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ORIGINAL ARTICLE

Correlation of PD-L1 expression with CD8+ T cells and oxidative stress-related molecules NRF2 and NQO1 in esophageal squamous cell carcinoma

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Abstract

Oxidative stress and the immune microenvironment both contribute to the pathogenesis of esophageal squamous cell carcinoma (ESCC). However, their interrelationships remain poorly understood. We aimed to examine the status of key molecules involved in oxidative stress and the immune microenvironment, as well as their relationships with each other and with clinicopathological features and prognosis in ESCC. The expression of programmed death-ligand 1 (PD-L1), CD8, nuclear factor erythroid-2 related factor-2 (NRF2), and NAD(P)H quinone oxidoreductase 1 (NQO1) was detected using immunohistochemistry in tissue samples from 176 patients with ESCC. We employed both combined positive score (CPS) and tumor proportion score (TPS) to evaluate PD-L1 expression and found a positive correlation between CPS and TPS. Notably, PD-L1 expression, as assessed by either CPS or TPS, was positively correlated with both NRF2 nuclear score and NQO1 score in stage II–IV ESCC. We also observed a positive correlation between the density of CD8+ T cells and PD-L1 expression. Furthermore, high levels of PD-L1 CPS, but not TPS, were associated with advanced TNM stage and lymph node metastases. Moreover, both PD-L1 CPS and the nuclear expression of NRF2 were found to be predictive of shorter overall survival in stage II–IV ESCC. By using the Mandard-tumor regression grading (TRG) system to evaluate the pathological response of tumors to neoadjuvant chemotherapy (NACT), we found that the TRG-5 group had higher NRF2 nuclear score, PD-L1 CPS, and TPS in pre-NACT biopsy samples compared with the TRG-3 + 4 group. The NQO1 scores of post-NACT surgical specimens were significantly higher in the TRG-5 group than in the TRG 3 + 4 group. In conclusion, the expression of PD-L1 is associated with aberrant NRF2 signaling pathway, advanced TNM stage, lymph node metastases, and unfavorable prognosis. The dysregulation of PD-L1 and aberrant activation of the NRF2 signaling pathway are implicated in resistance to NACT. Our findings shed light on the complex interrelationships between oxidative stress and the immune microenvironment in ESCC, which may have implications for personalized therapies and improved patient outcomes.

Keywords: PD-L1; CD8+ T cells; NRF2; NQO1; esophageal squamous cell carcinoma; tumor immune microenvironment; oxidative stress

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No conflicts of interest were declared.

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common types of cancer worldwide [1], ranking sixth in terms of incidence and fourth in terms of cancer-related deaths in PR China [2]. Despite advances in multidisciplinary therapies including surgery, chemotherapy, radiotherapy, and immunotherapy, the prognosis for ESCC patients remains unfavorable [3]. Therefore, further investigation is needed to clarify the specific roles of tumor-related factors in the development of ESCC, which will facilitate the exploration of innovative therapeutic strategies.

The tumor immune microenvironment and oxidative stress are interconnected and can interact in complex

ways to shape the cancer landscape. Reactive oxygen species (ROS)-induced oxidative stress has been found to play multifaceted roles in the tumor immune response, including the release of tumor-associated antigens, antigen presentation and recognition, immune cell differentiation, and immune infiltration of tumors, thereby regulating immunotherapeutic effects [4–6]. For instance, oxidative stress can impair the function of natural killer cells and cytotoxic T cells, making them less effective at recognizing and eliminating cancer cells [7,8]. Moreover, ROS can activate the Akt signaling pathway and lead to a substantial rise in the level of programmed death-ligand 1 (PD-L1) protein in tumor cells [5]. In turn, a dysregulated immune microenvironment can alter the redox status and induce oxidative stress, which can further compromise immune function and promote cancer progression. Besides, increasing evidence suggests that immunotherapies may intensify tumor oxidative stress, leading to ROS-dependent tumor rejection, with minimal toxicities to normal tissues [6]. Nuclear factor erythroid-2 related factor-2 (NRF2) is a vital transcription factor that regulates cellular redox homeostasis and responds to oxidative stress by its nuclear translocation and initiation of the transcription of a multitude of antioxidant genes, such as NAD(P)H quinone oxidoreductase 1 (NQO1) and proteins for glutathione synthesis [9]. Mutations in genes encoding NRF2 or its regulator Kelch-like ECH-associated protein 1 (KEAP1), which result in aberrant nuclear translocation of NRF2, have been found in ESCC and are associated with poor prognosis and resistance to chemotherapy and radiotherapy [10,11]. Although PD-L1-based immunotherapy has shown survival benefits in metastatic ESCC [3], it has been reported to be ineffective in treating NRF2-addicted non-small cell lung cancer (NSCLC) [12].

These findings highlight the importance of understanding the interrelationship between the tumor immune microenvironment and oxidative stress in ESCC and the need for developing new therapeutic strategies to overcome resistance to current mainstream cancer therapies. In the present study, we aimed to elucidate the status of PD-L1, NRF2, NQO1, and CD8+ T cells in ESCC. Additionally, we sought to explore the correlations between these factors and their associations with clinicopathological features and prognosis in patients who underwent esophagectomy with or without neoadjuvant chemotherapy (NACT), providing novel insights into the treatment and prognosis of ESCC.

Materials and methods

Clinical sample collection

A total of 176 ESCC patients who underwent esophageal mucosal biopsy or esophagectomy at Beijing Chao-Yang Hospital between January 2018 and October 2021 were retrospectively included. Of these patients, 64 had stage I ESCC, whereas 112 had stage II–IV ESCC. Fifteen patients underwent NACT with paclitaxel + cisplatin/carboplatin, and pre-NACT mucosal biopsy specimens and post-NACT surgical specimens were collected. None of these patients received immunotherapy. The TNM stage was defined in accordance with the eighth edition of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) TNM staging system. This study was approved by the institutional review boards of Beijing Chao-Yang Hospital, Capital Medical University. Informed consent was waived because of the retrospective and anonymous nature of this study.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using the Ventana BenchMark ULTRA platform (Roche Diagnostics, Indianapolis, IN, USA) and the following antibodies: PD-L1 (dilution 1:50, clone 22C3, Dako, Carpinteria, CA, USA), CD8 (dilution 1:150, clone SP16, Fuzhou Maixin Biotech Co., Ltd., Fuzhou, PR China), NRF2 (dilution 1:100, clone EP1808Y, Abcam, Cambridge, UK), and NQO1 (dilution 1:150, clone A-5, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

PD-L1 expression was evaluated using both the combined positive score (CPS) with a cutoff of 10 and the tumor proportion score (TPS) with a cutoff of 1%. CD8+ T cells were counted in six random fields in intratumoral areas and tumor-invasive margin at ×400 magnification, and an average density of ≥25/field was considered positive for CD8+ T cells.

Staining intensity for NRF2 and NQO1 was rated on a scale of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). IHC score was calculated by multiplying the staining intensity by the percentage of positive tumor cells, which ranged from 0 to 300. IHC staining of NRF2 was evaluated using a nuclear IHC score with a cutoff of 20, whereas IHC staining of NQO1 was assessed using an NQO1 score with a cutoff of 150 [10], as previously described.

Assessment of the pathological response to NACT

Post-NACT surgical specimens were evaluated using the Mandard tumor regression grading (Mandard-TRG) system [13]. TRGs were classified into five grades. TRG1: no residual cancer. TRG2: rare residual cancer cells. TRG3: fibrosis outgrowing residual cancer. TRG4: residual cancer outgrowing fibrosis. TRG5: absence of regressive changes.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA). Chi-square test or Fisher's exact test was used to compare categorical variables. Spearman correlation analysis was used to measure the strength of the association between two variables and the direction of the relationship. Differences between the two groups were assessed by *t*-test or Wilcoxon rank sum test. Kaplan–Meier curves and Cox regression analyses were performed for investigating the overall survival (OS) outcome and independent prognostic factors. $p < 0.05$ was considered to be statistically significant.

Results

Clinical characteristics

Among 176 patients with ESCC, 150 (85.2%) were males and 26 (14.8%) were females. There were 64 cases (36.4%) of stage I disease, 60 cases (34.1%) of stage II, 48 cases (27.3%) of stage III, and 4 cases (2.2%) of stage IV. For histopathological grade, 24 cases (13.9%) had well-differentiated ESCC (grade 1), 115 cases (66.5%) had moderately differentiated ESCC (grade 2), and 34 cases (19.6%) were poorly differentiated ESCC (grade 3). Sixty-four cases (36.4%) had vascular invasion, and lymph node metastases occurred in 57 cases (32.4%). A total of 112 patients (63.6%) had a smoking history and 102 (58.0%) had a history of alcohol consumption.

The expression of PD-L1, CD8, NRF2, and NQO1 in ESCC

All 64 stage I ESCC cases had pT1 lesions, without infiltration into the muscularis propria. Of the 64 patients, 16 (25.0%) had PD-L1 CPS ≥ 10, 54 (84.4%) had PD-L1 CPS ≥ 1, 36 (56.3%) had PD-L1 TPS ≥ 1%, 49 (76.6%) were positive for CD8+ T cells, 26 (40.6%) were positive for NRF2 nuclear expression and 37 (57.8%) were positive for NQO1 expression. Of the 112 patients with stage II–IV ESCC, 18 (16.1%) had PD-L1 CPS ≥ 10, 89 (79.5%) had PD-L1 CPS ≥ 1, 61 (54.4%) had PD-L1 TPS ≥ 1%, 69 (61.6%) were positive for CD8+ T cells, 31 (27.7%) were positive for NRF2 nuclear expression, and 36 (32.1%) were positive for NQO1 expression (Figure 1).

The interrelationships among PD-L1 CPS, PD-L1 TPS, CD8+ T cells, NRF2, and NQO1 in ESCC

We investigated the interrelationships among immune microenvironment-related factors and oxidative stress pathway-related molecules in stage I and stage II–IV of ESCC. We observed a robust positive correlation between PD-L1 CPS and PD-L1 TPS ($r = 0.635$, $p = 0.000$ in stage I; $r = 0.876$, $p = 0.000$ in stages II–IV). Additionally, the number of PD-L1 staining tumor cells correlated positively with the number of PD-L1 staining lymphocytes and macrophages ($r = 0.689$, $p = 0.000$ in stage I; $r = 0.627$, $p = 0.000$ in stages II–IV), and the density of CD8+ T cells ($r = 0.233$, $p = 0.014$ in stages II–IV). Furthermore, the density of CD8+ T cells was positively correlated with PD-L1 positivity when evaluated using either CPS ($r = 0.263$, $p = 0.036$ in stage I; $r = 0.248$, $p = 0.009$ in stages II–IV) or TPS ($r = 0.317$, $p = 0.011$ in stage I; $r = 0.220$, $p = 0.019$ in stages II–IV). NRF2 nuclear score was positively correlated with NQO1 score ($r = 0.474$, $p = 0.000$ in stage I; $r = 0.609$, $p = 0.000$ in stages II–IV), whereas no significant association was detected between the cytoplasmic expression of NRF2 and these markers ($p > 0.05$). Notably, in stages II–IV, we observed positive correlations between PD-L1 CPS and NRF2 nuclear score ($r = 0.256$, $p = 0.006$), as well as NQO1 score ($r = 0.269$, $p = 0.004$). Likewise, PD-L1 TPS showed positive correlations with NRF2 nuclear score ($r = 0.216$, $p = 0.022$), and NQO1 score ($r = 0.254$, $p = 0.007$) in stage II–IV ESCC (Tables 1 and 2).

The associations between PD-L1 CPS, PD-L1 TPS, CD8+ T cells, NRF2, NQO1 and the clinicopathological characteristics in stage II–IV ESCC

The PD-L1 CPS was found to be associated with TNM stage ($p = 0.021$). Specifically, 5 of 60 (8.3%) patients with stage II ESCC had PD-L1 CPS ≥ 10, compared with 13 of 52 (25.0%) patients with stages III and IV. Furthermore, the PD-L1 CPS was associated with lymph node metastasis, with 13 of 43 (30.2%) cases having PD-L1 CPS ≥ 10 in patients with lymph

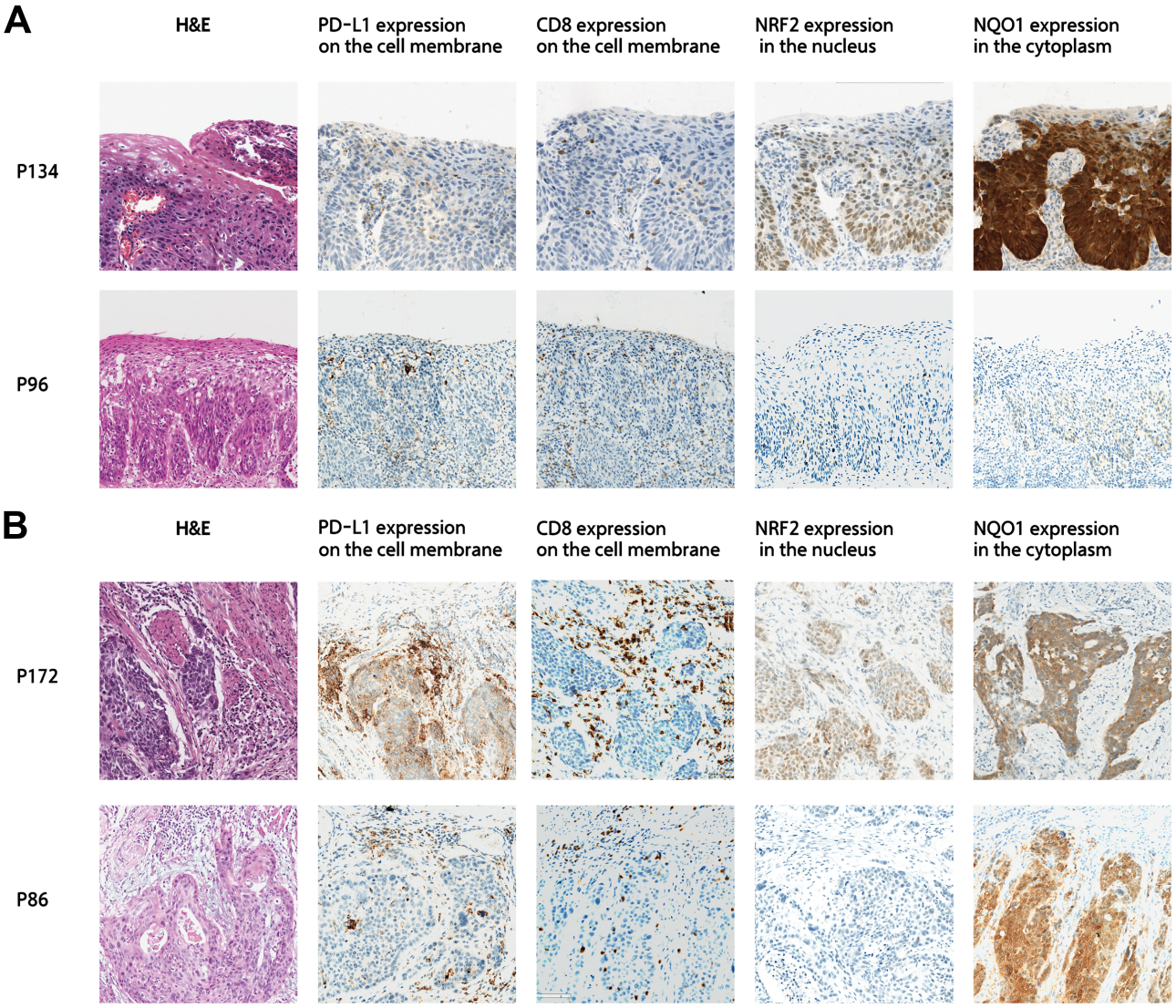

Figure 1. The expression of PD-L1, CD8+ T cells, NRF2, and NQO1 in ESCC. (A) Representative H&E and IHC staining for PD-L1, CD8+ T cells, NRF2, and NQO1 in stage I ESCC collected from two patients. P134: patient 134; P96: patient 96. (B) Representative H&E and IHC staining for PD-L1, CD8+ T cells, NRF2, and NQO1 in stage II–IV ESCC patients. P172: a patient with stage II ESCC; P86: a patient with stage III ESCC. All images were obtained at ×200 magnification.

node metastasis, versus 5 of 69 (7.2%) cases in patients without lymph node metastasis. In addition, CD8+ T cells were associated with vascular invasion. No significant associations were identified between PD-L1 TPS, NRF2, NQO1, and clinicopathological characteristics (Table 3).

Prognostic significance of NRF2 and PD-L1 expression in stage II–IV ESCC

In stage II–IV ESCC, our analysis revealed that patients with positive nuclear expression of NRF2 (nuclear IHC score ≥ 20) had a significantly shorter median OS compared with those with negative expression (24.0 months versus 48.0 months, $p = 0.007$, Figure 2A). Moreover, patients with a high proportion of NRF2 expression in the nucleus (more than 20%) had shorter median OS than others (17.0 months versus 48.0 months, $p = 0.000$, Figure 2B). Additionally, patients with positive PD-L1 expression (CPS ≥ 10) had shorter median OS than those with negative expression (22.0 months versus 48.0 months, $p = 0.013$, Figure 2C). However, there was no significant association between OS and NQO1 expression ($p = 0.191$,

Table 1. Relationships among PD-L1 CPS, PD-L1 TPS, CD8+ T cells, NRF2, and NQO1 in stage I ESCC

		NRF2	NQO1	CD8+ T cells	PD-L1TPS	PD-L1CPS	PD-L1 staining tumor cells	PD-L1 staining immune cells
NRF2	*r*	1.000	0.474**	0.039	0.069	0.071	0.195	0.027
	p	–	0.000	0.761	0.589	0.579	0.122	0.834
NQO1	*r*	0.474**	1.000	0.042	0.018	−0.169	0.162	−0.065
	p	0.000	–	0.740	0.890	0.183	0.202	0.610
CD8+ T cells	*r*	0.039	0.042	1.000	0.317*	0.263*	0.382**	0.436**
	p	0.761	0.740	–	0.011	0.036	0.002	0.000
PD-L1 TPS	*r*	0.069	0.018	0.317*	1.000	0.635**	0.660**	0.350**
	p	0.589	0.890	0.011	–	0.000	0.000	0.000
PD-L1 CPS	*r*	0.071	−0.169	0.263*	0.635**	1.000	0.617**	0.627**
	p	0.579	0.183	0.036	0.000	–	0.000	0.000
PD-L1 staining tumor cells	*r*	0.195	0.162	0.382**	0.660**	0.617**	1.000	0.689**
	p	0.122	0.202	0.002	0.000	0.000	–	0.000
PD-L1 staining immune cells	*r*	0.027	−0.065	0.436**	0.350**	0.627**	0.689**	1.000
	p	0.589	0.890	0.011	–	0.000	0.000	0.000

PD-L1 staining immune cells represent the number of PD-L1 staining lymphocytes and macrophages.
CPS, combined positive score; NQO1, NAD(P)H quinone dehydrogenase 1; NRF2, nuclear factor erythroid-2 related factor-2; *p*, significance; *r*, correlation coefficient; TPS, tumor proportion score.
*$p < 0.05$.
**$p < 0.01$.

Figure 2D), CD8+ T cells ($p = 0.612$, Figure 2E), or PD-L1 TPS ($p = 0.293$, Figure 2F). Multivariate Cox proportional hazards regression analysis identified positive nuclear expression of NRF2 (hazard ratio [HR]= 3.337, 95% CI, 1.593–6.988, $p = 0.001$) and PD-L1 CPS (HR = 2.604, 95% CI, 1.012–6.702, $p = 0.047$) as independent prognostic factors in stage II–IV ESCC.

Effects of NACT on expressions of PD-L1, CD8, NRF2, and NQO1

Compared with pre-NACT biopsy specimens, post-NACT surgical specimens showed a decrease in the nuclear positive score of NRF2 (median 80.0 versus 45.0, $p = 0.028$), PD-L1 CPS (median 8.0 versus 3.0, $p = 0.037$), and PD-L1 TPS (median 3% versus 1%, $p = 0.018$). However, CD8+ T cells (median 55.0 versus 110.0, $p = 0.367$) and NQO1 (median 210.0 versus 175.0, $p = 0.098$) did not demonstrate any statistically significant differences (Figure 3).

Post-NACT surgical specimens were evaluated using the Mandard-TRG system to assess the pathological response to NACT. According to this system, there were two (13.3%), five (33.3%), and eight (53.3%) cases in TRG-3, TRG-4, and TRG-5, respectively. The TRG-5 group exhibited significantly higher

Table 2. Relationships of PD-L1 CPS, PD-L1 TPS, CD8+ T cells, NRF2, and NQO1 in stage II–IV ESCC

		NRF2	NQO1	CD8+ T cells	PD-L1 TPS	PD-L1 CPS	PD-L1 staining tumor cells	PD-L1 staining immune cells
NRF2	*r*	1.000	0.609**	−0.012	0.216*	0.256**	0.098	0.069
	p	–	0.000	0.903	0.022	0.006	0.304	0.467
NQO1	*r*	0.609**	1.000	0.023	0.254**	0.296**	0.117	0.093
	p	0.000	–	0.809	0.007	0.004	0.218	0.330
CD8+ T cells	*r*	−0.012	0.023	1.000	0.220*	0.248**	0.233*	0.325**
	p	0.903	0.809	–	0.019	0.009	0.014	0.000
PD-L1 TPS	*r*	0.216*	0.254*	0.220*	1.000	0.876**	0.773**	0.412**
	p	0.022	0.007	0.019	–	0.000	0.000	0.000
PD-L1 CPS	*r*	0.256**	0.269**	0.248**	0.876**	1.000	0.701**	0.625**
	p	0.006	0.004	0.009	0.000	-	0.000	0.000
PD-L1 staining tumor cells	*r*	0.098	0.117	0.233*	0.773**	0.701**	1.000	0.627**
	p	0.304	0.218	0.014	0.000	0.000	–	0.000
PD-L1 staining immune cells	*r*	0.069	0.093	0.325**	0.412**	0.625**	0.627**	1.000
	p	0.467	0.330	0.000	0.000	0.000	0.000	–

PD-L1 staining immune cells represent the number of PD-L1 staining lymphocytes and macrophages.
CPS, combined positive score; NQO1, NAD(P)H quinone dehydrogenase 1; NRF2, nuclear factor erythroid-2 related factor-2; *p*, significance; *r*, correlation coefficient; TPS, tumor proportion score.
*$p < 0.05$.
**$p < 0.01$.

Table 3. The associations between PD-L1 CPS, CD8+ T cells, NRF2, and clinicopathological characteristics in stage II–IV ESCC

Characteristics	*n* (%)	PD-L1 CPS		*p* value	CD8+ T cells		*p* value	NRF2		*p* value
		Negative	Positive		Negative	Positive		Negative	Positive	
Gender										
Male	150 (85.2)	83 (84.7%)	15 (15.3%)	0.696	38 (38.8%)	60 (61.2%)	1.000	70 (71.4%)	28 (28.6%)	0.754
Female	26 (14.8)	11 (78.6%)	3 (21.4%)	–	5 (35.7%)	9 (64.3%)	–	11 (78.6%)	3 (21.4%)	–
Age										
≥60	107 (60.8)	57 (85.1%)	10 (14.9%)	0.794	25 (37.7%)	42 (62.7%)	0.844	49 (73.1%)	18 (26.9%)	0.832
<60	59 (39.2)	37 (82.2%)	8 (17.7%)	–	18 (40%)	27 (60%)	–	32 (71.1%)	13 (28.9%)	–
Histological grade										
High–medium	139 (80.4)	74 (87.1%)	11 (12.9%)	0.134	33 (38.8%)	52 (61.2%)	1.000	63 (74.1%)	22 (25.9%)	0.467
Low	34 (19.6)	20 (74.1%)	7 (25.9%)	–	10 (37.0%)	17 (63.0%)	–	18 (66.7%)	9 (33.3%)	–
AJCC stage										
II	60 (34.1)	55 (91.7%)	5 (8.3%)	0.021*	22 (36.7%)	38 (63.3%)	0.702	44 (73.3%)	16 (26.7%)	0.835
III + IV	52 (29.5)	39 (75.0%)	13 (25%)	–	21 (40.4%)	31 (59.6%)	–	37 (71.2%)	15 (28.8%)	–
Vascular invasion										
Yes	64 (36.4)	45 (84.9%)	8 (15.1%)	1.000	27 (50.9%)	26 (49.1%)	0.012*	35 (66.0%)	18 (34.0%)	0.205
No	112 (63.6)	49 (83.1%)	10 (16.9%)	–	16 (27.1%)	43 (72.9%)	–	46 (78.0%)	13 (22.0%)	–
Lymph node metastasis										
Yes	57 (32.4)	30 (69.8%)	13 (30.2%)	0.003**	15 (34.9%)	28 (65.1%)	0.690	30 (69.8%)	13 (30.2%)	0.668
No	119 (67.6)	64 (92.8%)	5 (7.2%)	–	28 (40.6%)	41 (59.4%)	–	51 (73.9%)	18 (26.1%)	–
Smoking history										
Yes	112 (63.6)	65 (84.4%)	12 (15.6%)	1.000	29 (37.7%)	48 (62.3%)	0.836	52 (67.5%)	25 (32.5%)	0.113
No	61 (34.7)	29 (82.9%)	6 (17.1%)	–	14 (40.0%)	21 (60.0%)	–	29 (82.9%)	6 (17.1%)	–
Unknown	3 (1.7)	–		–	–		–	–		–
Alcohol consumption										
Yes	102 (58.0)	55 (80.9%)	13 (19.1%)	0.307	24 (35.3%)	44 (64.7%)	0.431	48 (70.6%)	20 (29.4%)	0.670
No	71 (40.3)	39 (88.6%)	5 (11.4%)	–	19 (43.2%)	25 (56.8%)	–	33 (75.0%)	11 (25.0%)	–
Unknown	3 (1.7)	–		–	–		–	–		–

CPS, combined positive score; NRF2, nuclear factor erythroid-2 related factor-2.
*$p < 0.05$.
**$p < 0.01$.

NRF2 nuclear positivity scores (median 177.50 versus 30.0, $p = 0.014$), PD-L1 CPS (median 13.0 versus 3.0, $p = 0.029$), and PD-L1 TPS (median 12% versus 1%, $p = 0.040$) in pre-NACT biopsy samples compared with the TRG-3 + 4 group. However, there was no significant difference in NQO1 score (median 210.0 versus 210.0, $p = 0.378$) and CD8+ T cells (median 55.5 versus 55.0, $p = 0.728$) of pre-NACT samples between the two groups. On the other hand, the NQO1 scores of post-NACT surgical specimens were significantly higher in the TRG-5 group than in the TRG 3 + 4 group (median 232.50 versus 90.0, $p = 0.028$). Additionally, the TRG-5 group showed a trend of having higher NRF2 nuclear positivity scores in post-NACT specimens compared to the TRG-3 + 4 group (median 100.0 versus 0.0, $p = 0.060$). Nevertheless, there was no significant difference in PD-L1 CPS (median 4.0 versus 2.0, $p = 0.162$), PD-L1 TPS (median 2% versus 1%, $p = 0.310$) and CD8+ T cells (median 55.5 versus 55.0, $p = 0.643$) of post-NACT samples between the two groups.

Discussion

Oxidative stress and the immune microenvironment play crucial roles in the pathogenesis of ESCC, but their interplay remains unclear. Our study aimed to investigate the status of key molecules involved in oxidative stress and immune microenvironment, as well as their relationships in ESCC. We used both CPS and TPS methods to evaluate PD-L1 expression and found a positive correlation between the density of CD8+ T cells and PD-L1 positivity. Additionally, NRF2 nuclear score was positively correlated with NQO1 score. Most importantly, we discovered that the expression of PD-L1, as evaluated by either CPS or TPS, was positively correlated with both NRF2 nuclear score and NQO1 score in stage II–IV ESCC. We also observed a robust positive correlation between PD-L1 CPS and PD-L1 TPS. However, PD-L1 CPS, but not TPS, was associated with TNM stage and lymph node metastases. Moreover, PD-L1 CPS and positive nuclear expression of NRF2 could predict shorter OS in stage II–IV ESCC. Additionally,

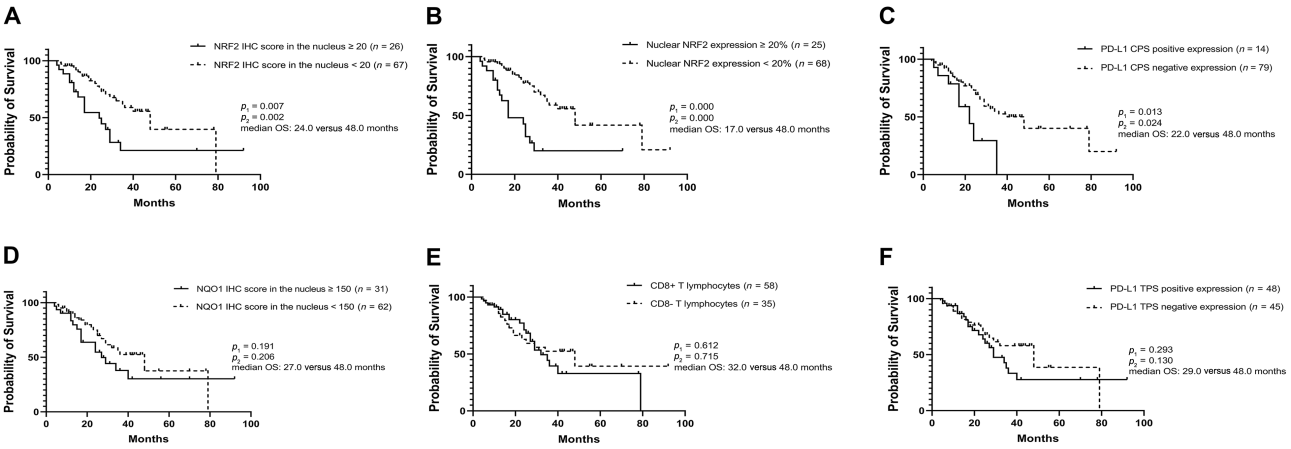

Figure 2. Overall survival by the expression level of NRF2, PD-L1, CD8, and NQO1 in stage II–IV ESCC. (A) Comparison of overall survival between patients with NRF2 nuclear IHC score ≥20 and those patients with NRF2 nuclear IHC score <20. (B) Comparison of overall survival between patients with the proportions of NRF2 expression in the nucleus more than 20% and those with less than 20%. (C) Comparison of overall survival between patients with PD-L1 CPS positive expression and those patients with negative expression. (D) Comparison of overall survival between patients with NQO1 IHC score ≥150 and those patients with NQO1 IHC score <150. (E) Comparison of overall survival between patients with CD8+ T cells positive expression and those patients with negative expression. (F) Comparison of overall survival between patients with PD-L1 TPS positive expression and those patients with negative expression. OS, overall survival. *p*1 and *p*2 indicate *p* values calculated without and with adjusting for age, gender, tumor stage, tumor grade, smoking, and drinking, respectively.

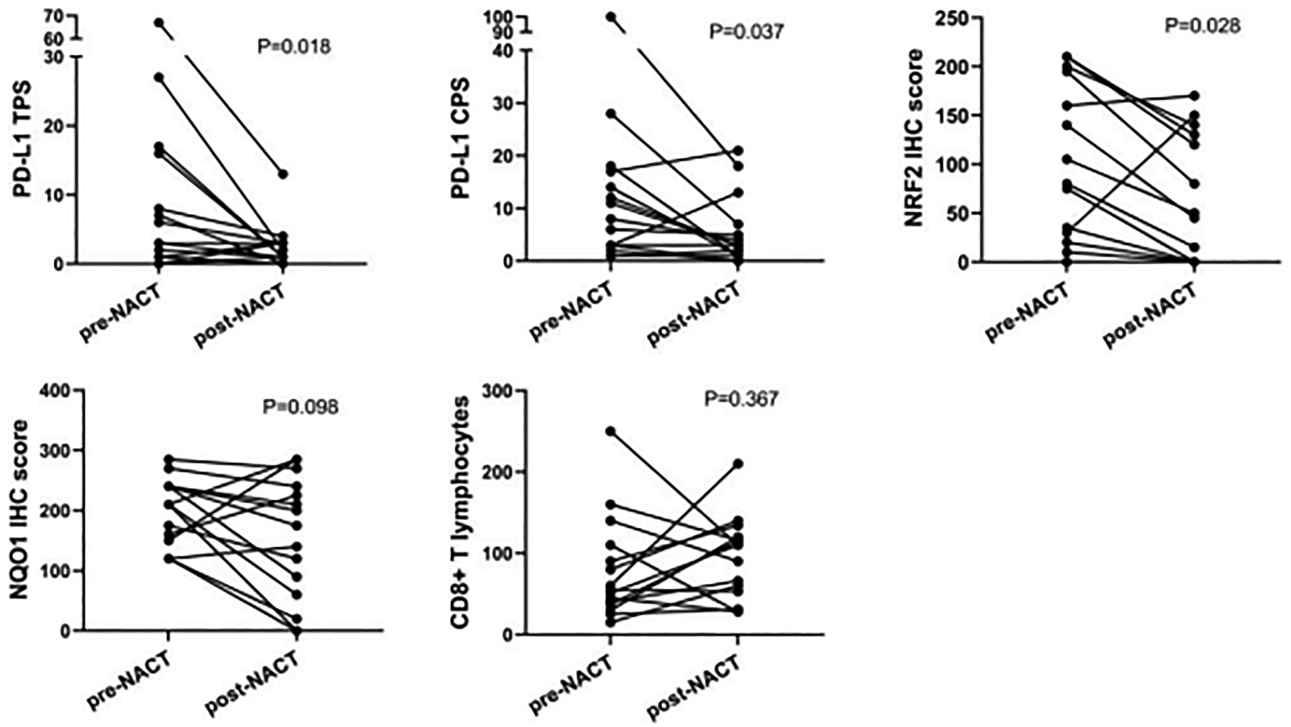

Figure 3. Comparison of the expression of PD-L1 TPS, PD-L1 CPS, CD8+ T cells, the nuclear positive score of NRF2, and the positive score of NQO1 before and after neoadjuvant chemotherapy in 15 patients with ESCC. pre-NACT, before neoadjuvant chemotherapy; post-NACT, after neoadjuvant chemotherapy.

the expression of PD-L1, NRF2, and NQO1 was found to be related to the pathological response to NACT. Our findings shed light on the complex interplay between oxidative stress and immune microenvironment in ESCC, which may have implications for personalized therapies and improved patient outcomes.

In recent years, the expression of PD-L1 has been extensively evaluated as a predictive biomarker for immune checkpoint inhibitors in various malignancies, including hepatocellular carcinoma [14], renal cell carcinoma [15], NSCLC [16], and ESCC [17]. Our study observed a robust positive correlation between PD-L1 CPS and TPS in ESCC. It has also been reported that CPS and TPS are strongly correlated in head and neck cancer [18]. Additionally, we found that the number of PD-L1 staining tumor cells correlated positively with the number of PD-L1 staining lymphocytes and macrophages, consistent with a previous study in a Chinese population with advanced ESCC [19]. Our study also showed a positive correlation between the density of CD8+ T cells and PD-L1 expression when evaluated by either CPS or TPS, which is similar to findings in endometrial serous carcinoma [20]. It has been documented that PD-L1 TPS (clone 73-10, 1% cutoff) was associated with deeper tumor invasion and nodal metastases [21], and PD-L1 TPS (clone SAB2900365, 5% cutoff) was associated with tumor location, grade, lymph node metastases, and TNM stage [22] in ESCC. Our results showed that PD-L1 CPS, but not TPS, was associated with TNM stage and lymph node metastases.

The abnormalities in the NRF2 pathway in ESCC are characterized by gene alterations such as mutations and/or amplification of *NRF2*, *KEAP1*, and *Cullin3* (*CUL3*). When NRF2 cannot bind to KEAP1 normally, it escapes ubiquitination and accumulates in the nucleus, activating downstream genes and enhancing the anti-oxidative stress capacity of tumor cells, as observed in lung cancer [23], squamous cell carcinoma of the skin [24], and ESCC [10]. In our previous study, we identified that high nuclear expression of NRF2 was associated with *NRF2* gene amplification or mutations within DLG and ETGE motifs, and IHC staining for NRF2 and its downstream target gene NQO1 can be used as alternative markers for predicting aberrant activation of the NRF2 signaling pathway in ESCC. In the present study, we observed a positive correlation between NQO1 expression score and NRF2 nuclear score, consistent with our previous findings [10]. Notably, in stage II–IV disease, we observed a positive correlation between PD-L1 expression, either CPS or TPS, and NRF2 nuclear score. Additionally, PD-L1 expression was also positively correlated with NQO1 score. Our analysis revealed that the overexpression of NRF2 in the nucleus (nuclear IHC score ≥ 20) and positive PD-L1 expression (CPS ≥ 10) were associated with poor OS in patients with stages II–IV ESCC. These findings indicate that the expression of PD-L1 may be associated with aberrant activation of NRF2 signaling pathway, providing new evidence for the intrinsic connection between the tumor immune microenvironment and oxidative stress. Further studies are needed to validate these findings in a large, multicenter cohort and clarify the underlying molecular mechanisms.

The tumor immune microenvironment and redox balance can be affected by chemotherapy. Studies have shown that the PD-L1 histoscore in ESCC significantly increased after neoadjuvant concurrent chemoradiotherapy, whereas it significantly decreased after NACT. However, the number of CD8+ T cells in ESCC samples remains unchanged after NACT [25]. Consistently, our study found that post-NACT surgical specimens had lower PD-L1 CPS, PD-L1 TPS, and nuclear positive score of NRF2 compared with paired pre-NACT biopsy specimens, whereas the density of CD8+ TILs remained unchanged. Drugs such as cisplatin, anthracyclines, and cyclophosphamide can achieve their chemotherapy effects by disrupting the redox balance in tumor cells, leading to apoptosis triggered by oxidative stress [26]. Our finding that the nuclear positive score of NRF2 decreased after NACT suggests that these surviving tumor cells may have enhanced adaptability to chemotherapy-induced oxidative stress.

Previous studies have revealed that abnormalities in the NRF2 pathway are associated with response and survival after chemoradiotherapy [27]. After evaluating the pathological response of tumors to NACT using the Mandard-TRG system, we found that the TRG-5 group had higher NRF2 nuclear positivity scores, PD-L1 CPS, and TPS in pre-NACT biopsy samples compared with the TRG-3 + 4 group, suggesting that high levels of NRF2 nuclear positivity score and PD-L1 CPS/TPS in pre-NACT biopsy samples may predict a poor response to NACT. The NQO1 scores of post-NACT surgical specimens were significantly higher in the TRG-5 group than in the TRG 3 + 4 group. Moreover, the TRG-5 group showed a trend of having higher NRF2 nuclear positivity scores in post-NACT specimens compared with the TRG-3 + 4 group. These findings suggest that aberrant activation of the NRF2

signaling pathway is implicated in the resistance to NACT. However, the sample size is small, and further analysis with larger data sets would provide more comprehensive insights.

Chemotherapy and NACT can induce changes in the tumor microenvironment, such as improving the immunogenicity of tumor cells, increasing the number and activity of antigen-presenting cells, improving the activity of T cells, and modifying the immune checkpoints [28,29]. These changes can potentially affect the efficacy of immunotherapy, which relies on the immune system's ability to recognize and attack cancer cells. Our study suggests that ESCC patients with high PD-L1 expression may have a poor pathological response to NACT, and that PD-L1 expression may decrease after NACT. Given the potential benefits of immunotherapy in patients with high PD-L1 expression [30–33], it is reasonable to consider administering immunotherapy in the preoperative setting, especially for those who are likely to benefit from it. Several clinical trials have shown improved efficacy when neoadjuvant immunotherapy is combined with chemotherapy or chemoradiotherapy [34,35]. Therefore, neoadjuvant immunotherapy combined with chemotherapy may be a more optimal strategy compared to NACT alone, especially for patients with high PD-L1 expression. However, careful evaluation of the risks and benefits of this approach is necessary, and ongoing clinical trials will provide important guidance for clinicians and patients.

Taken together, this study demonstrated that the expression of PD-L1 is positively correlated with both the nuclear expression of NRF2 and the expression of NQO1 in stage II–IV ESCC. Furthermore, high levels of PD-L1 CPS were associated with advanced TNM stage and lymph node metastases. Both PD-L1 CPS and the nuclear expression of NRF2 were found to be predictive of shorter OS in stage II–IV ESCC. Additionally, the expression of PD-L1, NRF2, and NQO1 was related to the pathological response to NACT. The study provides novel insights into the interrelationships between immune evasion, oxidative stress, and cancer progression in ESCC, highlighting the importance of considering the interaction between the PD-L1 and NRF2 pathways in developing innovative cancer therapies.

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Author contributions statement

XZ contributed data curation, data analysis and writing – original draft. YY performed data analysis and methodology. HZ, ZT, QC, YL, YG and QS carried out methodology and investigation. XH performed validation and methodology. MJ was involved in supervision, review and editing. XJ contributed supervision and writing – review and editing.

Data availability statement

The data used to support the results of this study can be obtained from the corresponding author.

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