Erythropoietin as a Potential Prognostic Biomarker Correlated With Immune Infiltration in Hepatocellular Carcinoma

Huange Zhu (hgzhu2012@163.com)
Xi'an Jiaotong University Second Affiliated Hospital https://orcid.org/0000-0002-4104-6975
Yanping Zhang
Xi'an Jiaotong University Second Affiliated Hospital
Jiafeng Yin
Xi'an Jiaotong University Second Affiliated Hospital
Jie Lu
Xi'an Jiaotong University Second Affiliated Hospital
Hailong Liu
Xi'an Jiaotong University Second Affiliated Hospital
Ting Zhang
Xi'an Jiaotong University Second Affiliated Hospital
Yan Geng
Xi'an Jiaotong University Second Affiliated Hospital

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Abstract

Background. Hepatocellular carcinoma (HCC) is a malignant tumor with poor prognosis. Immune checkpoint genes are considered novel tumor immunotherapy targets for many cancer types. Erythropoietin (EPO) is a protein secreted mainly in the liver and kidneys; in several cancer types, EPO expression stimulates tumor growth and corelates with patient survival. The study aimed to identify EPO as a prognostic biomarker correlated with immune infiltration in HCC.

Methods. We investigated the correlation between EPO expression and HCC patient clinical characteristics using data from The Cancer Genome Atlas. The relationship between EPO expression and HCC patient survival was investigated using Kaplan–Meier analysis. We also used R (v.3.6.3) to analyze the correlation between EPO expression and immune infiltration in HCC.

Results. We found that EPO expression was downregulated in HCC. Notably, EPO was upregulated in advanced-stage HCC and had a diagnostic value of 0.83 ($p < 0.001$) in HCC diagnosis. HCC patients with increased EPO expression had poor prognoses. Furthermore, altered EPO expression was associated with immune cell infiltration and immune checkpoint gene expression in HCC.

Conclusions. EPO expression was positively correlated with hepatocellular carcinoma stage, and negatively correlated with patient prognosis, with influening HCC immune cell infiltration and checkpoint gene expression. EPO can be as both a potential prognostic biomarker and a novel potential treatment target for HCC.

Our work is not a clinical trial, but preclinical basic study.

Introduction

Liver cancer is a common fatal malignancy worldwide [1, 2]. Hepatocellular carcinoma (HCC) is the most common liver cancer [3]. HCC progression is characterized by rapid infiltration and growth, early metastasis, and high malignancy. However, its treatment remains limited as curative strategies are only suitable for early-stage patients. Improving surveillance and early HCC detection may considerably improve patient prognosis. However, early HCC diagnosis is challenging as the disease is often asymptomatic with high associated medical costs and poor patient compliance with surveillance protocols. Therefore, early detection and more effective HCC treatments are urgently needed in addition to identifying prognostic HCC biomarkers and exploring the mechanisms underlying its development and progression.

HCC is typically preceded by liver damage and immune cell infiltration, and kinase and immune checkpoint inhibitors have demonstrated effectiveness in treating patients with advanced-stage HCC [4]. However, the HCC immune microenvironment is poorly characterized [5]. Therefore, it is necessary to characterize novel targets and prognostic predictors of HCC to develop effective therapeutic strategies.

Erythropoietin (EPO) is a glycosylated cytokine comprising four α-helical bundles [6]. It is a protein secreted by hypoxic livers and kidneys to stimulate red blood cell production. EPO is also expressed in the brain and eye, and its elevated expression has been observed in diabetic retinopathy and ocular hypertension patients [7-9]. Additionally, EPO stimulates tumor growth and promotes the survival of several cancer cell types [10-12]. Recombinant EPO is anti-apoptotic in several tissues and has been used to treat anemia and enhance cancer therapy effectiveness [13-15]. However, EPO expression and its role in HCC, including its diagnosis and prognostic value, remain unclear.

RNA sequencing and microarray technology have become essential to biomedical research, and public databases such as The Cancer Genome Atlas (TCGA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) have made bioinformatic analyses based on these data possible. Furthermore, interactive analyses of RNA sequencing data of tumor and normal samples from TCGA and Genotype-Tissue Expression Project (GTEx) with standard processing pipelines can improve our ability to research cancers.

In this study, we aimed to bioinformatically analyze EPO expression in HCC. We explored the correlation among EPO expression, HCC clinicopathological features, and HCC prognosis to evaluate the predictive value of EPO as an HCC biomarker, especially advanced-stage HCC. In addition, we investigated the correlation between EPO expression and immune cell infiltration and immune checkpoint gene expression in HCC to explore its potential regulatory mechanism in such patients.

Materials And Methods

Data collection

The gene expression data (Workflow Type: HTSeq-Counts) and corresponding clinical information were downloaded from TCGA official website (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) for the Uterine Corpus Endometrial Carcinoma Project (UCEC) [16]. The RNA-Seq gene expression level 3 HTSeq-FPKM data of HCC patients and clinical data were retained and further analyzed (table 1).

Diagnosis and survival analyses

The predictive value of EPO for diagnosing HCC was assessed by performing a receiver operating characteristic (ROC) curve analysis of EPO expression in HCC. The cancer cases in TCGA were divided into low and high expression groups based on the median value of EPO expression to investigate the correlation between its expression and the prognosis of patients with HCC. We also assessed the prognostic value of EPO for survival of patients using Kaplan–Meier survival analysis. Both univariate and multivariate analyses using Cox regression were performed for HCC patients in TCGA dataset.

Functional annotation

Analyzing differentially expressed genes (DEGs) is a common strategy to explore the potential biological roles of genes. We performed functional enrichment analyses for data from TCGA dataset to explore the underlying biological function of EPO in HCC. First, according to EPO expression level, the HCC cancer cases were divided into two groups: EPO-high and -low. Next, we compared the DEGs between the two groups using the criteria set of $|log2FC| > 1.5$ and adjusted $p < 0.05$. The DEGs were then identified using the limma package. The Cluster Profiler package was employed to conduct Gene Ontology (GO) and KEGG analyses, and adjusted $p < 0.05$ was considered to indicate significance [17].

Analysis of correlation between EPO expression and immune characteristics

The correlation between EPO and 24 types of immune cells or 18 immune checkpoint genes was analyzed using the GSVA Package with R [18]. Spearman correlation analysis was performed to assess the correlation between EPO expression and immune infiltration.

Statistical analysis

For all R packages, R (v.3.6.3) was used, and significance was defined as $p < 0.05$. The Kruskal–Wallis test was used to evaluate EPO levels in multiple tissues, and the t-test was used to compare EPO expression between normal and tumor tissues. The ROC and area under the ROC curve (AUC) were analyzed using the R packages pROC for analysis and ggplot2 for visualization [19]. Diagnostic value was considered significant when $p < 0.05$ and $AUC > 0.7$. Overall survival (OS) was estimated using Cox proportional hazards regression and Kaplan–Meier models for survival analysis. The cut-off value for EPO expression was determined based on its median value [16].

Results

EPO expression and its diagnostic value in HCC

We first compared EPO expression in TCGA datasets, and then correlated data in the GTEx dataset were added to supplement the healthy population for analysis to reduce bias. We found that EPO expression was overall dysregulated in human cancer (Fig. 1a, b). Notably, both results showed that EPO expression was downregulated in HCC (Fig. 1a, b, $p < 0.001$).

To further validate the results, we compared EPO expression in HCC tissues with that in both non-paired and paired normal tissues. As shown in Fig. 1c, d, both results validated the previous results, showing that EPO expression was downregulated in HCC. The diagnostic performance of EPO was examined by performing a ROC curve analysis. We also found that EPO was effective for diagnosing HCC as indicated by the AUC, which was 0.830 ($p < 0.001$) (Fig. 1e). With a cut-off value of 0.646, the sensitivity and specificity for diagnosing HCC was 66.6% and 91.2%, respectively.

Correlation of EPO with HCC patient survival

According to the clinical pathological stage, HCC can be divided into four groups. The clinical features of HCC patients are shown in table 1. Compared with normal tissue, EPO was downregulated in stage I ($p < 0.001$), stage II ($p < 0.001$), and stage III ($p < 0.01$), separately, but not in stage IV (Fig. 2a). As there were only five samples for stage IV, we reanalyzed after combining stages I and II as the early-stage and stages III and IV as the advanced stage. The results showed that EPO was downregulated in both early- and advance-stage HCC, while EPO expression in advanced-stage HCC (stages III and IV) was higher than that in early-stage HCC (stages I and II) (Fig. 2b).

EPO expression was significantly correlated with OS in patients with HCC. Kaplan–Meier survival analysis indicated that HCC patients with higher EPO expression had significantly reduced OS (hazard ratio [HR] = 1.65, confidence interval [CI] = 1.16–2.34, $p = 0.005$) (Fig. 2c). Univariate analysis using Cox regression revealed that some factors, including pathological stage (HR =2.090, $p < 0.001$), tumor status (HR = 2.317, $p < 0.001$), T stage (HR = 2.126, $p < 0.001$), and EPO (HR = 1.647, $p = 0.005$) were significantly associated with OS (Table 2). In multivariate analysis, high EPO expression (HR = 1.493, $p = 0.43$) and tumor status (HR = 1.844, $p = 0.003$) were independent prognostic factors of favorable prognosis (Table 2).

Based on the results, we concluded that EPO expression was correlated with HCC pathological stage and negatively correlated with HCC prognosis. Advanced-stage HCC patients with high EPO expression are more likely to have poor prognosis than those at the early stage.

Correlation and functional analyses of EPO in HCC

The volcano plot showed 731 DEGs, of which 201 were downregulated and 530 were upregulated (Fig. 3a). GO enrichment analysis of the DEGs predicted their functional roles based on biological processes, cellular components, and molecular functions. We found that GO:0006959 (humoral immune response), GO:0019724 (B cell-mediated immunity), GO:0016064 (immunoglobulin mediated immune response), GO:0002455 (humoral immune response mediated by circulating immunoglobulin), GO:0006958 (complement activation, classical pathway), GO:0006910 (phagocytosis, recognition), GO:0007218 (neuropeptide signaling pathway), GO:0006956 (complement activation), GO:0019814 (immunoglobulin complex). GO:0042571 (immunoglobulin complex, circulating), GO:0005179 (hormone activity), GO:0034987 (immunoglobulin receptor binding), has04974 (Protein digestion and absorption), and hsa04080 (Neuroactive ligand-receptor interaction) were significantly correlated with altered EPO expression (Fig. 3b). This indicated that EPO is closely related with the immune microenvironment in the development of diseases. Furthermore, correlation analysis revealed that EPO and EPOR expression in HCC were significantly positively correlated ($r = 0.25$, $p < 0.001$) (Fig. 3c).

Correlation of EPO expression with immune characteristics in HCC

Tumor-infiltrating lymphocytes are an independent predictor of cancer, and immune checkpoint blockade is a novel approach for cancer therapy [20], which has been shown to gradually improve outcomes for different cancer types [21,22]. Functional analysis revealed a potential correlation between EPO

expression and immune responses. We compared the level of 24 lymphocyte types in HCC (Fig. 4a) and examined the relationship between EPO expression and enrichment of these tumor-infiltrating lymphocyte types in HCC (Fig. 4b). The results overlapped 11 types of cells. Among them, the enrichment scores of eight immune cells (activated DCs (aDC), B cells, immature DCs (iDC), macrophages, NK $CD56^{bright}$ cells, T cells, T follicular helper (Tfh), and Th2) were increased with higher EPO level in HCC, and the infiltration of all of them was positively correlated with EPO expression (Fig. 4c–j). The enrichment scores of the other three immune cells (central memory CD8+ T (T cm), T helper 17 (Th17), and T gamma delta (Tgd) cells) were decreased with higher EPO level in HCC, the infiltration of which was negatively correlated with EPO expression (Fig. 4k–m).

Next, we analyzed the correlation between EPO and 18 common immune-related genes in HCC (Fig. 5). EPO expression was associated with 11 of them: D80, CD86, VTCN1, HHLA2, TNFRSF14, NECTIN2, LGALS9, TNFSF9, TNFSF4, CD70 (all $p < 0.001$), and CD48 ($p < 0.01$). These results strongly indicated that EPO plays a crucial role in tumor immunity.

Discussion

HCC is a major cause of cancer-related death globally. Recent advances in its diagnosis and treatment have improved its clinical outcome. Immunotherapy has gained popularity in cancer treatment [23,24]. However, knowledge of the HCC immune microenvironment, including immune cell infiltration and immunoregulatory mechanisms, is limited [5]. Therefore, it is important to identify diagnostic and prognostic biomarkers and their potential underlying action mechanisms in HCC.

We explored the relationship between EPO expression and various HCC parameters and found that EPO expression was downregulated in HCC compared with that in controls. Interestingly, EPO expression was higher in advanced-stage (stage III and IV) than that in early-stage (stage I and II) HCC, and its expression was positively correlated with HCC pathological stage, suggesting that EPO expression and function in HCC development are complex and may have different mechanistic influences during various stages of tumor development. The AUC indicated that EPO expression had a predictive value of 0.830 ($p < 0.001$) for HCC diagnosis. Furthermore, high EPO expression was correlated with poor HCC prognosis and was an independent risk factor for HCC. All these results are consistent with those of previous studies showing EPO dysregulation in cancers. For example, EPO and EPOR are reported to be expressed in 88% and 92% of cervical cancer samples; cervical cancers with high EPO expression showed significantly reduced OS.[10] Further, the co-expression of EPO and EPOR was observed in several human cancers [12, 25-27]. In accordance with these findings, we found that EPO and EPOR expression in HCC was positively correlated. All together, these findings suggest that EPO functions in HCC tumorigenesis and development in either an autocrine or paracrine fashion.

Immune checkpoint genes are novel tumor immunotherapy targets and improve the outcomes of several cancers [21, 28]. We found that the DEGs in this study positively affected humoral, immunoglobulin-mediated, and B cell-mediated immune responses and neuroactive ligand-receptor interactions. This

provides evidence of directions for subsequent mechanistic studies. We also explored the relationship between EPO expression and immune cell infiltration and immune checkpoint gene expression in HCC. EPO expression was negatively correlated with Tcm, Tgd, and Th17 cell infiltration. Tcm cells are vital to immunity against infectious agents, in cancer immunotherapy, and in adoptive treatments of malignant and viral diseases [29, 30]. Our results are consistent with previous results showing that HCC patients with high EPO expression are likely to have poor prognoses [10]. In contrast, EPO expression was significantly positively correlated with both innate immune cells (NK $CD56^{bright}$, macrophages, aDC, and iDC) and adaptive immune cell (B cells, T cells, Tfh, and Th2) infiltration. $CD56^{bright}$ NK cells are an NK subset with characteristic high CD56 expression, cytokine secretion, immunomodulatory functions, and anti-fibrotic activity. In common conception, $CD56^{dim}$ NK cells are antitumorigenic, whereas $CD56^{bright}$ NK cells are involved in immunomodulation. However, a previous study also identified $CD56^{bright}$ NK cells as potent antitumor effectors and cancer immunotherapy targets [31]. Tfh cells, which help B-cell-mediated immune responses, are strongly correlated with B cells. Both B and T cells, as the main cells in adaptive immune response, play important roles in the occurrence and development of tumors. A previous study showed that most T cells decrease in tumor progression, while Tfh cells and innate cells increase, and B cells have dual effects on tumor progression and patient survival [18].

Th2 cells are CD4+ T cells. A Th1/Th2 imbalance in the tumor microenvironment is often associated with a predominant Th2 cell state. This imbalance exists in many tumors and may be related to the immune escape of tumor cells. A previous study indicated that CD4+ T cell-mediated allograft rejection is associated with a dominant Th2 cell response in the absence of CD8+ T cell activation [32].

We next found that EPO expression was associated with the expression of the immune checkpoint genes CD80, CD86, VTCN1, HHLA2, TNFRSF14, NECTIN2, LGALS9, TNFSF9, TNFSF4, CD70, and CD48. As immune checkpoint blockade has been highly successful in treating several other cancers, the correlation between EPO and immune checkpoint gene expression further confirmed the correlation of EPO with the immune microenvironment in HCC, suggesting a new immune therapy target in HCC.

EPO expression was higher in advanced- than early-stage HCC and showed good predictive value in HCC diagnosis with poor prognosis. Altered EPO expression was found to be associated with HCC tumor infiltration by CD84+ T, CD4+ T, and NK cells and the expression of several immune checkpoint genes. These results strongly suggest that EPO expression influences HCC immune cell infiltration and checkpoint gene expression and has the potential to be a novel prognostic biomarker of HCC, serving as a new immune therapy target in HCC.

Nevertheless, the limitations of this study must be noted. First, the data included are from public databases, and the findings need to be verified with cohort studies. Second, since EPO is a secreted cytokine and can function in either an autocrine or paracrine manner, its expression and role in the blood or fluids in the body should be studied to better understand its role in the pathophysiological process of HCC. Furthermore, as cells infiltrating tumors are heterogeneous, and analyses of tumor infiltration of

immune cells have often yielded contradictory results, more comprehensive studies are needed to fully understand the tumor immune microenvironment.

Declarations

Funding None.

Competing Interests None declared.

Availability of data and material

The datasets analyzed during the study are available from TCGA and GTEx databases.

Code availability Not applicable.

Authors' contributors

Huange Zhu, Yanping Zhang and Yan Geng contributed to the study conception and design. Material preparation, data collection and analysis were performed by Huange Zhu, Jiafeng Yin, Jie Lu, Hailong Liu and Ting Zhang. The first draft of the manuscript was written by Huange Zhu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Characteristics of hepatocellular carcinoma patients in The Cancer Genome Atlas (TCGA).

Characteristic	Low expression of EPO	High expression of EPO
n	187	187
T stage, n (%)		
T1	99 (26.7%)	84 (22.6%)
T2	48 (12.9%)	47 (12.7%)
T3	34 (9.2%)	46 (12.4%)
T4	4 (1.1%)	9 (2.4%)
N stage, n (%)		
N0	126 (48.8%)	128 (49.6%)
N1	1 (0.4%)	3 (1.2%)
M stage, n (%)		
M0	131 (48.2%)	137 (50.4%)
M1	1 (0.4%)	3 (1.1%)
Pathologic stage, n (%)		
Stage I	94 (26.9%)	79 (22.6%)
Stage II	43 (12.3%)	44 (12.6%)
Stage III	34 (9.7%)	51 (14.6%)
Stage IV	1 (0.3%)	4 (1.1%)
Tumor status, n (%)		
Tumor free	110 (31%)	92 (25.9%)
With tumor	70 (19.7%)	83 (23.4%)
Gender, n (%)		
Female	53 (14.2%)	68 (18.2%)
Male	134 (35.8%)	119 (31.8%)
Age, n (%)		
<=60	83 (22.3%)	94 (25.2%)
>60	104 (27.9%)	92 (24.7%)
AFP(ng/ml), n (%)		
<=400	124 (44.3%)	91 (32.5%)

Characteristic	Low expression of EPO	High expression of EPO
>400	22 (7.9%)	43 (15.4%)
Child-Pugh grade, n (%)		
A	121 (50.2%)	98 (40.7%)
B	10 (4.1%)	11 (4.6%)
C	0 (0%)	1 (0.4%)
Fibrosis ishak score, n (%)		
0	45 (20.9%)	30 (14%)
1/2	19 (8.8%)	12 (5.6%)
3/4	12 (5.6%)	16 (7.4%)
5/6	45 (20.9%)	36 (16.7%)
OS event, n (%)		
Alive	132 (35.3%)	112 (29.9%)
Dead	55 (14.7%)	75 (20.1%)
Age, meidan (IQR)	62 (54.5, 70)	60 (51, 68)

Table 2. Univariate and multivariate Cox analyses of erythropoietin (EPO) expression and overall survival (OS) in patients with hepatocellular carcinoma (HCC) in The Cancer Genome Atlas (TCGA) dataset.

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
T stage (T2&T3&T4 vs.T1)	370	2.126 (1.481-3.052)	**<0.001**	0.865 (0.118-6.310)	0.886
Pathologic stage (Stage II& III& IV vs. Stage I)	349	2.090 (1.429-3.055)	**<0.001**	2.162 (0.291-16.052)	0.451
Tumor status (Yes vs. No)	354	2.317 (1.590-3.376)	**<0.001**	1.844 (1.236-2.751)	**0.003**
Gender (Male vs. Female)	373	0.793 (0.557-1.130)	0.200		
Age (>60 vs. <=60)	373	1.205 (0.850-1.708)	0.295		
AFP(ng/ml) (>400 vs.<=400)	279	1.075 (0.658-1.759)	0.772		
Fibrosis ishak score (5/6&3/4&1/2 vs. 0)	214	0.772 (0.465-1.281)	0.316		
Child-Pugh grade (B&C vs. A)	240	1.643 (0.811-3.330)	0.168		
Vascular invasion (Yes vs. No)	317	1.344 (0.887-2.035)	0.163		
EPO (High vs. Low)	373	1.647 (1.161-2.336)	**0.005**	1.493 (1.013-2.199)	**0.043**

Figures

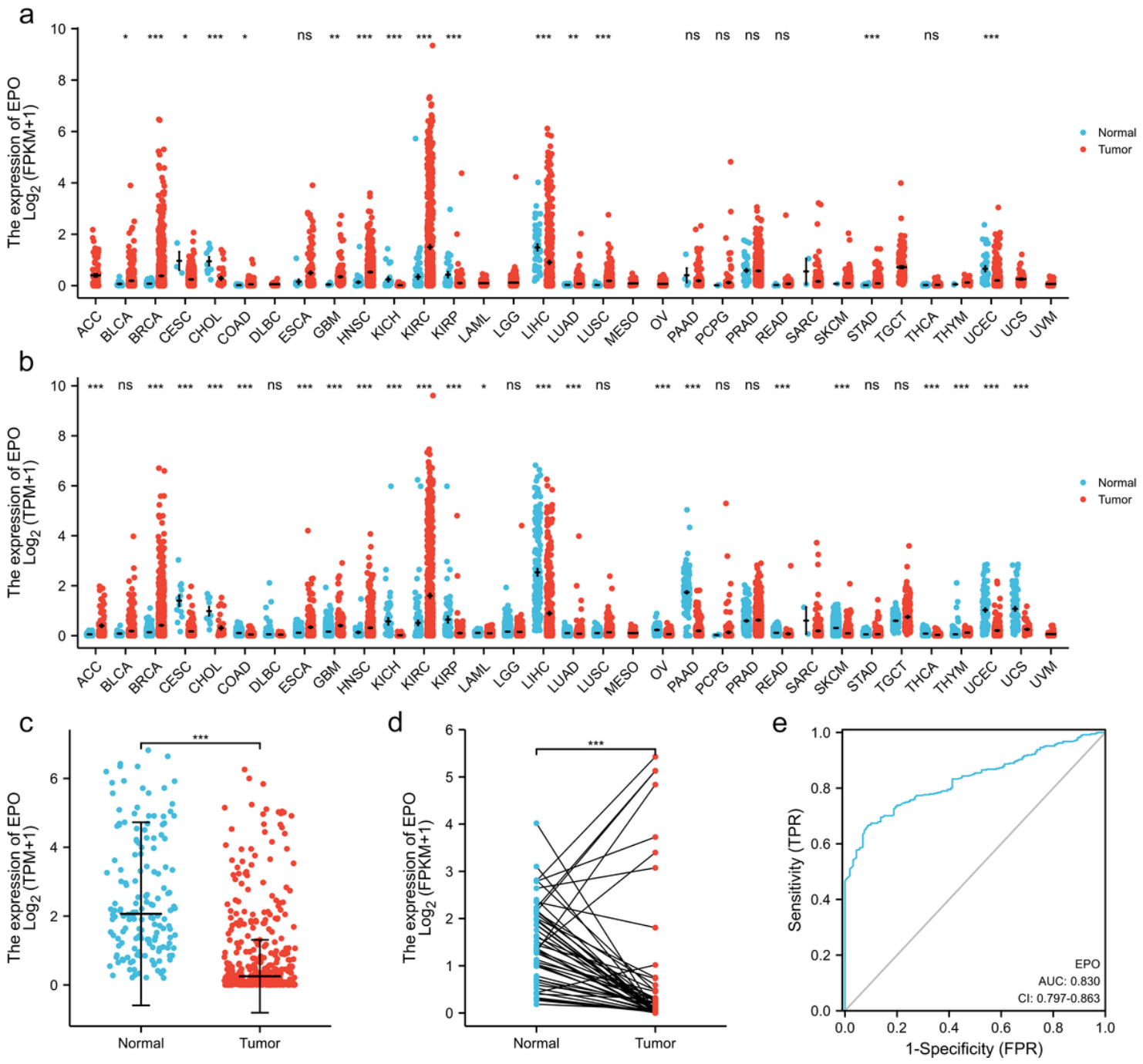

Figure 1

Erythropoietin (EPO) expression and association with clinical characteristics in hepatocellular carcinoma (HCC) patients (a) EPO expression in different cancer types based on The Cancer Genome Atlas (TCGA). (b) EPO expression in different cancer types based on the Genotype-Tissue Expression Project (GTEx) database and TCGA. (c) EPO expression in HCC and nonpaired normal tissues. (d) EPO expression in HCC and paired normal tissues. (e) Receiver operating characteristics (ROC) curve of EPO based on the GTEx database and TCGA.

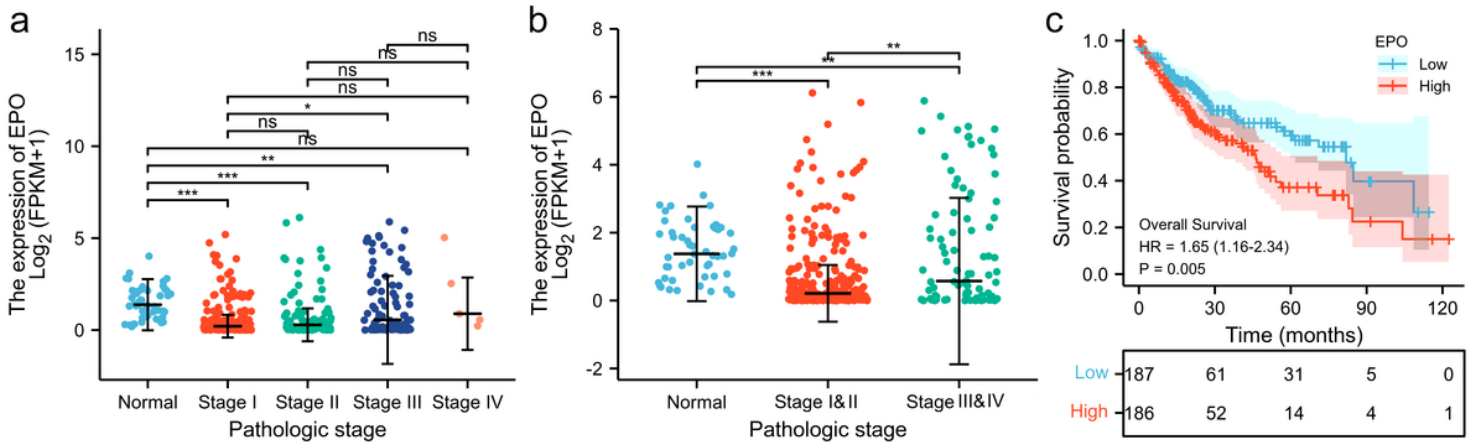

Figure 2

Erythropoietin (EPO) expression and association with overall survival (OS) of hepatocellular carcinoma (HCC) patients (a, b) EPO expression in different pathological stages. (c) Prognostic performance of EPO in terms of OS in HCC.

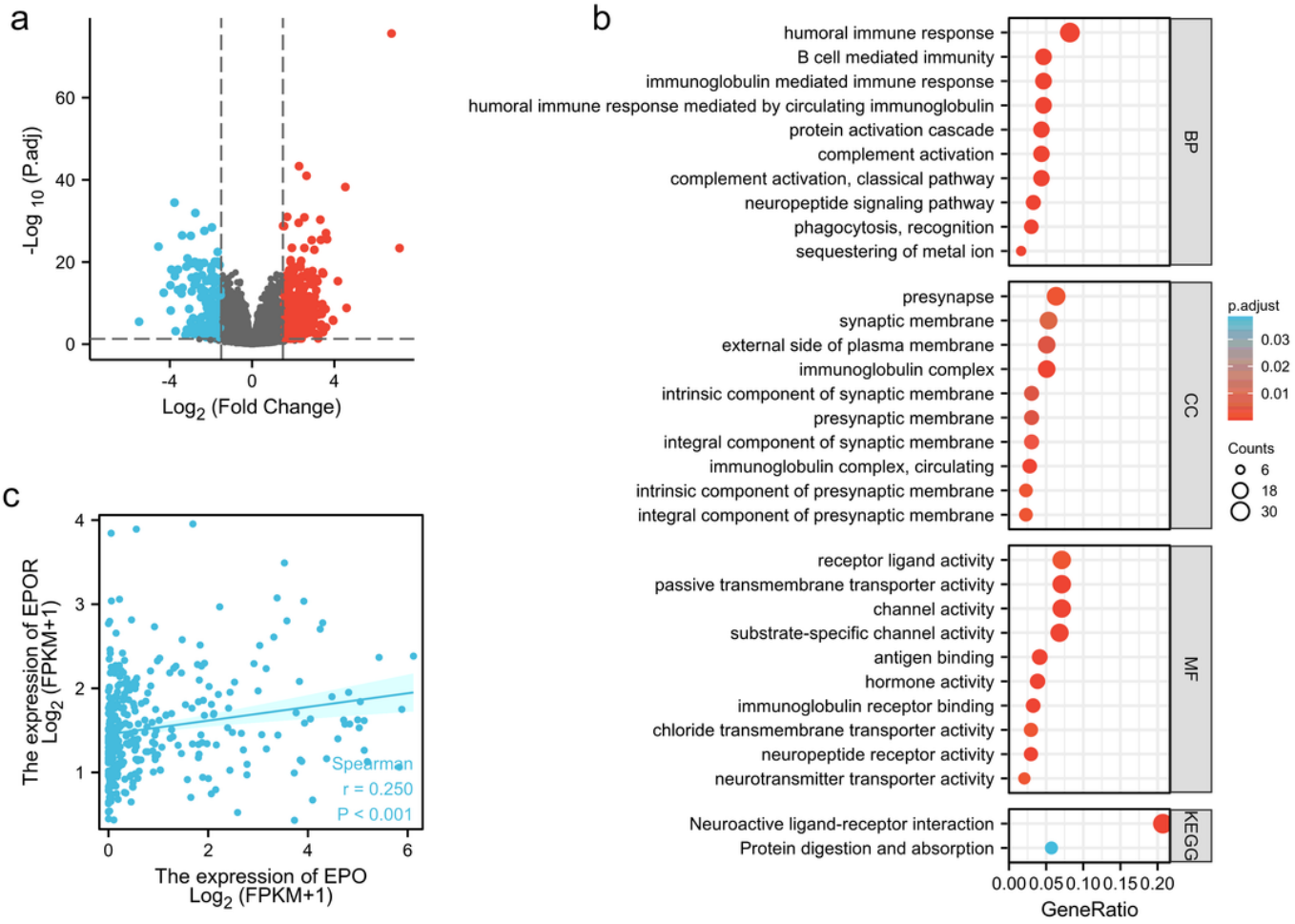

Figure 3

Enrichment analysis of erythropoietin (EPO) expression-correlated differentially expressed genes (DEGs) in hepatocellular carcinoma (HCC) (a) Volcano plot of EPO-correlated DEGs in HCC. (b) Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses for EPO-correlated DEGs. (c) Correlation analysis of EPO and EPOR expression in HCC.

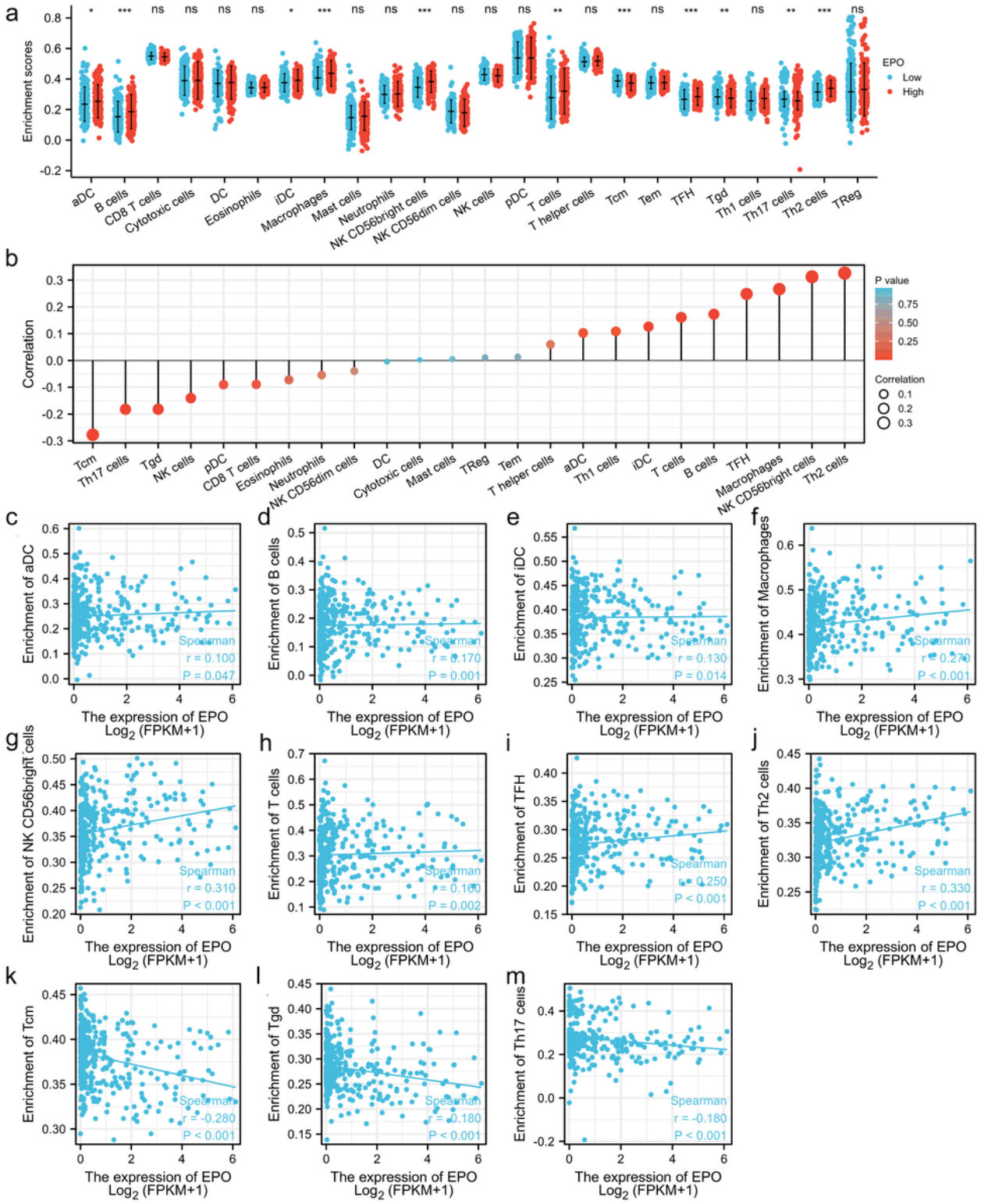

Figure 4

Analysis of correlation between erythropoietin (EPO) expression and tumor-infiltrating lymphocytes (TILs) in hepatocellular carcinoma (HCC) (a) Enrichment of TILs in HCC. (b) Relationship landscape between EPO expression and TILs in HCC. (c–m) Significant correlation between EPO and TILs in HCC, separately.

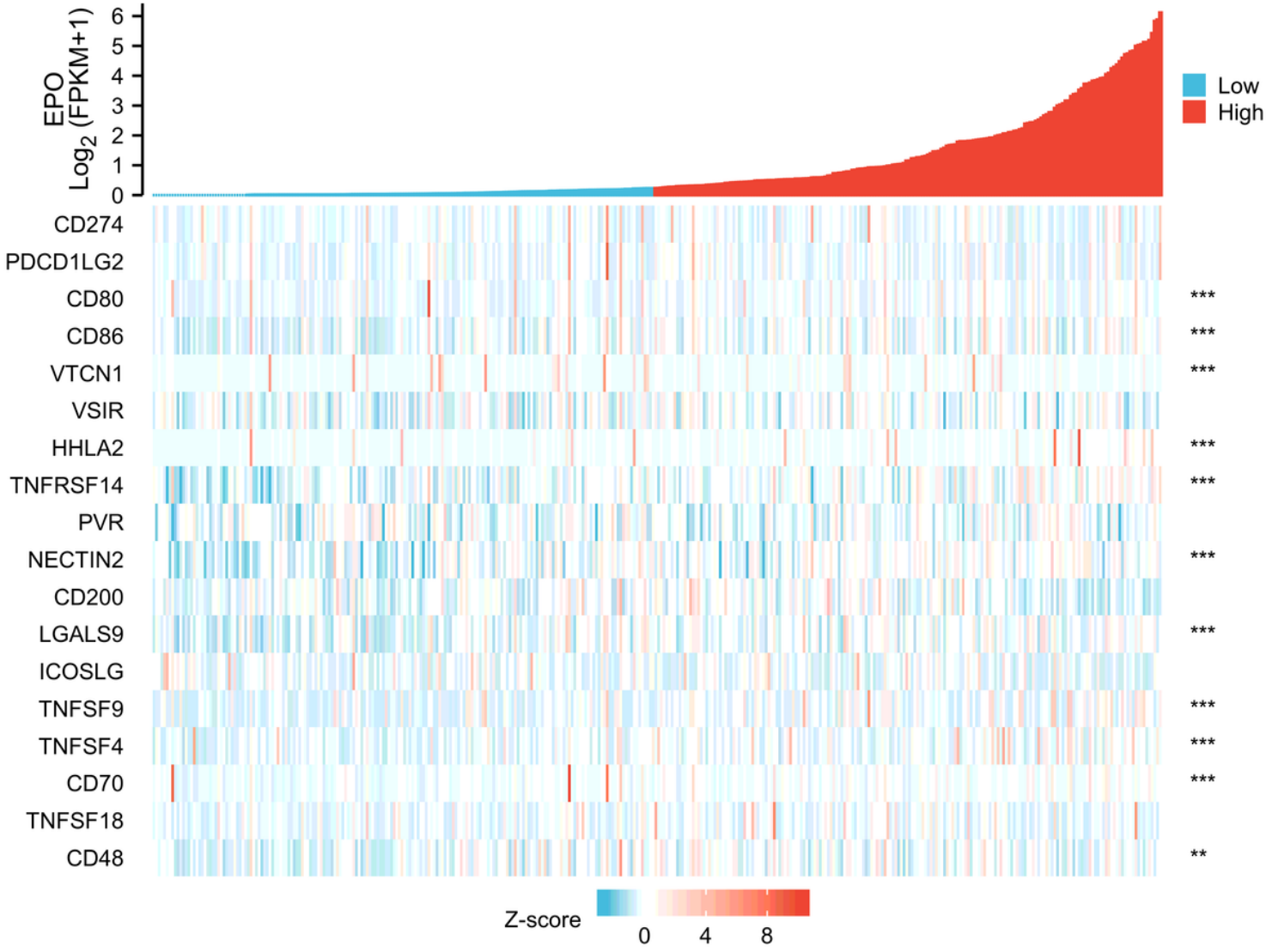

Figure 5

Analysis of correlation between erythropoietin (EPO) and immune checkpoint gene expression EPO expression was positively related with CD80, CD86, VTCN1, HHLA2, TNFRSF14, NECTIN2, LGALS9, TNFSF9, TNFSF4, CD70, and CD48 in hepatocellular carcinoma (HCC). **: $p < 0.01$; ***: $p < 0.001$.