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Clinicopathological characteristics and the relationship of PD-L1 status, tumor mutation burden, and microsatellite instability in patients with esophageal carcinoma

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Abstract

Background Despite significant advancements in the field of immunotherapy for esophageal cancer in recent years, only a minority of patients respond to these treatments, and effective predictive biomarkers remain elusive. Biomarkers such as programmed cell death 1 ligand 1 (PD-L1), tumor mutational burden (TMB), and microsatellite instability (MSI) are pivotal in guiding immune checkpoint inhibitor therapies. This study aimed to explore the correlation between the three biomarkers in patients with esophageal carcinoma.

Methods We collected one hundred esophageal squamous cell carcinoma (ESCC) tumor samples from patients who have been undergoing radical resection of esophageal carcinoma. Each tissue sample was divided into two parts for next-generation sequencing (NGS) and immunohistochemical staining. Mutations were identified using the NGS database, and TMB was calculated. Multiplex PCR targeting five loci (NR21, NR24, NR27, BAT25, and BAT26) was used to evaluate MSI. PD-L1 expression was determined through immunohistochemical analysis.

Results Among the 100 ESCC patients, 54% (54/100) exhibited positive PD-L1 expression, 57% (57/100) demonstrated high TMB (TMB-H), and only 1% (1/100) had high MSI (MSI-H). Within the subset of TMB-H cases, 32 showed positive PD-L1 expression, with a single case displaying high expression of all three biomarkers, and 21 cases displaying low expression of all three biomarkers. There was no statistical association between PD-L1 expression levels and TMB. Further analysis showed a significant correlation between TNM staging and PD-L1 expression levels in ESCC tissues, with higher positive rates of PD-L1 expression observed in advanced stages. Similarly, a significant relationship was observed between TMB and lymph node metastasis.

Conclusions Based on our preliminary results, TMB and PD-L1 can serve as potential early screening clinical biomarkers and molecular targets for immune treatment in ESCC. However, there is no apparent statistical association between TMB and PD-L1 expression levels. Furthermore, PD-L1 and TMB may independently influence the efficacy

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of immunotherapy, highlighting the inadequacy of single-marker detection in effectively predicting treatment outcomes.

Keywords Esophageal squamous cell carcinoma, Biomarkers, Tumor mutational burden, Programmed cell death 1 ligand 1, Immunotherapy, Immune checkpoint inhibitors

Introduction

Being responsible for more than 90% of oesophageal cancer cases, esophageal squamous cell carcinoma (ESCC) is one of the most prevalent types of esophageal cancer in Asia [1]. Despite advancements in surgery, radiotherapy, and chemotherapy, ESCC is still associated with high mortality and low survival rates, posing significant threats to patients and imposing heavy burdens on families and society [2]. The advent of immune checkpoint inhibitors (ICIs) has heralded a new era in the ESCC therapy, vastly improving the overall survival of patients [3, 4]. However, some patients continue to experience inadequate responses to ICIs, emphasizing the need for reliable biomarkers to predict treatment efficacy.

Programmed cell death 1 ligand 1 (PD-L1) is one of the most widely recognized biomarkers for immunotherapy in clinical practice [5, 6]. Clinical studies have found a strong relationship between PD-L1 expression levels in tumor microenvironments and immunotherapy effectiveness, however patients with lower PD-L1 expression levels can still benefit from these treatments [7]. This suggests that PD-L1 alone might not be a sufficient prognostic marker. Additionally, a higher tumor mutation burden (TMB) has been linked to more pronounced responses to immunotherapy across various tumor types, highlighting its potential as a universal biomarker [8, 9]. Microsatellite instability (MSI), which represents genetic hypermutability caused by the mismatch repair gene inactivation, is another important biomarker for immunotherapy [10]. Evaluating MSI status is crucial for cancer prognosis, therapeutic decisions, and assessing familial cancer risk [11, 12].

Despite the individual significance of PD-L1, TMB, and MSI as biomarkers [13, 14], the interrelationship among them and their combined utility in predicting immunotherapy outcomes in ESCC remain underexplored [15, 16]. In our study, we plan to bridge this divide by assessing the expression of PD-L1, TMB, and MSI in ESCC patients and analyzing their combined efficacy in predicting responses to immunotherapy. Our findings indicate that a multiplex biomarker approach may provide a more accurate prediction of treatment outcomes compared to single-marker assessments.

Materials and methods

Patients and tumor samples

One hundred ESCC patients who underwent radical resection between June 2018 and June 2020 at Taizhou People's Hospital were selected. Eligible patients had to have a diagnosis of ESCC confirmed by postoperative histology and sufficient tissue material for PD-L1 immunohistochemistry and TMB analysis. Patients with complications from other tumors, severe underlying diseases, immune system disorders, or those who had received any preoperative radiation, chemotherapy, or immunotherapy were excluded. Tumor staging criteria followed the internationally recognized UICC TNM staging system (8th edition), specifically for esophageal malignancies, as issued by the American Joint Committee on Cancer in 2017.

The collected tumor tissue samples underwent fixation in 10% formalin at room temperature for 24 to 72 h. This preparatory process was essential before proceeding with formalin-fixed paraffin embedding (FFPE), a technique that preserves the tissue for further analysis. The study received ethical approval from the Institutional Ethics Committee under the reference number CXTDA2017042, ensuring that the research adhered to established ethical standards. All participants involved in the study provided informed consent, affirming their voluntary participation. Detailed accounts of the clinic features of the patients, along with the methods employed for tissue collection, can be found in Tables 2 and 3.

PD-L1 immunohistochemistry assessment

PD-L1 expression was assessed using the Ventana SP263 antibody clone on formalin-fixed paraffin-embedded (FFPE) tumor specimens. The combined positive score (CPS) was calculated as the total number of PD-L1-positive tumor cells, lymphocytes, and macrophages divided by the total number of tumor cells, multiplied by 100. Tumors with CPS ≥ 1% were classified as PD-L1-positive, while those with CPS < 1% were classified as PD-L1-negative.

Immunohistochemical analysis of FFPE tumor specimens was performed using the Ventana BenchMark ULTRA automated staining platform (Ventana Medical Systems, Tucson, AZ, USA). Heat-induced epitope retrieval (HIER) was conducted with Cell Conditioning Solution (CC1, Tris-EDTA buffer, pH 8.0) for 64 min at 95 °C. A pre-diluted (5 μg/mL) mouse anti-human PD-L1 antibody (Clone SP263, Suzhou Xuguang Kexing Company) was applied as the primary antibody. Detection was achieved using the OptiView DAB IHC Detection Kit, and slides were counterstained with hematoxylin. Phosphate-buffered saline (PBS, pH 7.4) was used for rinsing

between steps. The combined positive score (CPS) was calculated as the total number of PD-L1-positive tumor cells, lymphocytes, and macrophages divided by the total number of tumor cells, multiplied by 100. Tumors with CPS ≥ 1% were classified as PD-L1-positive, while those with CPS < 1% were classified as PD-L1-negative. Previous studies have used either a three-tier or two-tier approach for categorizing PD-L1 TPS and CPS [17–19].

Next-generation sequencing

For the extraction of genomic DNA, FFPE tissue slices were prepared, with each slice measuring between 6 and 10 mm. The genomic DNA extracted from these samples underwent purification using the Qiagen AllPrep DNA/RNA FFPE Kit, which is specifically designed for this purpose by Qiagen based in Venlo, The Netherlands. To determine the concentration of the isolated DNA, the Qubit dsDNA HS Assay Kit was employed, sourced from Thermo Fisher Scientific in Waltham, MA, USA. Following adjustments to the manufacturer's protocol, an input of 120 nanograms of DNA was utilized for the library construction process. Furthermore, the integrity, size, and overall quality of the genomic DNA were assessed using Genomic DNA ScreenTape on the Agilent 2200 TapeStation system, a method that allows for precise evaluation of DNA quality prior to downstream applications.

MSI and TMB analysis

The NGS panel used for TMB detection showed a strong correlation with TMB results from whole-exome sequencing, confirming that selective sequencing of a limited genome region is sufficient to understand a patient's TMB and forecast the efficacy of ICIs. The samples were sent to Jiangsu Kangwei Century Laboratory for NGS detection. The TMB values were obtained using the a550AR NGS sequence based on the Illumina sequencing platform. TMB was computed by taking the number of somatic missense mutations, nonsense mutations, and coding chimeras and dividing it by the amount of exonic bases with at least 60-fold coverage, represented as the number of mutations for every megabase. TMB levels were categorized into microsatellite instability-high (MSI-H) and microsatellite instability-low (MSI-L), with quantile values > 80% and ≤ 80% muts/Mb, respectively.

The percentage of microsatellite unstable loci in patient samples was determined by detecting specific changes in microsatellite alleles within the genome and analyzing them with bioinformatics algorithms. This value might be influenced by sequencing data quality, tumor cell content, degree of tumor cell necrosis, and other factors.

Statistical analysis

Statistical software SPSS (Statistics 22.0 version, IBM) was used to analyze the data. The correlation between PD-L1 + and TMB-H and other clinicopathological variables was evaluated by the chi-square test or Fisher's exact test. For all statistical tests, two-sided tests were performed, and a P-value less than 0.05 indicates statistical significance.

Results

The overall rate of MSI-H in this cohort was 1.0%. Among the cases we studied, 57% were classified as TMB-H, and 54% showed positive PD-L1 expression. Interestingly, 32% of the PD-L1-positive cases also exhibited TMB-H. Only 1% of cases were positive for all three markers (PD-L1, TMB-H, and MSI-H), while 21% were negative for all three markers. This indicates that PD-L1 and TMB are more commonly expressed in esophageal cancer patients and have greater prognostic value. Due to the limited expression rate of MSI-H (only 1%), conducting meaningful correlation tests was not feasible.

PD-L1 expression

Detailed analysis of PD-L1 expression proved that it was present in the tumor cell cytoplasm and membrane, as well as in the surrounding infiltrating tissues. The expression of PD-L1 was remarkably more abundant in tumor tissues than in paraneoplastic tissues, with 54% (54/100) compared to 22% (22/100), respectively ($P < 0.001$). The results are presented in Table 1; Fig. 1.

PD-L1 expression and clinicopathological features

PD-L1 expression in tumor tissues from ESCC patients was investigated in relation to various clinicopathological features. PD-L1 expression was not significantly correlated with gender, age, smoking history, lesion site, degree of differentiation, EGFR mutation status, degree of invasion, or lymph node metastasis. Notably, there was a significant correlation between PD-L1 expression and TNM stage ($P = 0.019$), indicating that PD-L1 expression varied according to the stage of ESCC (Table 2).

Table 1 Comparison of expression differences of PD-L1 in esophageal squamous cell carcinoma between tumor tissues and paracancerous tissues

Variables	*n*	PD-L1(+)	PD-L1(-)	Positive rate	χ^2	*P* value
Tumor tissue	100	54	46	54%	21.732	< 0.001
Paracancerous tissue	100	22	78	22%		

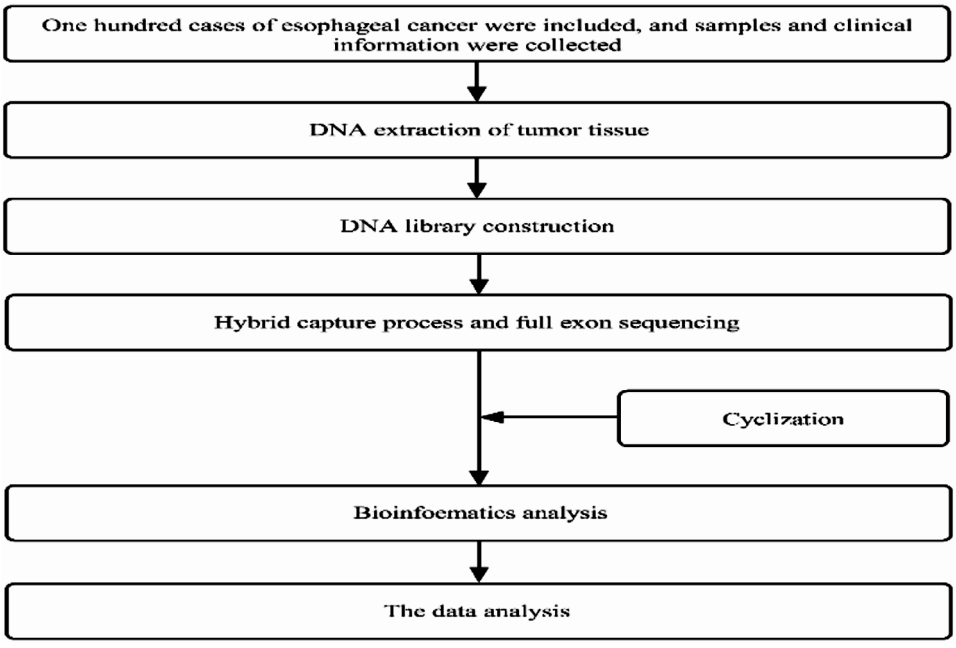

Fig. 1 Expression of PD-L1 in tumor tissue and immune infiltrating cells

TMB and clinicopathological characteristics

The clinicopathological factors associated with TMB were also statistically analyzed (Table 2). TMB in tumor tissues did not significantly correlate with gender, age, smoking history, pathological changes, degree of differentiation, EGFR mutation status, infiltration depth, or TNM stage. However, a significant correlation was found between TMB and lymph node metastasis ($P < 0.001$), indicating that a higher mutational burden in tumor cells may decrease the likelihood of lymph node metastasis.

Relationship between TMB and PD-L1 expression

To further examine the relationship between TMB and PD-L1 expression, the 100 samples were categorized into TMB-H and TMB-L groups. PD-L1 expression was documented in both groups. The positivity rate of PD-L1 expression in the TMB-H ESCC samples was 56.1%, while the positivity rate in the TMB-L ESCC samples was 51.2%. The difference of the two groups was not statistically significant ($p = 0.625$).

Additionally, the 100 samples were further categorized into four groups according to their TMB and PD-L1 status: TMB-H and PD-L1+, TMB-H and PD-L1-, TMB-L and PD-L1+, and TMB-L and PD-L1-. The proportions for each group were 32%, 25%, 22%, and 21%, respectively. Notably, the TMB-H and PD-L1 + groups accounted for the highest proportion (Tables 4 and 5; Fig. 2).

A: PD-L1 was negatively expressed in tumor cells; B: PD-L1 was weakly positive in tumor cells; C: PD-L1 was strongly positive in tumor cells; D: Expression of PD-L1 in immunoinfiltrated cells E: Expression of PD-L1 in precancerous region (IHC×400, SP staining).

Discussion

ICIs have significantly revolutionized the treatment of various malignancies, making the identification of suitable biomarkers crucial for molecular-based therapies such as immunotherapy and targeted therapy [20]. This study was designed to analyze the correlation of PD-L1 expression with TMB and MSI and to explore how these biomarkers relate to the effectiveness of ICIs in ESCC. We investigated the clinicopathological characteristics and relationships among PD-L1 status, TMB, and MSI in esophageal carcinoma patients, assessing PD-L1, MSI, and TMB expression as prediction biomarkers for therapy response to ICIs.

As the first widely used clinical predictive biomarker, PD-L1 has been continuously developed and improved. However, PD-L1 alone is insufficient for predicting treatment effects [21]. Summary analyses of anti-PD-1/PD-L1 antibody tests suggest that the predictive value of PD-L1 is limited [22]. Our results show that PD-L1 expression levels were significantly greater in tumor tissues compared to paraneoplastic tissues, with rates of 54% and 23%, respectively. It is worth noting that we observed a relatively high PD-L1 expression rate (22%) in the paracancerous tissue compared to the tumor tissue (54%). This phenomenon may be explained by field cancerization, a well-documented concept in esophageal cancer [23]. Field cancerization refers to molecular alterations in histologically normal-appearing tissues adjacent to the

Table 2 Correlation analysis of PD-L1 expression in esophageal squamous cell carcinoma and clinicopathological characteristics of patients

Variables	PD-L1(+)	PD-L1(-)	χ^2	*P*	
Sex					
male (*n* = 72)	35	37	3.006	0.083	
female (*n* = 28)	19	9			
Age					
≤ 60 (*n* = 14)	9	5	0.693	0.405	
> 60 (*n* = 86)	45	41			
Smoking					
yes (*n* = 21)	8	13	2.707	0.100	
no (*n* = 79)	46	33			
Alcohol drinking					
yes (*n* = 35)	15		20	2.692	0.101
no (*n* = 65)	39		26		
Lesions part					
upper (*n* = 11)	7	4	0.838	0.658	
middle (*n* = 74)	38	36			
lower (*n* = 15)	9	6			
Degrees of differentiation					
low (*n* = 20)	12	8	0.966	0.617	
middle (*n* = 60)	30	30			
high (*n* = 20)	12	8			
EGFR Status*					
yes (*n* = 70)	37	33	0.123	0.726	
no (*n* = 30)	17	13			
T					
T1 + T2 (*n* = 51)	25	26	1.039	0.308	
T3 + T4 (*n* = 49)	29	20			
N					
lymph node negative (*n* = 54)	27	27	0.756	0.385	
lymph node positive (*n* = 46)	27	19			
TNM					
I/II stage (*n* = 57)	25	32	5.487	0.019	
III/IV stage (*n* = 43)	29	14			

*EGFR status was assessed via immunohistochemistry (IHC) for protein overexpression, not genetic mutation analysis

tumor, which are at increased risk for malignant transformation. The elevated PD-L1 expression in the paracancerous region could reflect early immune evasion mechanisms in precancerous tissues, potentially contributing to tumor progression. Additionally, PD-L1 expression in ESCC tissues was associated with TNM staging, with higher levels detected in later disease stages. These discoveries align with an earlier study by Leng et al., who also reported a positive correlation between PD-L1 expression and the TNM stage in ESCC [24, 25]. What's more, while we used a CPS cut-off of ≥ 1% to define PD-L1 positivity, it is noteworthy that higher cut-offs (e.g., CPS ≥ 5 or CPS ≥ 10) may provide additional predictive value in certain clinical contexts. Future studies should explore the utility of these higher CPS thresholds in ESCC patients receiving immune checkpoint inhibitors.

Similarly, TMB is another critical biomarker for predicting response to ICIs [26]. Tumor cells with higher somatic mutation rates could generate neoantigens that are recognized by the immune system, thereby enhancing T cell-mediated antitumor responses [27–30]. The response rate of the nivolumab group in TMB-H patients was greater than that of the chemotherapy group in the CheckMate 026 trial, providing support for this theory [31]. Current research on TMB levels primarily focuses on melanoma and lung squamous cell cancer; however, its potential in ESCC is still worth exploring. In our cohort, the rate of high TMB in ESCC tissues was 57%, suggesting that TMB could be a valuable clinical biomarker for screening ESCC patients. Moreover, TMB levels were not significantly correlated with the majority of clinicopathological factors, including gender, age, smoking history, lesion site, degree of differentiation, EGFR mutation status, depth of invasion, and TNM

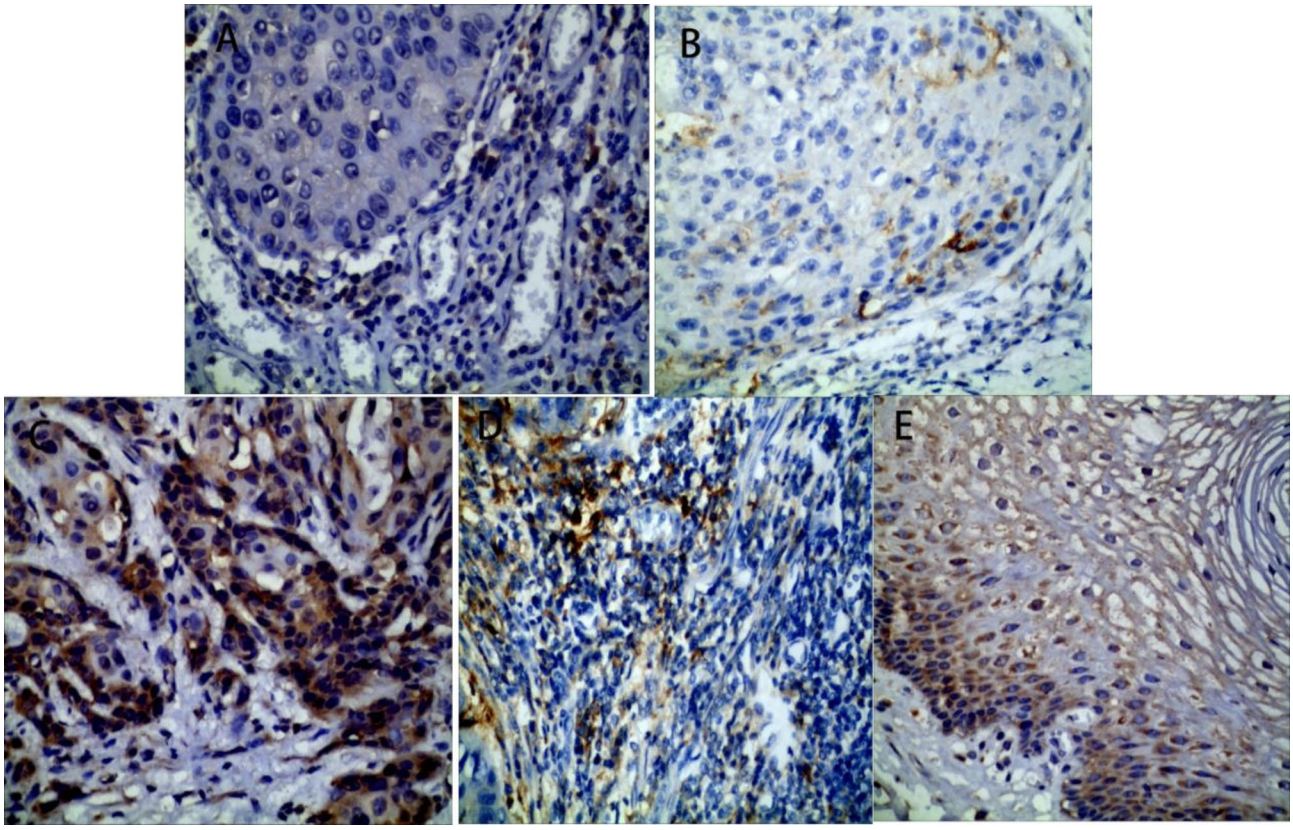

Fig. 2 Correlation pie chart of PD-L1, TMB and MSI

stage. However, an inverse correlation was observed with lymph node metastasis, where a higher TMB level was associated with fewer lymph node metastases. This finding has not been previously reported and warrants further investigation.

Besides PD-L1 and TMB, MSI resulting from deficient mismatch repair (dMMR) is also known to be a relevant predictive biomarker for therapy response to ICIs [32]. It results in the accumulation of mutations and the formation of neoantigens, which enhances antitumor immunity [33]. However, our study identified only one MSI-H case, limiting our ability to draw strong conclusions about its role in ESCC. Given the low prevalence of MSI-H in our cohort, its utility as a predictive biomarker in ESCC remains uncertain [34].

Despite the lack of synergistic effects between TMB and PD-L1 in multiple trials, high expressions of both markers have been associated with better outcomes in monotherapy against PD-1 and PD-L1. Hellmann and colleagues revealed that patients with non-small cell lung cancer with both high PD-L1 and high TMB expressions exhibited the highest efficacy with nivolumab and ipilimumab combined immunotherapy, achieving a clinical benefit rate of 52.5% [35]. This suggests that these biomarkers can be used together to optimize treatment strategies. Our study, alongside recent evidence from Zhao et al. [36], highlights the unique biomarker landscape of ESCC, where PD-L1 and TMB exhibit independent roles. While prior studies in gastric and biliary tract cancers [37] suggest synergistic biomarker interactions, ESCC biology appears distinct, likely due to differences in tumor etiology and microenvironment. These findings advocate for tumor-specific biomarker evaluation. Furthermore, recent studies in lung cancer have demonstrated that focal amplifications of PD-L1 are strong predictors of ICI response. Although we did not evaluate PD-L1 copy number variations in this study, future research should investigate the role of PD-L1 CNVs in esophageal carcinoma to better understand its potential as a biomarker for immunotherapy efficacy [38].

This study has several limitations. Firstly, MSI-H cases constituted a small proportion of positives in the cohort, which limits the generalizability of our findings. Secondly, due to the retrospective nature of our study, we were unable to retrospectively evaluate additional PD-L1 scores, such as tumor proportion score (TPS), immune cell (IC) score, or tumor area positivity (TAP). These scores may provide additional insights in future prospective studies. Additionally, our study focused exclusively on ESCC, and the relationships between biomarkers observed may differ in other subtypes of esophageal carcinoma or other malignancies. Further in-depth

Table 3 Correlation analysis of TMB expression in esophageal squamous cell carcinoma and clinicopathological characteristics of patients

Variables	TMB-H	TMB-L	χ^2	*P* value	
Sex					
male (*n* = 72)	42	30	0.187	0.666	
female (*n* = 28)	15	13			
Age					
≤ 60 (*n* = 14)	9	5	0.353	0.553	
> 60 (*n* = 86)	48	38			
Smoking					
yes (*n* = 21)	12	9	0.000	0.988	
no (*n* = 79)	45	34			
Alcohol drinking					
yes (*n* = 32)	17		15	0.288	0.591
no (*n* = 68)	40		28		
Lesions part					
upper (*n* = 11)	6	5	3.822	0.148	
middle (*n* = 74)	39	35			
lower (*n* = 15)	12	3			
Degrees of differentiation					
low (*n* = 20)	10	10	1.333	0.514	
middle (*n* = 60)	37	23			
high (*n* = 20)	10	10			
EGFR status *					
yes (*n* = 70)	20	31	0.157	0.692	
no (*n* = 30)	18	12			
T					
T1 + T2 (*n* = 51)	29	22	1.785	0.618	
T3 + T4 (*n* = 49)	28	21			
N					
Lymph node negative (*n* = 54)	36	18	6.456	0.011	
Lymph node positive (*n* = 46)	19	27			
TNM					
I/II stage(*n* = 57)	31	26	0.370	0.543	
III/IV stage (*n* = 43)	26	17			

*EGFR status was assessed via immunohistochemistry (IHC) for protein overexpression, not genetic mutation analysis

Table 4 The expression and proportion of pathological factors in 100 patients

		MSI	TMB	PD-L1	MSI-H, TMB-H	MSI-H, PD-L1+	TMB-H, PD-L1+	MSI-H, TMB-H, PD-L1+	None
	N	MSI-H (%)	TMB-H (%)	PD-L1+ (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Esophageal	100	1(1)	57(57)	54(54)	1(1)	1(1)	32(32)	1(1)	21(21)

Table 5 Correlation diagram of PD-L1 and TMB

Variables	*n*	PD-L1(+)	PD-L1(-)	χ^2	*P* value
TMB-H	57	32	25	0.244	0.621
TMB-L	43	22	21		

researches are required to explore the biological links and mechanisms underlying the clinical significance of these markers.

Conclusions

In this clinical investigation, we detected and validated the expression levels of PD-L1, TMB, and MSI in ESCC. Our findings show that PD-L1, TMB, and MSI are prevalent in this carcinoma subtype. Additionally, higher levels of PD-L1 expression were correlated with more advanced stages of ESCC, while more abundant TMB was correlated with fewer instances of lymph node metastasis. These results indicate that elevated levels of PD-L1 and TMB may predict better efficacy of immunotherapy in advanced esophageal carcinoma. Therefore, more

researches are warranted to explore the biological mechanisms behind these clinical correlations.

Supplementary Information

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Supplementary Material 1

Author contributions

Suyao Li, Yongling Yu and Yirong Xu wrote the main manuscript text. Junxing Huang, Jinghao Jia and Yue Zhou prepared Tables 1, 2, 3, 4 and 5, Suyao Li and Junxing Huang prepared Figs. 1 and 2. All authors reviewed the manuscript.

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Data availability

All data generated and analysed during this study are included in supplementary information files.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Department of Medical Oncology, Taizhou People's Hospital, Taizhou, China (No. KY20189401), and informed consent was obtained from all patients. The authors confirm that the reporting of human experimentation and the use of human tissue samples were conducted in accordance with relevant guidelines and regulations.

Consent for publication

All authors and hospitals agreed to publish this article. Informed consent for publication of images were obtained from all participants.

Competing interests

The authors declare no competing interests.

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