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# Molecular profiles of different PD-L1 expression in patients with esophageal squamous cell carcinoma

Songchen Zhao[a], Xintong Hu[b], Peiwen Zhou[b], Ang Li[b], Liguo Chen[b], Duo Wang[b], Jiaxue He[b], and Yanfang Jiang[b]

[a]Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China; [b]Key Laboratory of Organ Regeneration & Transplantation of the Ministry of Education, Genetic Diagnosis Center, The First Hospital of Jilin University, Changchun, China

**ABSTRACT**

**Background:** PD-1/PD-L1 inhibitors are approved treatments for patients with esophageal squamous cell carcinoma (ESCC). The present investigation aspired to explore the interrelation between molecular phenotype and PD-L1 expression in ESCC.

**Methods:** PD-L1 testing and targeted next-generation sequencing (NGS) were performed on tumoral tissues from 139 ESCC patients. Tumor-infiltrating lymphocytes (TILs) were scrutinized using a tyramide signal amplification system combined with immunohistochemistry.

**Results:** Among enrolled patients, 36.7% displayed high PD-L1 expression (combined positive score [CPS] ≥10). BRCA1 and NF1 gene mutations were significantly associated with high PD-L1 expression ($p < .05$) while TGFβ pathway alterations were linked to low PD-L1 expression ($p = .02$). High copy number instability (CNI) and copy number alterations (CNA) were correlated with low PD-L1 expression. Patients with CDKN2A deletion exhibited higher PD-L1 expression. Varying types of TILs were observed across different PD-L1 expression groups. The ratio of $CD8^+PD\text{-}L1^+$ T cells and $CD8^+PD\text{-}1^+$ T cells to $CD8^+$ T cells remained comparable in both tumoral and stromal regions, but the ratio of $CD68^+PD\text{-}L1^+$ macrophages to $CD68^+$ macrophages was higher than the ratio of $CD68^+PD\text{-}1^+$ macrophages to $CD68^+$ macrophages. CPS was significantly correlated with $PD\text{-}L1^+$ lymphocytes and $CD68^+$ macrophages in the tumoral region. $CD8^+$ T cell infiltration was positively correlated with $PD\text{-}1^+$ cells in both tumoral and stromal regions.

**Conclusion:** In this study, we presented the prevalence rates of PD-L1 expression in Chinese ESCC patients. The association of genetic profiles with PD-L1 expression levels also provide the clue that genomic phenotype may interact with the immunologic phenotype in ESCC.

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ESCC; PD-L1; PD-1; NGS; immunotherapy

## Introduction

Esophageal cancer (EC) ranks as the eighth most prevalent cancer and the sixth leading cause of cancer-related mortality, with a relatively modest five-year overall survival (OS) rate.[1,2] Based on histological characteristics, EC is typically classified into two main subtypes: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC).[2] In China, ESCC is the major subtype of EC, accounting for more than 90% of all EC cases.[3] Currently, treatment options for ESCC remain limited, underscoring the urgent imperative to identify more precise biomarkers and effective therapeutic targets to improve the treatment outcomes for these patients.

Immune checkpoint inhibitors (ICIs) have been shown to improve the clinical outcomes in patients with many tumor types. ICIs are divided into two main categories, targeting programmed death-1/programmed death ligand-1 (PD-1/PD-L1) and cytotoxic T lymphocyte antigen 4 (CTL-4), respectively. PD-1/PD-L1 blockade has demonstrated significant responses and clinical benefits across multiple malignancies, including ESCC.[4–7] PD-L1 is expressed on various cells within the tumor microenvironment (TME), encompassing immune cells and tumor cells. It couples with PD-1 to restrain the function of T cells in the immune system and is involved in preventing autoimmune responses and excessive inflammation.[8] Detection of PD-L1 expression through immunohistochemistry (IHC) has been proposed as a prospective predictive biomarker to identify patients who may derive substantial benefits from anti-PD-1/PD-L1 therapy. This evidence has led to the Food and Drug Administration (FDA) approving its clinical use in a variety of tumor types.[9–11]

PD-1/PD-L1 inhibitors have shown promising anti-tumor activity in patients with advanced EC.[12–14] In the phase III KEYNOTE-181 trial, pembrolizumab did not confer a significant survival benefit over chemotherapy as a second or subsequent line of treatment. However, in the subgroup of patients with a PD-L1 combined positive score (CPS) ≥10, encompassing both PD-L1 expression on both tumors and immune cells, treatment with pembrolizumab was associated with improved overall response rate (ORR) and survival.[15] Moreover, in the KEYNOTE-590 study, a noteworthy survival benefit was observed when pembrolizumab was combined with cisplatin-fluoropyrimidine chemotherapy in ESCC

**CONTACT** Yanfang Jiang yanfangjiang@jlu.edu.cn Key Laboratory of Organ Regeneration & Transplantation of the Ministry of Education, Genetic Diagnosis Center, The First Hospital of Jilin University, Changchun 130021, China

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patients with CPS ≥ 10.[16] Meanwhile, the ATTRACTION-3 and ESCORT studies showed that the PD-1 antibodies nivolumab and camrelizumab were associated with a statistically significant and clinically meaningful improvement in OS, compared to chemotherapy in patients with advanced ESCC.[17,18]

In this study, the researchers conducted PD-L1 testing and targeted NGS on tumor tissue samples from ESCC patients. The primary objectives were to explore the correlation between the molecular profile and patterns of PD-L1 expression and to identify potential genomic alterations that may contribute to favorable responses to anti-PD-1/PD-L1 immunotherapy.

## Materials and methods

### Patient information

The samples and data of 139 patients with histologically confirmed ESCC at the First Hospital of Jilin University from 2019 to 2022 were reviewed. The clinical characteristics of the patients were presented in Table S1. Only patients who had both formalin-fixed paraffin-embedded (FFPE) tissues and matched blood samples available for analysis underwent testing using a designed 543-gene NGS panel to evaluate for somatic and germline mutations. Our study adhered to the tenets of the Declaration of Helsinki and was approved by the Ethical Review Board of the First Hospital of Jilin University (No.2018–467).

### DNA extraction and sequencing

To identify somatic single nucleotide variants (SNVs) and indel mutations, FFPE tumor samples were tested, and the matched blood samples were used as controls. VarScan2 (version 2.4.2) was used for SNV calling and the identified variants were annotated by ANNOVAR. The exclusion criteria of variants were as follows: (a) sequencing coverage < 30×, (b) silent mutations in non-reference alleles, (c) support reads were < 5, (d) allele frequency ≥ 0.005 in the Exome Aggregation Consortium (ExAC) or Genome Aggregation Database (gnomAD) database, and (e) allele frequency < 0.02 in the tumor sample.

### Tumour mutational burden (TMB)

TMB (mutations/Mb) was calculated using a previously established algorithm.[19] Nonsynonymous somatic mutations at the exonic and splicing regions were quantified to determine the TMB value of a sample, with a variant frequency of not less than 0.7%. The total number of mutations counted was divided by the size of the coding region of the target panel to calculate the TMB per megabase.

### Copy number instability (CNI)

GC content and length of target region were first corrected using proprietary algorithms for each region. Subsequently, read counts were transformed into log2 ratios and further transformed to Z-score based on Gaussian transformations, comparing them against a normal control group ($n = 30$). Finally, those target regions were retained whose Z-scores were greater than the 95th percentile and whose absolute standard deviation was twice that of the normal control group. The remaining Z-scores were summed as the CNI score.

### Copy number analysis

For copy number analysis, a set of 30 normal blood samples served as controls to calculate the gene specificity score (GCS). A joint statistical significance test involving GCS and the absolute value of the copy number was employed to determine CNV. If the copy number value was > 4, it was defined as CNV gain, and if the copy number value was < 1, it was defined as CNV loss, respectively. The copy number alteration (CNA) burden was estimated based on the total number of genes exhibiting copy number gains and losses across the samples under investigation.

### PD-L1 expression

IHC staining was performed to assess the PD-L1 expression on the surface of tumor cells (TC) and tumor-infiltrating immune cells (IC). FFPE tissue blocks were sliced up into 4-mm thick sections and stained with anti-PD-L1 22C3 primary antibody (1:50, Dako, M3653) using a Ventana GX automated system (Ventana, AZ, USA). PD-L1 CPS was calculated as the number of PD-L1 positive cells (tumor cells, macrophages and lymphocytes) divided by the total number of tumor cells.

### Multiplex immunohistochemistry

For multiplex immunohistochemical staining, FFPE tissue blocks were sliced up into 4-mm thick sections. The immune biomarker panel included CD68 (1:500, Beijing Zhongshan Golden Bridge Biotechnology, ZM0060), CD8 (1:100, Beijing Zhongshan Golden Bridge Biotechnology, ZA0508), PD-1 (1:50, Beijing Zhongshan Golden Bridge Biotechnology, ZM0381) and PD-L1 (1:25, Roche Diagnostics, 740–4859). TSA visualization was performed using an Opal ™ 7-color IHC Kit (PerkinElmer Inc., Boston, MA, USA) according to the manufacturer's instructions. The fluorophores used in the experiment were Opal 520 (CD8), Opal 570 (PD-L1), Opal 650 (CD68) and Opal 690 (PD-1), with intensity values fixed at 81%. Nuclear counterstaining was performed with DAPI. A PerkinElmer Vectra imaging system (Vectra3.0.5; PerkinElmer, Massachusetts, USA) was used to scan the sections. Regions of interest (ROI) were selected using the Phenochart viewer (Akoya Bioscience) analyzed using inForm Advanced Image Analysis software (inForm 2.3.0; PerkinElmer, Massachusetts, USA). The density of positively stained cells was counted for tumor parenchyma (tumor), distant stroma and total regions respectively.

### Statistical analysis

All statistical analyses were performed using the R software (version 4.1.1; https://www.r-project.org/, Institute of Statistics

and Mathematics, Vienna, Austria). Fisher's exact test was used for comparisons between two categorical variables, and Mann – Whitney U test was used for comparisons between two continuous variables. All differences with $P < .05$ were considered statistically significant.

## Results

### Basic information and PD-L1 expression distribution in our cohort

A total of 139 ESCC patients were eligible for this study. Their samples were subjected to targeted NGS and PD-L1 IHC staining. Out of these patients, 27 patients had sufficient samples for multiplex immunofluorescence (mIF).

Findings from KEYNOTE-181 and KEYNOTE-590 showed that only patients with a high CPS (≥10) benefited from the PD-1 blockades. In this study, a good correlation was observed between CPS and tumor proportion score (TPS) ($R = 0.941$, $p < 2.2e-16$) (Figure 1a). Therefore, we used CPS to represent PD-L1 expression and used CPS = 10 as the cutoff to divide patients into two groups, the high PD-L1 and low PD-L1 groups. We observed that 36.7% of the ESCC patients in this study had high PD-L1 expression (CPS ≥10) while the rest (63.3%) were in the range of 0 to 10 (Figure 1b,c), which is consistent with the observations from a previous study.[20] The distribution of age, sex and stage was not significantly different between the high and low PD-L1 expression groups (Table S1).

### Mutation profiles of the high and low PD-L1 groups

The mutational profiles of patients in the two groups were examined, and the frequencies of mutated genes were compared (Figure 2a). Similar to a previous study,[21] the most frequently mutated genes in this study were *TP53* (91%), *NOTCH1* (30%), *KMT2D* (22%) and *PIK3CA* (21%). Notably, mutations in *BRCA1* and *NF1* genes were the only two genes that were more significantly enriched in patients with high PD-L1 expression than in patients with low PD-L1 expression (*BRCA1*, $p = .02$; *NF1*, $p = .03$) (Figure 2b). Furthermore, the PD-L1 expression in patients with *BRCA1* or *NF1* mutations was higher than in those with wild-type (WT) tumors (*BRCA1*, $p = .06$; *NF1*, $p = .035$) (Figure 2c–d).

TMB has previously been shown to independently predict ICI response[22,23] but was not associated with PD-L1 expression in several cancer types.[24,25] In this study, we observed a similar finding, where PD-L1 expression did not show any significant association with TMB, whether considered as a continuous or categorical variable (Figure 2e,f).

A pathway analysis was then performed to assess the alterations between the two groups (Figure 3a). Similar to another report,[21] most mutations were found to be enriched in the p53 pathway (91%), followed by the Notch (50%) and Ras (46%) pathways. Alterations in the TGFβ pathway were observed more frequently in the low PD-L1 group than in the high PD-L1 group ($p = .02$) (Figure 3b). Furthermore, lower PD-L1 expression was observed in patients with TGFβ pathway alterations (Figure 3c). *TGFBR2*, *SMAD4* and *TGFBR1* were included in the TGFβ pathway (Figure 3d) and their mutations were also found to be enriched in the low PD-L1 group compared to the high PD-L1 group (Figure 3e–g).

### CNA in the high and low PD-L1 groups

As CNI and CNA are predictive of response to ICIs responses,[25–28] the CNI and CNA of the two groups were assessed. Our findings showed that both CNI and CNA were negatively associated with PD-L1 expression ($R = -0.285$, $p = .00451$;$R = -0.193$, $p = .0194$) (Figure 4a, c), and the CNI and CNA levels of the low PD-L1 group were higher than those of the high PD-L1 group ($p = .044$; $p = .036$) (Figure 4b, d). The CNA profiles of the two groups are illustrated in Figure 4e. *CDKN2A* deletion emerged as one of the most prevalent CNAs observed in the ESCC patients, accounting for 29% of cases. Interestingly, we observed that PD-L1 expression was significantly lower in patients with *CDKN2A* deletion (Figure 4f). Additionally, we identified that amplifications in the *TP63* and *PRKCI* genes were enriched in the PD-L1 low expression group (Figure 4g, h).

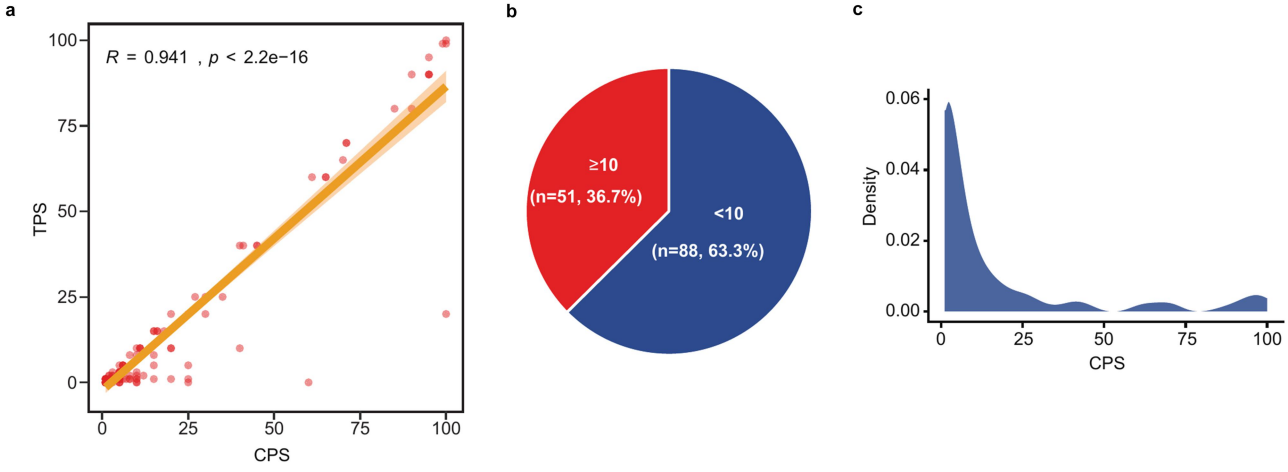


**Figure 1.** Distribution of PD-L1 expression in ESCC patients. (a) correlation between PD-L1 CPS and tumor proportion score (TPS). (b) the distribution of PD-L1 expression (high [≥10] and low [<10]) in ESCC patients. (c) the distribution of PD-L1 expression based on CPS.

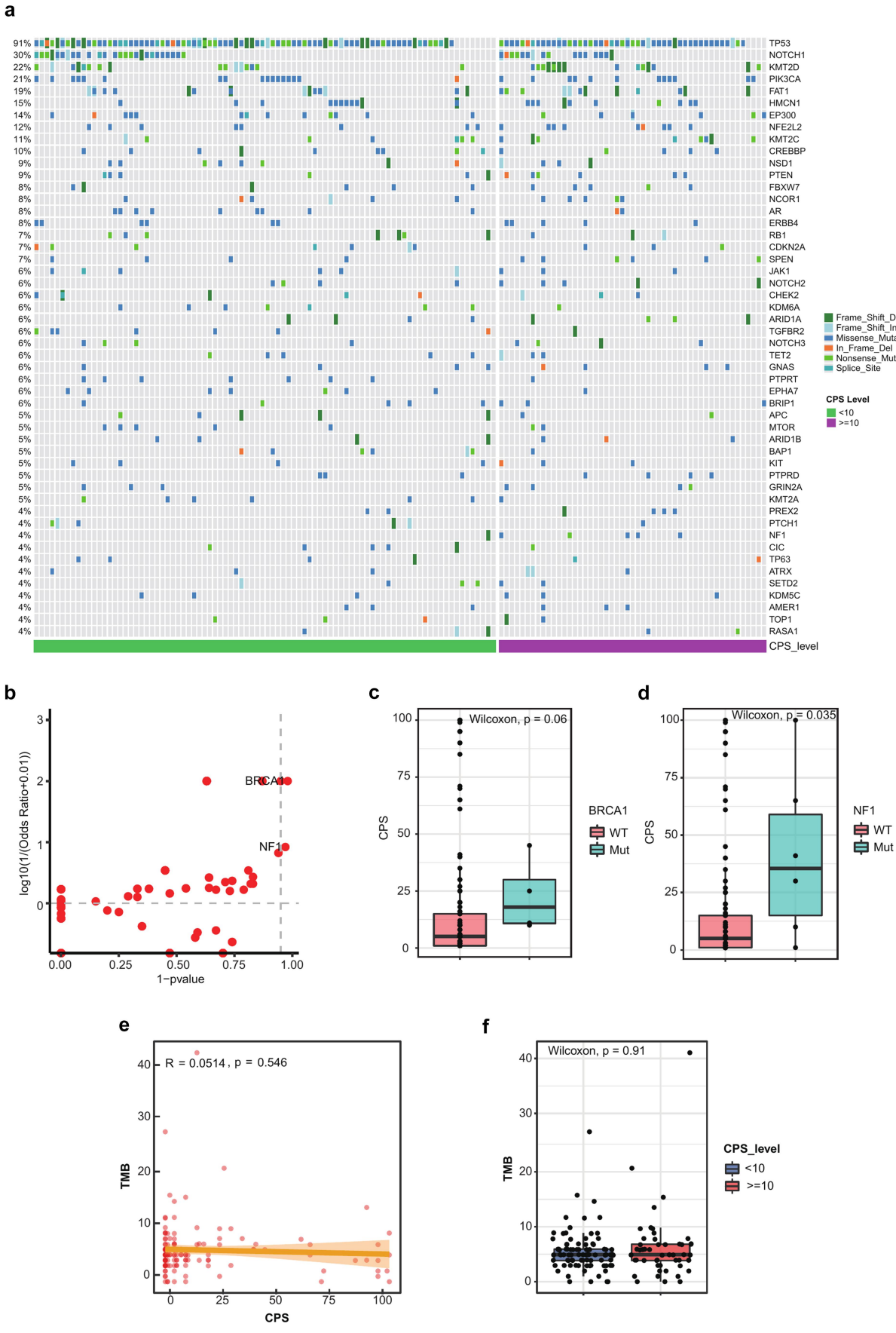


**Figure 2.** Somatic mutations within the PD-L1 subgroups. (a) mutational landscape of the high PD-L1 and low PD-L1 groups. (b) comparison of the frequency of mutated genes in the high PD-L1 and low PD-L1 groups. (c) expression of PD-L1 in patients with and without *BRCA1* mutations. (d) expression of PD-L1 in patients with and without *NF1* mutations. (e) correlation between CPS and TMB. (f) comparison of TMB in the high PD-L1 and low PD-L1 groups.

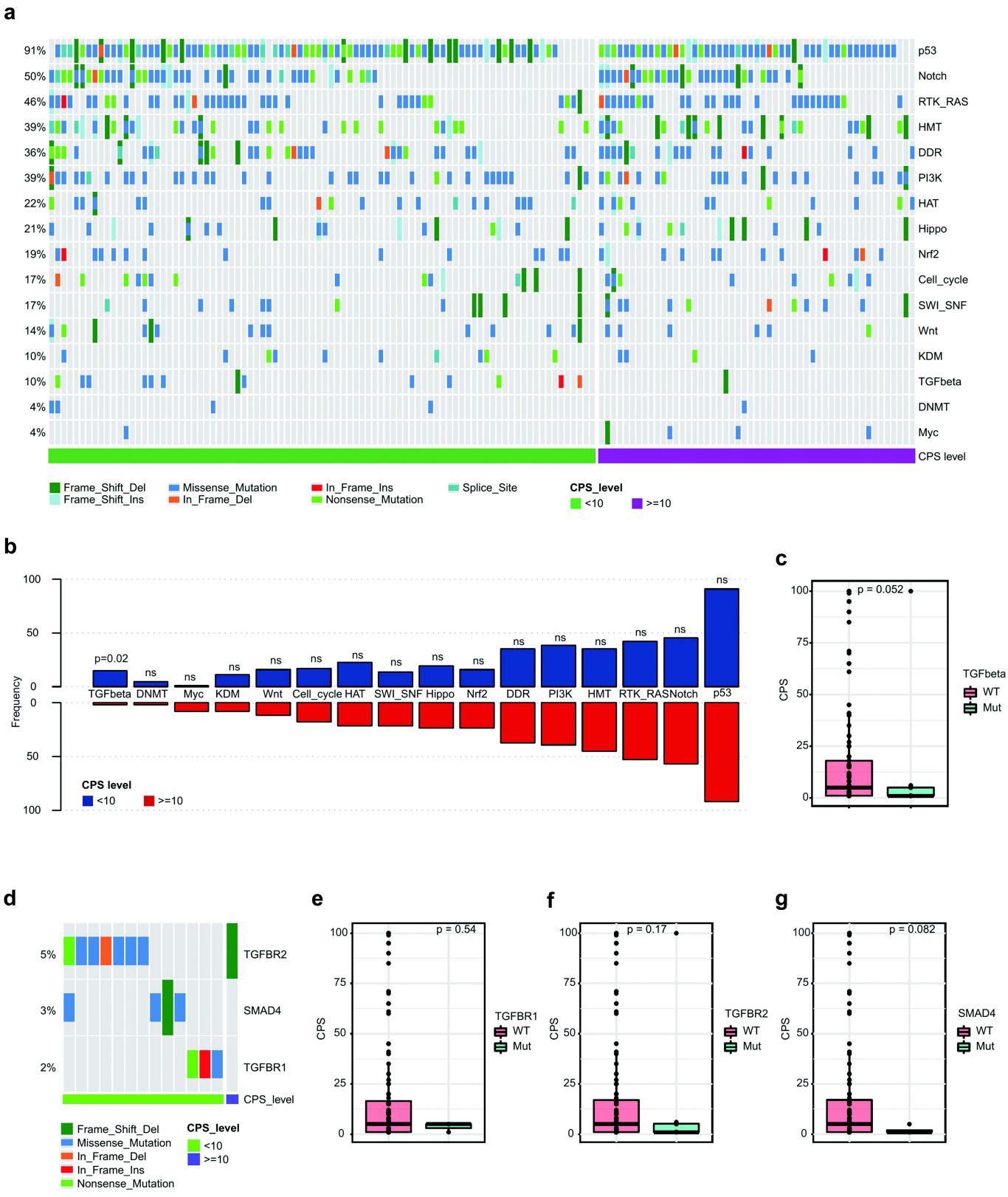


**Figure 3.** Pathway alterations of the PD-L1 subgroups. (a) pathway alteration landscape of the high PD-L1 and low PD-L1 groups. (b) comparison of the frequency of altered pathways in the high PD-L1 and low PD-L1 groups. (c) expression of PD-L1 in patients with and without TGFβ pathway alteration. (d) comparison of the frequency of mutated genes involved in TGFβ pathway in the high PD-L1 and low PD-L1 groups. (e – g) expression of PD-L1 in patients with and without *TGFBR1*, *TGFBR2* or *SMAD4* mutations.

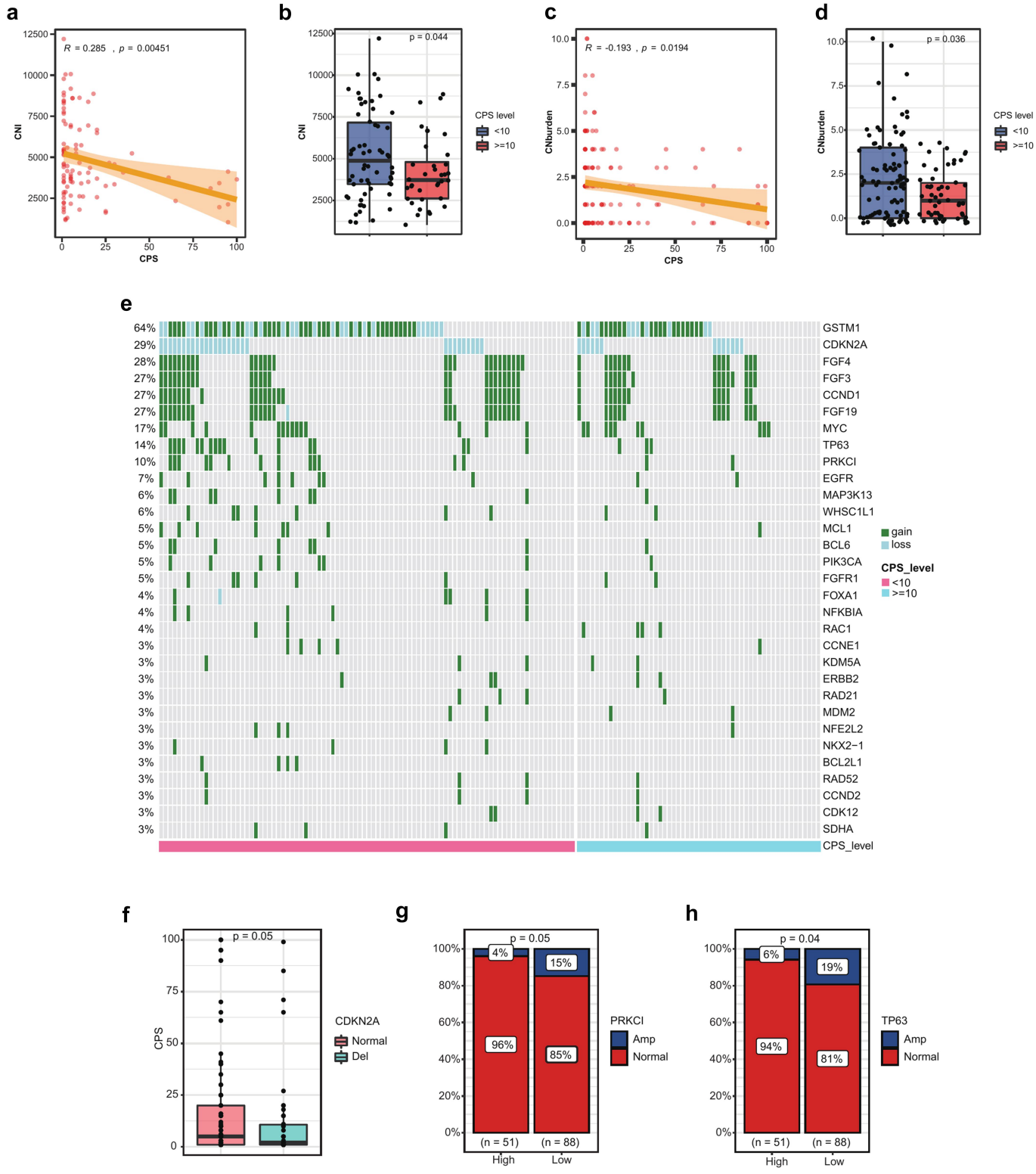


**Figure 4.** Copy number alterations (CNA) in the PD-L1 subgroups. (a) correlation between copy number instability (CNI) and CPS. (b) comparison of CNI in the high PD-L1 and low PD-L1 groups. (c) correlation between CNA and CPS. (d) comparison of CNA in the high PD-L1 and low PD-L1 groups. (e) copy number alteration landscape of the high PD-L1 and low PD-L1 groups. (f) expression of PD-L1 in patients with and without deletion of *CDKN2A*. (g) comparison of the frequency of *PRKCI* amplification in the high PD-L1 and low PD-L1 groups. (h) comparison of the frequency of *TP63* amplification in the high PD-L1 and low PD-L1 groups.

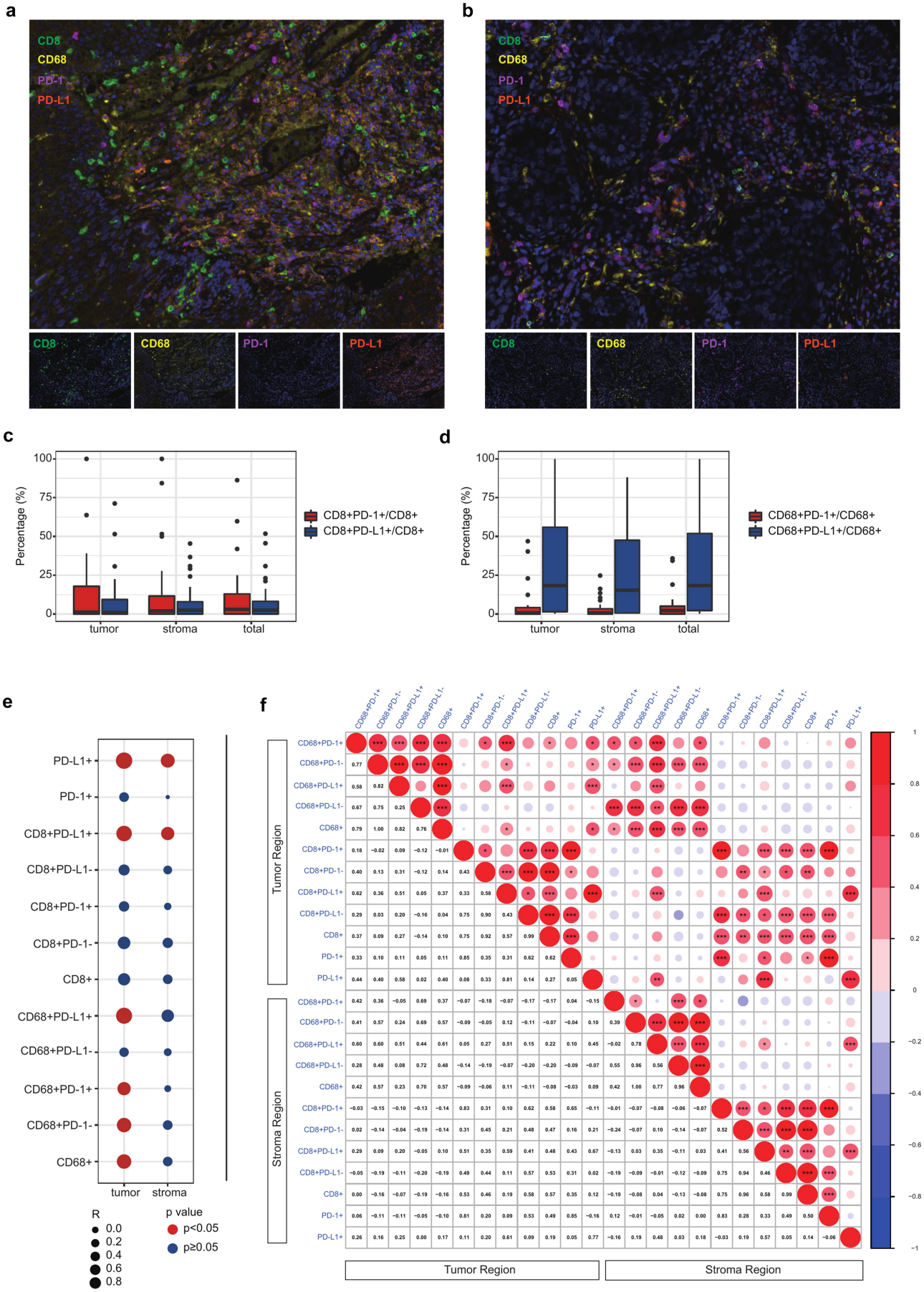


**Figure 5.** Association between tumor-infiltrating lymphocytes (TILs) and PD-L1 expression. (a) Representative mIHC staining figures of the group with high PD-L1 expression. (b) Representative mIHC staining figures of the group with low PD-L1 expression. (c) the ratio of PD-L1$^+$ or PD-1$^+$ CD8$^+$ T cells to CD8$^+$ T cells. (d) the ratio of PD-L1$^+$ or PD-1$^+$ CD68$^+$ macrophage to CD68$^+$ macrophage. (e) correlation between CPS and TILs in the tumoral and stromal region. (f) correlation matrix between TILs in tumor and stroma region. (***, $p < .001$; **, $p < .01$; *, $p < .05$).

### *Tumour-infiltrating lymphocytes (TILs) and PD-L1 expression*

Tumor-infiltrating lymphocytes play an important role in the tumor immune environment, and PD-L1 is expressed on the membrane of various TILs, including B and T lymphocytes, dendritic cells, macrophages and mast cells.[29] Immunohistochemical staining for multiple markers, including CD8, CD68, PD-L1 and PD-1, was performed to assess the infiltration of TILs in 27 ESCC patients (Figure 5a, b).

First, the ratio of PD-L1$^+$ or PD-1$^+$ CD8$^+$ T cells to CD8$^+$ T cells and PD-L1$^+$ or PD-1$^+$ CD68$^+$ macrophages to CD68$^+$ macrophages was assessed, respectively. The ratio of CD8$^+$PD-L1$^+$ T cells and CD8$^+$PD-1$^+$ T cells to CD8$^+$ T cells was found to be similar in both the tumoral and stromal regions (Figure 5c), whereas the ratio of CD68$^+$PD-L1$^+$ macrophages to CD68$^+$ macrophages was higher than the ratio of CD68$^+$PD-1$^+$ macrophages to CD68$^+$ macrophages (Figure 5d).

Second, we examined the association between PD-L1 expression and TILs. Correlation analysis showed that CPS was significantly correlated with the density of PD-L1$^+$ lymphocytes and CD68$^+$ cells in the tumoral region but not in the stromal region (Figure 5d). The correlation analysis between TILs is shown in Figure 5e.

In a previous study, the abundance of CD8$^+$ T cells was found to be associated with a favorable response to anti-PD-1/PD-L1 therapy.[30] In this study, we found that the infiltration of CD8$^+$ T cells was positively correlated with PD-1$^+$ cells in both the tumoral and stromal regions. However, CD8$^+$PD-L1$^+$ T cells were strongly correlated with CD68$^+$ macrophages (including CD68$^+$PD-1$^+$ and CD68$^+$PD-L1$^+$ macrophages) in the tumor region only (Figure 5f).

## Discussion

Understanding the correlation between molecular alterations and PD-L1 expression may help identify more clinically reliable factors associated with anti-PD-1/PD-L1 treatment response and improve the clinical outcomes. This study describes diverse molecular features and TILs profiles at different PD-L1 expression levels in ESCC patients, providing suggestions to further enable precision therapy with anti-PD-1/PD-L1 treatment.

This study identified several molecular alterations associated with differential PD-L1 expression. Mutations in single genes including *BRCA1* and *NF1* demonstrated significant correlations with high PD-L1 expression in ESCC patients. The *NF1* gene encodes neurofibromin, which is ubiquitously expressed in human tissues and is involved in the Hippo-*YAP* and RAS signaling pathway[31–33] (*YAP* has been shown to be involved in the positive regulation of CD274 transcription.[34,35] The inactive mutations of *NF1* may lead to *YAP* translocation to the nucleus and activates the transcription of CD274 (PD-L1), which probably explains the association between *NF1* mutation and PD-L1 expression. It has been reported that melanoma patients with mutated *NF1* gene showed favorable responses to anti-PD–1/PD-L1,[36] suggesting that *NF1* mutations may correlate with the outcome of ESCC patients receiving anti-PD-1/PD-L1 therapy. According to previous studies, only alterations in the *BRCA2* gene, but not *BRCA1*, were observed to predict ICIs response in some cancers.[37,38] Conversely, in our study, only *BRCA1* mutation was associated with high PD-L1 expression, suggesting an inconsistent impact of *BRCA1/2* alterations in different cancer types.

In this study, we have additionally ascertained that alterations in the TGFβ pathway were associated with low PD-L1 expression. TGFβ shapes the tumor microenvironment by limiting T-cell infiltration and attenuating the tumor response to PD-L1 blockade.[39] In a two-part, single-arm, multinational, Phase Ib study,[40] the combination of the TGFβ RI inhibitor galunisertib with PD-L1 inhibitor durvalumab in patients with pancreatic cancer demonstrated promising clinical activity with a DCR of 25.0% and an ORR of 3.1%. The results of this study suggest that ESCC patients with high PD-L1 expression and TGFβ pathway mutation may benefit from this novel treatment strategy. Mechanically, exogenous TGFβ can elevate CD274 transcription through Smad binding elements.[41,42] Conversely, the inactivation of TGFβ would result in the downregulation of CD274 transcription, subsequently leading to decreased PD-L1 expression. Our findings align harmoniously with the aforementioned studies.

TMB has previously been shown to independently predict anti-PD-1/PD-L1 response[22,23] but was not associated with PD-L1 expression in several cancers.[24,25] Similarly, our present investigation revealed that the TMB of ESCC patients possessing high and low PD-L1 expression levels remained comparable.

The predictive role of CNA for outcomes in patients with various cancers undergoing ICIs has been reported.[25–27] For instance, a lower burden of copy number loss (CNloss) was observed in responders to ICIs treatment in melanoma.[25] In gastrointestinal cancer, lower CNA was associated with better response to ICIs[43] including PD-1/PD-L1 blockade. In our study, CNI and CNburden were both significantly but negatively correlated with PD-L1 expression, indicating that CNI and CNburden might also be predictors of PD-1/PD-L1 blockade in ESCC. Notably, *CDKN2A* CNloss is more common in patients with PD-L1 low expression. *CDKN2A* represents a tumor suppressor gene located on chromosome 9p21.3, encoding proteins that govern the cell cycle.[44] This gene ranks among the most frequently altered genes in human cancers, with its deletion being reported in over 30% of ESCC in the Asian population,[45] a proportion closely resembling our findings. Genomic deletions, so-called “deep deletions” or homozygous deletions, which usually extend to other genes on 9p21.3, are the most common alterations affecting *CDKN2A*.[46] Loss of *CDKN2A* could induce the activation of CDK4/CDK6 which could contribute to destabilization of PD-L1 protein.[47] Several studies have examined the impact of *CDKN2A* alterations on the tumor immune microenvironment and the ICI response.[39,48] In melanoma, immune checkpoint blockade is less effective when *CDKN2A* is absent.[49,50] In a study of 909 NSCLC tumor specimens, *CDKN2A* pure deletion was significantly associated with negative PD-L1 expression.[51] In uroepithelial cancers, *CDKN2A* deletion was found to be associated with a reduced benefit from ICI therapy.[52] In our study, PD-L1 expression was significantly higher in patients with *CDKN2A* deletion than in those with

normal *CDKN2A*, suggesting that *CDKN2A* deletion might be associated with the response to anti-PD-1/PD-L1 immunotherapy in ESCC patients.

TILs have been shown to play an important role in the tumor immune environment. It was previously reported that PD-L1 was expressed in both tumor epithelial cells and TILs. In our study, we found that PD-L1 expression was positively correlated with the infiltration of $CD8^+PD\text{-}L1^+$ T cells and $CD68^+PD\text{-}L1^+$ macrophages. $CD8^+$ T cells have been reported to play a key role in killing tumor cells. Moreover, a comprehensive meta-analysis has demonstrated that $CD8^+$ T cells hold the ability to prognosticate treatment outcomes in patients undergoing ICIs, be it as a standalone therapy or in conjunction with other treatment modalities, spanning diverse types of cancers.[53] Besides, in an immunotherapy cohort, higher infiltration of $CD8^+PD\text{-}L1^+$ T cells was significantly associated with better response to anti-PD-1 treatment in NSCLC patients.[54] High infiltration of $CD68^+PD\text{-}L1^+$ macrophages has been reported to be associated with improved survival in primary testicular lymphoma and triple-negative breast cancer.[55,56] These reports suggest that PD-L1 expression and infiltration of $PD\text{-}L1^+$ macrophages might also contribute to favorable responses with PD-L1/PD-L1 blockade in ESCC patients.

## Conclusion

This study highlights the correlation between PD-L1 expression and molecular alterations in patients with ESCC. It delves into the status of TILs concerning various patterns of PD-L1 expression, suggesting that these factors may influence the outcomes of anti-PD-1/PD-L1 immunotherapy. The significance of these findings warrants validation through future investigations involving more extensive cohorts of ESCC patients. The interpretation of potential biological connections between genomic profiling and PD-L1 expression might provide new insights to further improve precision therapy with PD-L1 blockade.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Notes on contributors

***Songchen Zhao*** is currently working toward the Ph.D. degree on Oncology in Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, People's Republic of China.

***Xintong Hu*** received the B.S. degree in Medical examination from Beihua University, Jilin, China, in 2014. And the Ph.D. degree in Clinical laboratory diagnostics from Jilin University, Changchun, China, in 2020. Her research interests include tumor gene research and precision medicine.

***Peiwen Zhou*** graduated from Jilin University with a bachelor's degree in clinical medicine. Changchun in 2021. Pursuing PhD in Jilin University, Changchun. Her research interests include tumor gene research and precision medicine.

***Ang Li*** received the medical master degree in Clinical Laboratory Diagnostics from Jiangsu University. Jiangsu. China. in 2018 and the medical doctor degree in Clinical Laboratory Diagnostics from Jilin University. Changchun. China in 2023. His research interests include tumor gene research and precision medicine.

***Liguo Chen*** received the B.S. degree in bioengineering from Nanjing Agricultural University. Nanjing. China, in 2012. His research interests include sequencing and analysis of tumor genes.

***Duo Wang*** received the B.S. degree in Medical Laboratory Technology from Beihua University. Jilin. China, in 2018 and the M.S. degree in Clinical Laboratory Diagnostics from Beihua University.Jilin. China, in 2021. Her research interests include sequencing and analysis of tumor genes.

***Jiaxue He*** received the B.S. degree in Animal Medical Science from Jilin Agricultural University. Changchun. China. in 2006 and the M.S. degree in Preventive Veterinary Medicine from Jilin Agricultural University. Changchun, China in 2009, the Ph.D. degree in Pathology and Pathophysiology from Jilin University. Changchun. China in 2013. Her research interests include tumor gene research and precision medicine.

***Yanfang Jiang*** received the B.S. degree in Medicine and Japanese from Norman Bethune Medical University. Changchun. China, in 1995 and the M.S. degree in Medicine from Norman Bethune Medical University. Changchun. China, in 1998. She received the Ph.D. degree in Medicine from Jilin University. Changchun. China in 2005. Her research interests include precision medicine, molecular diagnosis and immunology.

## ORCID

Yanfang Jiang http://orcid.org/0000-0002-2478-1521

## Author contributions

All authors contributed to the study conception and design. Study design and material preparation were performed by XH, SZ and YJ. Data collection and analysis were performed by XH, SZ, PZ, AL, LC, DW, JH and YJ. The first draft of the manuscript was written by XH and SZ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Ethics statement

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the ethical review board of the First Hospital of Jilin University (No.2018–467).

## Data availability statement

The data that support the findings of this study are available from the corresponding author Yanfang Jiang, upon reasonable request.

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