based on 5-year OS identified cutoff values of 310, 213, 647, and 519 cells/mm^2 for CD3 CT, CD8 CT, CD3 IM, and CD8 IM, respectively, with corresponding Youden index of 0.43, 0.31, 0.33, and 0.37.

Accordingly, the five-category IS distribution in the training set was I0 in 14.2% of patients (18/127), I1 in 10.2% (13/127), I2 in 29.9% (38/127), I3 in 18.9% (24/127), and I4 in 26.8% (34/127). Under the three-category classification, 24.4% of patients (31/127) had ISLo, 48.8% (62/127) had ISInt, and 26.8% (34/127) had ISHi. Under the two-category classification, 31 patients (24.4%) had ISLo and 96 (75.6%) had ISInt+Hi. The three-category IS was significantly associated with T stage, TNM stage, and recurrence (*P*<0.05) (**Table 2**).

Association between the IS and outcomes in patients with ESCC

The prognostic value of the IS in patients with ESCC was evaluated in the training set (n=127) and subsequently examined in the validation set (n=128). In the training set, patients with ISInt+Hi had a significantly lower risk of recurrence than those with ISLo (5-year RFS: 61.7% vs. 9.7%; unadjusted HR for ISInt+Hi vs. ISLo, 0.48; 95% CI, 0.39-0.59; *P*<0.001) and significantly prolonged OS (5-year OS: 52.8% vs. 12.9%; unadjusted HR, 0.67; 95% CI, 0.58-0.77; *P*<0.001) (**Figure 3A** and **3B**; **Table 3**).

The two-category IS was evaluated in the validation set using the same methodology and cutoff values. Patients with ISInt+Hi had a significantly lower risk of recurrence than those with ISLo (5-year RFS: 59.4% vs. 4.9%; unadjusted HR, 0.57; 95% CI, 0.45-0.74; *P*<0.001) and significantly prolonged OS (5-year OS: 54.0% vs. 20.1%; unadjusted HR, 0.53; 95% CI, 0.42-0.64; *P*<0.001) (**Figure 3C** and **3D**; **Table 3** and Supplementary Table 1).

Under the three-category classification, patients with ISHi, ISInt, and ISLo in the training set had 5-year RFS rates of 90.1%, 44.7%, and 9.7%, respectively (unadjusted HR for ISHi vs. ISLo, 0.29; 95% CI, 0.18-0.50; P<0.001), and 5-year OS rates of 78.9%, 37.4%, and 12.9%, respectively (unadjusted HR, 0.53; 95% CI,

Table 1. Comparison of clinicopathological variables between training set and validation set (n=255)

Characteristics	Cases (N=255)	Training set (N=127, 49.8%)	Validation set (N=128, 50.2%)	*P*
Gender, n (%)				0.569[a]
Male	165 (64.7%)	80 (63.0%)	85 (66.4%)	
Female	90 (35.3%)	47 (37.0%)	43 (33.6%)	
Age (year), Mean ± SD	65.66 ± 7.75	65.36 ± 7.78	66.95 ± 7.74	0.544[b]
Differentiation, n (%)				0.860[a]
Poor	14 (5.5%)	7 (5.5%)	7 (5.5%)	
Moderately	220 (86.3%)	109 (85.8%)	111 (86.7%)	
Well	21 (8.2%)	11 (8.7%)	10 (7.8%)	
Location, n (%)				0.706[a]
Upper	38 (14.9%)	22 (17.3%)	16 (12.5%)	
Middle	138 (54.1%)	65 (51.2%)	73 (57.0%)	
Lower	79 (31.0%)	40 (31.5%)	39 (30.5%)	
Perineural invasion, n (%)				0.148[a]
Yes	18 (7.1%)	6 (4.7%)	12 (9.4%)	
No	237 (92.9%)	121 (95.3%)	116 (90.6%)	
Venous emboli, n (%)				0.613[a]
Yes	49 (19.2%)	26 (20.5%)	23 (18.0%)	
No	206 (80.8%)	101 (79.5%)	105 (82.0%)	
T stage, n (%)				0.134[a]
T1a	22 (8.6%)	15 (11.8%)	7 (5.5%)	
T1b	51 (20.0%)	26 (20.5%)	25 (19.5%)	
T2	48 (18.8%)	24 (18.9%)	24 (18.8%)	
T3	134 (52.5%)	62 (48.8%)	72 (56.3%)	
N stage, n (%)				0.229[a]
N0	156 (61.2%)	81 (63.8%)	75 (58.6%)	
N1	61 (23.9%)	32 (25.2%)	29 (22.7%)	
N2	28 (11.0%)	11 (8.7%)	17 (13.3%)	
N3	10 (3.9%)	3 (2.4%)	7 (5.5%)	
TNM, n (%)				0.146[a]
IA	21 (8.2%)	14 (11.0%)	7 (5.5%)	
IB	41 (16.1%)	20 (15.7%)	21 (16.4%)	
IIA	31 (12.2%)	16 (12.6%)	15 (11.7%)	
IIB	73 (28.6%)	37 (29.1%)	36 (28.1%)	
IIIA	21 (8.2%)	10 (7.9%)	11 (8.6%)	
IIIB	58 (22.7%)	27 (21.3%)	31 (24.2%)	
IVA	10 (3.9%)	3 (2.4%)	7 (5.5%)	
Recurrence, n (%)				0.560[a]
Yes	150 (58.8%)	77 (60.6%)	73 (57.0%)	
No	105 (41.2%)	50 (39.4%)	55 (43.0%)	

[a]Pearson χ^2 test, [b]Independent Samples T Test.

0.39-0.73; P<0.001) (**Figure 3E** and **3F**; **Table 3**). In the validation set, patients with ISHi, ISInt, and ISLo had 5-year RFS rates of 83.7%, 44.7%, and 4.9%, respectively (unadjusted HR for ISHi vs. ISLo, 0.29; 95% CI, 0.16-0.52; *P*<0.001), and 5-year OS rates of 77.5%, 40.1%, and 20.1%, respectively (unadjusted HR, 0.34; 95% CI, 0.20-0.47; P<0.001) (**Figure 3G** and **3H**). Similar results were observed for the five-category IS (**Table 3** and Supplementary Table 1).

Table 2. Correlation between immunoscore and clinicopathological variables in training set patients (n=127)

Characteristics	Cases (N=127)	3-categories Immunoscore			*P*
		[a]ISLo (N=31, 24.4%)	[b]ISInt (N=62, 48.8%)	[c]ISHi (N=34, 26.8%)	
Gender, n (%)					[d]0.555
Male	80 (63.0%)	22 (71.0%)	38 (61.3%)	20 (58.8%)	
Female	47 (37.0%)	9 (29.0%)	24 (38.7%)	14 (41.2%)	
Age (year), Mean ± SD	65.36 ± 7.78	63.87 ± 8.64	65.82 ± 7.48	65.88 ± 7.56	[e]0.474
Differentiation, n (%)					[d]0.150
Poor	7 (5.5%)	4 (12.9%)	3 (4.8%)	0 (0.0%)	
Moderately	109 (85.8%)	23 (74.2%)	54 (87.1%)	32 (94.1%)	
Well	11 (8.7%)	4 (12.9%)	5 (8.1%)	2 (5.9%)	
Location, n (%)					[d]0.267
Upper	22 (17.3%)	8 (25.8%)	10 (16.1%)	4 (11.8%)	
Middle	65 (51.2%)	16 (51.6%)	28 (45.2%)	21 (61.8%)	
Lower	40 (31.5%)	7 (22.6%)	24 (38.7%)	9 (26.5%)	
Perineural invasion, n (%)					[d]0.326
Yes	6 (4.7%)	3 (9.7%)	2 (3.2%)	1 (2.9%)	
No	121 (95.3%)	28 (90.3%)	60 (96.8%)	33 (97.1%)	
Venous emboli, n (%)					[d]0.064
Yes	26 (20.5%)	10 (32.3%)	13 (21.0%)	3 (8.8%)	
No	101 (79.5%)	21 (67.7%)	49 (79.0%)	31 (91.2%)	
T stage, n (%)					[d]0.003
T1a	15 (11.8%)	4 (12.9%)	7 (11.3%)	4 (11.8%)	
T1b	26 (20.5%)	2 (6.5%)	9 (14.5%)	15 (44.1%)	
T2	24 (18.9%)	4 (12.9%)	15 (24.2%)	5 (14.7%)	
T3	62 (48.8%)	21 (67.7%)	31 (50.0%)	10 (29.4%)	
N stage, n (%)					[d]0.215
N0	81 (63.8%)	14 (45.2%)	41 (66.1%)	26 (76.5%)	
N1	32 (25.2%)	11 (35.5%)	16 (25.8%)	5 (14.7%)	
N2	11 (8.7%)	5 (16.1%)	4 (6.5%)	2 (5.9%)	
N3	3 (2.4%)	1 (3.2%)	1 (1.6%)	1 (2.9%)	
TNM, n (%)					[d]0.007
IA	14 (11.0%)	4 (12.9%)	6 (9.7%)	4 (11.8%)	
IB	20 (13.7%)	1 (3.2%)	7 (11.3%)	12 (35.3%)	
IIA	16 (12.6%)	2 (6.5%)	10 (16.1%)	4 (11.8%)	
IIB	37 (29.1%)	7 (22.6%)	21 (33.9%)	9 (26.5%)	
IIIA	10 (7.9%)	3 (9.7%)	7 (11.3%)	0 (0.0%)	
IIIB	27 (21.3%)	13 (41.9%)	10 (16.1%)	4 (11.8%)	
IVA	3 (2.4%)	1 (3.2%)	1 (1.6%)	1 (2.9%)	
Recurrence, n (%)					[d]<0.001
Yes	77 (60.6%)	31 (100.0%)	36 (58.1%)	10 (29.4%)	
No	50 (39.4%)	0 (0.0%)	26 (41.9%)	24 (70.6%)	

[a]Low Immunoscore, [b]Intermediate Immunoscore, [c]High Immunoscore, [d]Pearson χ^2 test, [e]One-Way ANOVA test.

Multivariable Cox regression analyses in the training and validation sets were adjusted for age, gender, perineural invasion, IS, tumor location, tumor differentiation, TNM stage and venous tumor emboli. Both the two-category and three-category IS classifications retained

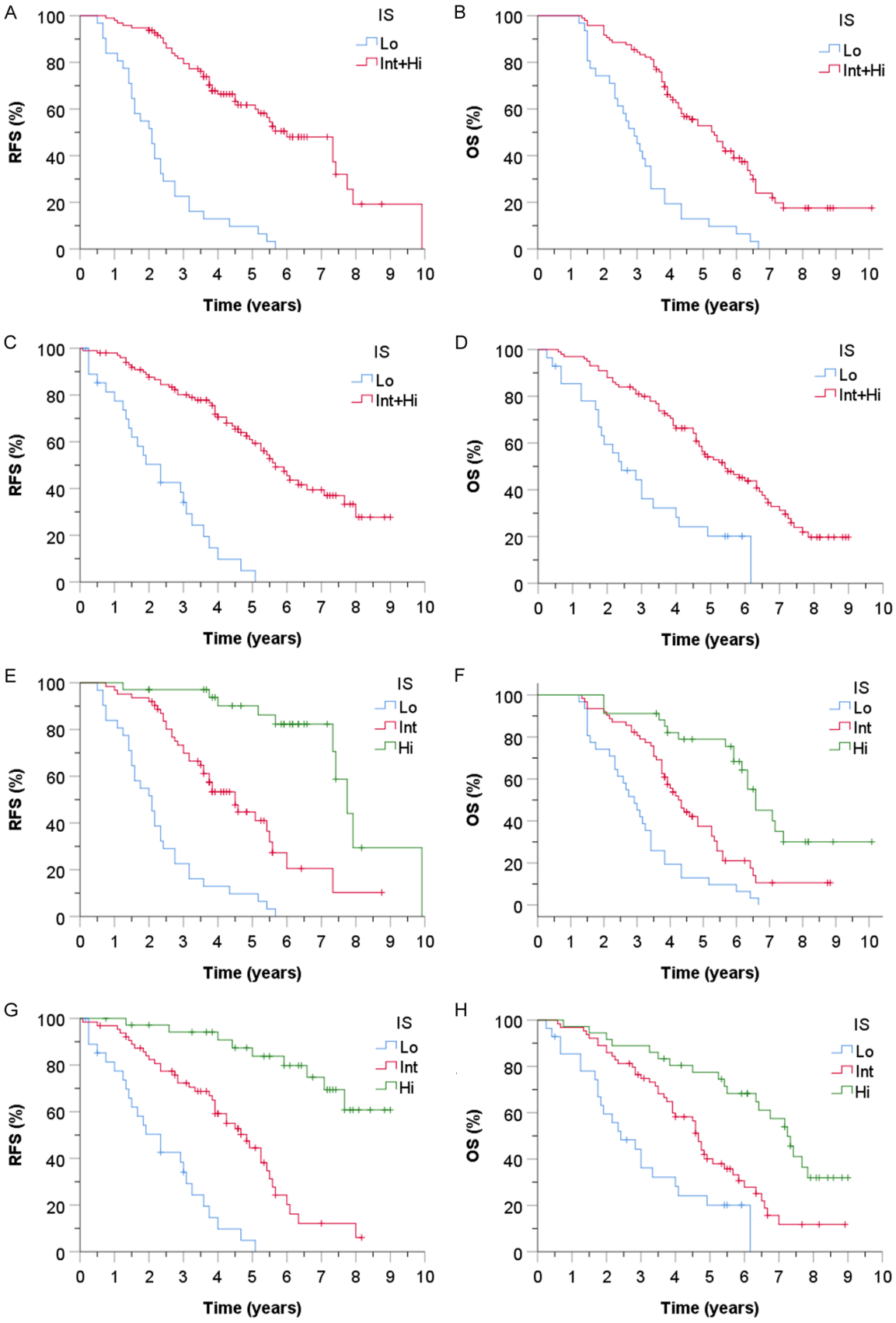
A
RFS (%)
Time (years)
IS
Lo
Int+Hi
B
OS (%)
Time (years)
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Figure 3. Kaplan-Meier survival curves for the impact of IS on the outcome of patients with ESCC. For 2-categories IS, Recurrence free survival (RFS) in (A) training set patients, (C) validation set patients, and overall survival (OS) in (B) training set patients, (D) validation set patients. For 3-categories IS, RFS in (E) training set patients, (G) validation set patients, and overall survival (OS) in (F) training set patients, (H) validation set patients (all $P<0.001$).

Table 3. Univariate analysis of RFS and OS using immunoscore category in training set patients (n=127)

Prognostic indicators	Immunoscore Category	No. of Patients, n (%)	[a]Rate at 3 Years (%) (95% CI)*	[a]Rate at 5 Years (%) (95% CI)*	Hazard Ratio (95% CI)*	[b]*P*
RFS	2-categories IS					
	Lo	31 (24.4)	22.6 (15.1 to 30.1)	9.7 (4.4 to 15.0)	1.00 (Reference)	
	Int+Hi	96 (75.6)	81.7 (77.7 to 85.7)	61.7 (56.3 to 67.1)	0.48 (0.39 to 0.59)	<0.001
RFS	3-categories IS					
	Lo	31 (24.4)	22.6 (15.1 to 30.1)	9.7 (4.4 to 15.0)	1.00 (Reference)	
	Int	62 (48.8)	73.3 (67.6 to 79.0)	44.7 (37.5 to 51.9)	0.58 (0.47 to 0.72)	<0.001
	Hi	34 (26.8)	97.1 (94.2 to 100)	90.1 (84.6 to 95.6)	0.29 (0.18 to 0.50)	<0.001
RFS	5-categories IS					
	0	18 (14.2)	16.7 (7.9 to 25.5)	11.1 (3.7 to 18.5)	1.00 (Reference)	
	1	13 (10.2)	30.8 (18.0 to 43.6)	7.7 (0.3 to 15.1)	NA	NA
	2	38 (29.9)	64.6 (56.7 to 72.5)	29.4 (20.6 to 38.2)	0.71 (0.58 to 0.87)	0.011
	3	24 (18.9)	78.3 (69.7 to 86.9)	67.0 (56.4 to 77.6)	0.38 (0.22 to 0.63)	<0.001
	4	34 (26.8)	97.1 (94.2 to 100)	90.1 (84.6 to 95.6)	0.29 (0.18 to 0.50)	<0.001
OS	2-categories IS					
	Lo	31 (24.4)	45.2 (36.3 to 54.1)	12.9 (6.9 to 18.9)	1.00 (Reference)	
	Int+Hi	96 (75.6)	84.4 (80.7 to 88.1)	52.8 (47.5 to 58.1)	0.67 (0.58 to 0.77)	<0.001
OS	3-categories IS					
	Lo	31 (24.4)	45.2 (36.3 to 54.1)	12.9 (6.9 to 18.9)	1.00 (Reference)	
	Int	62 (48.8)	82.3 (77.4 to 87.2)	37.4 (30.8 to 44.0)	0.72 (0.64 to 0.86)	0.002
	Hi	34 (26.8)	91.2 (86.3 to 96.1)	78.9 (71.8 to 86.0)	0.53 (0.39 to 0.73)	<0.001
OS	5-categories IS					
	0	18 (14.2)	33.3 (22.2 to 44.4)	5.6 (0.2 to 11.0)	1.00 (Reference)	
	1	13 (10.2)	61.5 (48.0 to 75.0)	15.4 (5.4 to 25.4)	NA	NA
	2	38 (29.9)	81.6 (75.3 to 87.9)	37.0 (28.9 to 45.1)	0.71 (0.58 to 0.87)	0.011
	3	24 (18.9)	79.2 (70.9 to 87.5)	40.5 (29.7 to 51.3)	0.79 (0.65 to 0.97)	0.039
	4	34 (26.8)	91.2 (86.3 to 96.1)	78.9 (71.8 to 86.0)	0.53 (0.39 to 0.73)	<0.001

[a]Kaplan-Meier test, [b]Crosstab. *Confidence Interval.

significant prognostic value (**Table 4**). The three-category IS had greater predictive power for RFS and OS than TNM, perineural invasion, and tumor differentiation. The variables with the largest relative contributions to RFS, based on the proportion of the total χ^2 statistic, were the three-category IS, TNM stage, perineural invasion and tumor differentiation. For OS, the largest relative contributions were observed for the three-category IS, perineural invasion, age, TNM and tumor differentiation. The remaining variables made relatively small contributions. Similar findings were obtained for the two-category IS.

Association between the IS and outcomes in patients receiving postoperative chemotherapy

The efficacy of postoperative chemotherapy varies substantially among patients with ESCC. Therefore, the prognostic value of the IS was investigated among patients who received postoperative chemotherapy (**Figure 4**). In the training set, patients received postoperative

Table 4. Cox multivariable regression analysis of RFS and OS combining the 2-categories and 3-categories immunoscore with clinical parameters (n=255)

Analysis	Variable	Wald	HR (95% CI)	*P*
RFS combining the 2-categories Immunoscore with clinical parameters	2-categories IS	54.89	0.23 (0.16 to 0.34)	<0.001
	TNM	46.03	1.54 (1.36 to 1.75)	<0.001
	Perineural invasion	6.02	2.35 (1.19 to 4.65)	0.014
	Differentiation	4.71	0.63 (0.42 to 0.96)	0.030
RFS combining the 3-categories Immunoscore with clinical parameters	3-categories IS	87.23	0.27 (0.20 to 0.35)	<0.001
	TNM	44.69	1.55 (1.37 to 1.77)	<0.001
	Perineural invasion	4.67	2.11 (1.07 to 4.15)	0.031
	Differentiation	3.97	0.67 (0.45 to 0.99)	0.046
OS combining the 2-categories Immunoscore with clinical parameters	2-categories IS	25.04	0.39 (0.27 to 0.57)	<0.001
	Perineural invasion	10.50	2.77 (1.50 to 5.14)	0.001
	TNM	8.22	1.17 (1.05 to 1.30)	0.004
	Age	7.35	1.03 (1.01 to 1.05)	0.007
	Differentiation	6.13	0.61 (0.41 to 0.90)	0.013
OS combining the 3-categories Immunoscore with clinical parameters	3-categories IS	46.63	0.44 (0.35 to 0.56)	<0.001
	Perineural invasion	9.07	2.56 (1.39 to 4.73)	0.003
	Age	8.03	1.03 (1.01 to 1.05)	0.005
	TNM	7.20	1.16 (1.04 to 1.29)	0.007
	Differentiation	5.70	0.63 (0.43 to 0.92)	0.017

chemotherapy (n=87) who had ISInt+Hi experienced significantly prolonged RFS compared with those who had ISLo (5-year RFS: 57.0% vs. 12.5%; unadjusted HR for ISInt+Hi vs. ISLo, 0.57; 95% CI, 0.46-0.71; P<0.001). They also had significantly prolonged OS (5-year OS: 48.9% vs. 12.5%; unadjusted HR, 0.75; 95% CI, 0.65-0.86; P<0.001) (**Figure 4A** and **4B**; **Table 5**).

In the validation set, patients received postoperative chemotherapy (n=82) who had ISInt+Hi experienced significantly prolonged RFS compared with those who had ISLo (5-year RFS: 48.5% vs. 6.5%; unadjusted HR for ISInt+Hi vs. ISLo, 0.54; 95% CI, 0.36-0.82; P=0.017) and significantly prolonged OS (5-year OS: 55.6% vs. 22.2%; unadjusted HR, 0.67; 95% CI, 0.58-0.61; P<0.001) (**Figure 4C** and **4D**). Similar findings were obtained using the three-category IS in both the training set (**Figure 4E** and **4F**; **Table 5**) and the validation set (**Figure 4G** and **4H**; Supplementary Table 2).

Multivariable Cox regression analyses of all patients receiving postoperative chemotherapy in the training and validation sets (n=169) showed that the three-category IS, TNM stage, and age made the largest relative contributions to RFS, whereas tumor differentiation made a relatively small contribution. For OS, the variables with the largest relative contributions to risk, based on the proportion of the total χ^2 statistic, were the three-category IS, TNM stage, and age, whereas location and tumor differentiation made relatively small contributions. Similar findings were obtained for the two-category IS (**Table 6**).

Discussion

In this study, we evaluated the prognostic value of the IS, based on the densities of $CD3^+$ and cytotoxic $CD8^+$ T lymphocytes, in patients with ESCC. Using resected specimens from a large cohort of patients with previously untreated ESCC (n=255), we found that the IS was significantly associated with patient outcomes. Multivariable analyses identified the IS as an independent prognostic factor for RFS and OS. We further evaluated the IS among patients who received postoperative chemotherapy and found that a high IS (ISHi) was associated with a significantly lower risk of recurrence and prolonged OS.

The immune system contributes to antitumor responses by recognizing and eliminating cancer cells [18]. TILs have been associated with

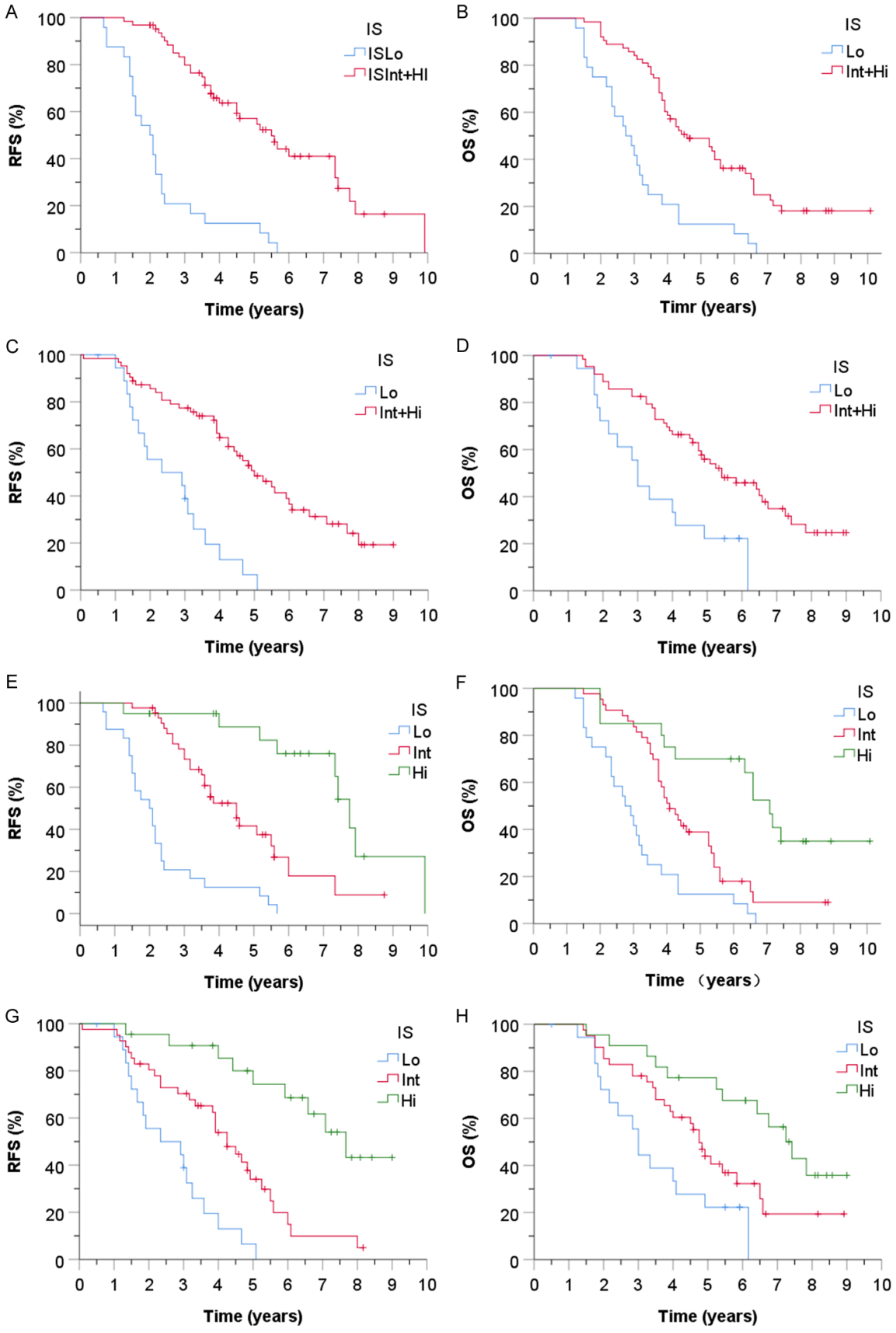
A
RFS (%)
Time (years)
IS
ISLo
ISInt+HI
B
OS (%)
Timr (years)
IS
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Figure 4. Kaplan-Meier survival curves for the impact of IS on outcome of ESCC patients with postoperative chemotherapy. For 2-categories IS, RFS in (A) training set patients, (C) validation set patients, and OS in (B) training set patients, (D) validation set patients. For 3-categories IS, RFS in (E) training set patients, (G) validation set patients, and OS in (F) training set patients, (H) validation set patients (all $P<0.001$).

Table 5. Univariate analysis of RFS and OS using immunoscore category in training set patients with postoperative chemotherapy (n=87)

Prognostic indicators	Immunoscore Category	No. of Patients, n (%)	[a]Rate at 3 Years (%) (95% CI)*	[b]Rate at 5 Years (%) (95% CI)*	[b]Hazard Ratio (95% CI)*	[b]*P*
RFS	2-categories IS					
	Lo	24 (27.6)	20.8 (12.5 to 29.1)	12.5 (5.7 to 19.3)	1.00 (Reference)	
	Int+Hi	63 (72.4)	83.3 (78.5 to 88.1)	57.0 (50.2 to 63.8)	0.57 (0.46 to 0.71)	<0.001
	3-categories IS					
	Lo	24 (27.6)	20.8 (12.5 to 29.1)	12.5 (5.7 to 19.3)	1.00 (Reference)	
	Int	43 (49.4)	73.3 (66.4 to 80.2)	41.7 (33.3 to 50.1)	0.63 (0.50 to 0.79)	0.001
	Hi	20 (23.0)	95.0 (90.1 to 99.9)	88.7 (81.1 to 96.3)	0.45 (0.28 to 0.73)	<0.001
OS	2-categories IS					
	Lo	24 (27.6)	41.7 (31.6 to 51.8)	12.5 (5.7 to 19.3)	1.00 (Reference)	
	Int+Hi	63 (72.4)	84.1 (79.5 to 88.7)	48.9 (42.6 to 55.2)	0.75 (0.65 to 0.86)	0.006
	3-categories IS					
	Lo	24 (27.6)	41.7 (31.6 to 51.8)	12.5 (5.7 to 19.3)	1.00 (Reference)	
	Int	43 (49.4)	83.7 (78.1 to 89.3)	38.9 (31.4 to 46.4)	0.81 (0.71 to 0.94)	0.024
	Hi	20 (23.0)	85.0 (77.0 to 93.0)	70.0 (59.8 to 80.2)	0.60 (0.42 to 0.86)	0.001

[a]Kaplan-Meier test, [b]Crosstab. *Confidence Interval.

Table 6. Cox multivariable regression analysis of RFS and OS combining the 2-categories IS, 3-categories IS with clinical parameters in patients with postoperative chemotherapy (n=169)

Analysis	Variable	Wald	HR (95% CI)	*P*
RFS combining the 2-categories IS with clinical parameters	2-categories IS	42.48	0.23 (0.15 to 0.36)	<0.001
	TNM	38.75	1.74 (1.46 to 2.07)	<0.001
	Differentiation	5.35	0.58 (0.36 to 0.92)	0.021
RFS combining the 3-categories IS with clinical parameters	3-categories IS	62.01	0.29 (0.21 to 0.39)	<0.001
	TNM	41.25	1.82 (1.52 to 2.18)	<0.001
	Age	5.31	1.03 (1.01 to 1.06)	0.021
	Differentiation	4.53	0.62 (0.40 to 0.96)	0.033
OS combining the 2-categories IS with clinical parameters	2-categories IS	19.26	0.39 (0.25 to 0.59)	<0.001
	TNM	16.91	1.40 (1.19 to 1.64)	<0.001
	Age	10.63	1.05 (1.02 to 1.08)	0.001
	Differentiation	4.68	0.59 (0.37 to 0.95)	0.031
OS combining the 3-categories IS with clinical parameters	3-categories IS	31.29	0.45 (0.34 to 0.59)	<0.001
	TNM	17.65	1.42 (1.20 to 1.67)	<0.001
	Age	10.90	1.05 (1.02 to 1.08)	0.001
	Location	5.19	0.73 (0.56 to 0.96)	0.023
	Differentiation	3.89	0.63 (0.40 to 0.99)	0.049

favorable outcomes in various cancers [19]. T cells, a major subset of lymphocytes, exert cytotoxic effects against tumor cells and secrete cytokines that inhibit cancer cell proliferation and metastasis. Based on this concept, the IS, which quantifies T-cell density, was

developed as an effective method for evaluating immune infiltration within tumors [20]. $CD8^+$ T cells have been identified as an important prognostic component of immune scoring systems in ESCC and esophageal adenocarcinoma [13]. A recent meta-analysis showed that $CD3^+$ and $CD8^+$ T-cell infiltration in both the CT and IM was associated with improved survival across different cancer stages [21]. In recent years, the IS, based on the densities of $CD3^+$ and cytotoxic $CD8^+$ T lymphocytes, has emerged as a promising tool for improving the management of various cancers [22]. The prognostic significance of the TME and the IS has been demonstrated in precancerous lesions, primary tumors, and metastatic disease [23-27].

To date, relatively few studies have investigated the clinical relevance of the IS in EC. The IS in surgical specimens and TIL density in pretreatment biopsy specimens have been proposed as potential predictors of clinical outcomes in patients receiving neoadjuvant chemotherapy for EC [20]. The IS has also been associated with pathological response in locally advanced ESCC [28]. In these studies, $CD3^+$ and $CD8^+$ lymphocytes were automatically counted at ×200 magnification using a BZ-X710 digital microscope and hybrid cell-counting software, and the mean density across five hotspots was used to determine the IS cutoff. In contrast, Conroy et al. reported that CD3 or CD8 expression was not significantly associated with survival in patients with esophageal adenocarcinoma or ESCC [29]. In the present study, we also established an IS model for ESCC. $CD3^+$ and $CD8^+$ lymphocytes were counted at ×400 magnification using an Olympus CX41 microscope, and the optimal cutoff values were determined by ROC curve analysis based on 5-year OS and the maximum Youden index. Our findings suggest that the IS can provide valuable and accurate prognostic information for patients with ESCC.

In addition to its prognostic value, the IS may contribute to predicting outcomes following chemotherapy. Postoperative chemotherapy is commonly administered to reduce the risks of tumor recurrence and metastasis. However, responses to postoperative chemotherapy vary substantially among patients. Therefore, novel biomarkers are needed to support decisions regarding the administration of chemotherapy. Accumulating evidence suggests that the efficacy of chemotherapy may be mediated, at least in part, through the modulation of local and systemic immune responses. In rectal cancer, the densities of stromal $CD3^+$ and $CD8^+$ T cells increase after neoadjuvant treatment [30]. However, evidence regarding this association in ESCC remains limited [31]. In EC, patients who respond to neoadjuvant chemotherapy have higher densities of both $CD3^+$ and $CD8^+$ cells in pretreatment biopsy specimens, and $CD3^+$ cell density has been proposed as an independent prognostic factor [20]. In the present study, patients with intermediate or high IS values who received postoperative chemotherapy had better RFS and OS than those with a low IS. These findings may have implications for elderly patients with multiple comorbidities or poor tolerance of standard chemotherapy. However, whether patients with ISHi could benefit from reduced chemotherapy intensity or whether chemotherapy should be used more cautiously in patients with ISLo and a high risk of toxicity requires further prospective investigation.

Overcoming chemoresistance, particularly resistance to 5-fluorouracil (5-FU), remains a major challenge in the treatment of ESCC. In 5-FU-resistant ESCC, the TME may be actively remodeled to promote immune evasion. Therapeutic strategies should therefore target both resistant cancer cells and the immunosuppressive TME, potentially providing new approaches to overcoming chemoresistance in ESCC [16]. Multi-omics approaches also have considerable potential in drug discovery, particularly for predicting drug sensitivity [32]. Our IS analysis further suggests that $CD3^+$ and cytotoxic $CD8^+$ T lymphocytes may be involved in the response to postoperative chemotherapy. With continued technological advances, multi-omics analyses of the TME may facilitate the development of strategies to overcome chemoresistance in ESCC.

This study has several limitations. First, patients in both the training and validation sets were recruited from the same institution, and the findings were not validated in an independent external cohort. Second, because this was a retrospective cohort study, the available medical records were not sufficiently detailed to include certain potentially relevant factors,

such as family history and genetic characteristics, which may have affected the results. Third, owing to the limited sample size, the cutoff values established in this study may not be generalizable to all patients with ESCC. Large, multicenter prospective studies are therefore needed to validate these findings.

Conclusions

A high IS was significantly associated with prolonged RFS and OS in patients with ESCC, including those who received postoperative chemotherapy. These findings suggest that an IS based on the densities of $CD3^+$ and cytotoxic $CD8^+$ T lymphocytes may improve prognostic stratification and the assessment of outcomes following chemotherapy. The IS may therefore serve as a useful complement to the TNM staging system for patients with operable ESCC.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Univariate analysis of RFS and OS using immunoscore category in validation set patients (n=128)

Prognostic indicators	Immunoscore Category	No. of Patients, n (%)	[a]At 3 Years (%) (95% CI)*	[a]At 5 Years (%) (95% CI)*	[b]Hazard Ratio (95% CI)*	[b]*P*
RFS	2-categories IS					
	Lo	28 (21.9)	34.1 (24.7 to 43.5)	4.9 (0.2 to 9.6)	1.00 (Reference)	
	Int+Hi	100 (78.1)	80.1 (76.0 to 84.2)	59.4 (53.9 to 64.9)	0.57 (0.45 to 0.74)	0.001
	3-categories IS					
	Lo	28 (21.9)	34.1 (24.7 to 43.5)	4.9 (0.2 to 9.6)	1.00 (Reference)	
	Int	64 (50.0)	72.3 (66.6 to 78.0)	44.7 (37.5 to 51.9)	0.73 (0.57 to 0.93)	0.026
	Hi	36 (28.1)	94.1 (90.1 to 98.1)	83.7 (77.0 to 90.4)	0.29 (0.16 to 0.52)	<0.001
	5-categories IS					
	I0	16 (12.5)	25.8 (13.5 to 38.1)	8.6 (0.5 to 16.7)	1.00 (Reference)	
	I1	12 (9.4)	41.7 (27.5 to 55.9)	0 (NA)	1.13 (0.84 to 1.51)	0.436
	I2	33 (25.8)	61.2 (52.4 to 70.0)	26.4 (17.4 to 36.0)	0.76 (0.68 to 0.84)	0.048
	I3	31 (24.2)	83.5 (76.8 to 90.2)	61.1 (51.0 to 71.2)	0.54 (0.42 to 0.66)	0.003
	I4	36 (28.1)	94.1 (90.1 to 98.1)	83.7 (77.0 to 90.4)	0.21 (0.12 to 0.30)	<0.001
OS	2-categories IS					
	Lo	28 (21.9)	36.2 (26.8 to 45.6)	20.1 (12.1 to 28.1)	1.00 (Reference)	
	Int+Hi	100 (78.1)	79.9 (75.9 to 83.9)	54.0 (48.9 to 59.1)	0.53 (0.42 to 0.64)	0.001
	3-categories IS					
	Lo	28 (21.9)	36.2 (26.8 to 45.6)	20.1 (12.1 to 28.1)	1.00 (Reference)	
	Int	64 (50.0)	74.8 (69.4 to 80.2)	40.1 (33.7 to 46.5)	0.69 (0.55 to 0.86)	0.023
	Hi	36 (28.1)	88.9 (83.7 to 94.1)	77.5 (70.5 to 84.5)	0.34 (0.20 to 0.47)	<0.001
	5-categories IS					
	I0	16 (12.5)	39.1 (26.2 to 52.0)	23.4 (11.9 to 34.9)	1.00 (Reference)	
	I1	12 (9.4)	50.0 (35.6 to 64.4)	16.7 (5.9 to 27.5)	1.11 (0.76 to 1.62)	0.595
	I2	33 (25.8)	66.5 (58.3 to 74.7)	32.5 (23.8 to 41.2)	0.87 (0.75 to 0.96)	0.044
	I3	31 (24.2)	83.7 (77.0 to 90.4)	48.2 (38.8 to 57.6)	0.58 (0.50 to 0.65)	0.001
	I4	36 (28.1)	88.9 (83.7 to 94.1)	77.5 (70.5 to 84.5)	0.23 (0.14 to 0.31)	<0.001

[a]Kaplan-Meier test, [b]Crosstab. *Confidence Interval.

Supplementary Table 2. Univariate analysis of RFS and OS using immunoscore Category in training set patients with postoperative chemotherapy (n=82)

Prognostic indicators	Immunoscore Category	No. of Patients, n (%)	[a]Rate at 3 Years (%) (95% CI)*	[a]Rate at 5 Years (%) (95% CI)*	[b]Hazard Ratio (95% CI)*	[b]*P*
RFS	2-categories IS					
	Lo	19 (23.2)	38.9 (27.4 to 50.4)	6.5 (0.3 to 12.7)	1.00 (Reference)	
	Int+Hi	63 (76.8)	77.4 (72.1 to 82.7)	48.5 (41.7 to 55.3)	0.54 (0.36 to 0.82)	0.017
	3-categories IS					
	Lo	19 (23.2)	38.9 (27.4 to 50.4)	6.5 (0.3 to 12.7)	1.00 (Reference)	
	Int	41 (50.0)	70.4 (63.2 to 77.6)	34.0 (25.7 to 42.3)	0.65 (0.41 to 0.84)	0.032
	Hi	22 (26.8)	90.7 (84.4 to 97.0)	74.3 (64.3 to 84.3)	0.38 (0.15 to 0.53)	0.001
OS	2-categories IS					
	Lo	19 (23.2)	55.6 (43.9 to 67.3)	22.2 (12.4 to 32.0)	1.00 (Reference)	
	Int+Hi	63 (76.8)	82.5 (77.7 to 87.3)	55.9 (49.5 to 62.3)	0.67 (0.56 to 0.81)	<0.001
	3-categories IS					
	Lo	19 (23.2)	55.6 (43.9 to 67.3)	22.2 (12.4 to 32.0)	1.00 (Reference)	
	Int	41 (50.0)	78.0 (71.5 to 84.5)	40.0 (32.0 to 48.0)	0.73 (0.60 to 0.89)	0.001
	Hi	22 (26.8)	90.9 (84.8 to 97.0)	77.3 (68.4 to 86.2)	0.50 (0.38 to 0.73)	<0.001

[a]Kaplan-Meier test, [b]Crosstab. *Confidence Interval.