

Erythropoietin receptor is a risk factor for prognosis: a potential biomarker in lung adenocarcinoma

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Abstract

Background: Lung cancer has the highest mortality rate of all cancers, and LUAD' s survival rate is particularly poor. Erythropoietin receptor (EPOR) can be detected in lung adenocarcinoma (LUAD), however, the expression levels and prognostic value of EPOR in LUAD are still unclear.

Methods: Multiple bioinformatics databases, immunohistochemistry and clinicopathological data of 92 LUAD patients between January 2008 and June 2016 were used to explore the EPOR expression, gene mutations affecting EPOR expression, the correlation of EPOR expression with prognosis, immune microenvironment and so on. All statistical analyses were performed in the R version 4.1.1.

Results: The EPOR mRNA expression in LUAD tissues was possibly downregulated compared with that in normal lung tissues, but the EPOR protein expression in LUAD tissues was higher than that in paired normal lung tissues. Mutations in five genes including DDX60L, resulted in downregulation of EPOR expression, mutations in 10 genes including C1orf168, resulted in upregulation of EPOR expression. The KM Plotter and PrognoScan databases consistently concluded that EPOR was associated with prognosis in LUAD patients. Our clinicopathological data showed that high EPOR expression was associated with poorer OS (29.5 vs 46 months) and had a good predictive ability for 5-year survival probability.

Conclusions: EPOR expression might be downregulated at the mRNA levels and significantly upregulated at the protein levels in LUAD, which showed that the mRNA and protein levels of EPOR are inconsistent. The high expression of EPOR was associated with poor prognosis and is expected to be a potential new prognostic marker for LUAD.

Introduction

According to the latest statistics, cancer remains the leading cause of human death. Compared to other cancers, lung cancer has a relatively low survival rate, 80% of which is non-small cell lung cancer (NSCLC) [1]. Adenocarcinoma (AD) is the most common histological subtype of NSCLC [2, 3]. In recent years, despite the achievements in medical treatment and the inclusion of targeted drugs in the treatment of patients with this type of cancer, the survival rate of lung adenocarcinoma (LUAD) is still very poor, only 4–17% [4]. This may be due to the high heterogeneity of LUAD and the lack of effective prognostic markers [5, 6]. The prognosis of LUAD has been a hot topic of medical research in recent years [7–10], it is not difficult to see the urgent pursuit of improving survival rate, so there is still a long way to go to explore the prognostic markers of LUAD. Erythropoietin receptor (EPOR) is a member of the cytokine class I receptor family, that mediates erythropoietin (EPO) - induced proliferation and differentiation of erythroblasts, with a structural motif consisting of two extracellular immunoglobulin-like domains, four similarly spaced cysteine residues and the sequence WSXWS, lacking tyrosine kinase activity and binding to JAK kinase, forming homodimers, heterodimers or heterotrimer complexes [11]. Upon EPO stimulation, binding to its homologous dimer receptor complex, induces the activation of Janus kinase 2 (JAK2), a non-receptor tyrosine kinase [12]. Activation of JAK2 results in the activation of specific downstream

effectors, such as STAT1, STAT3, or STAT5 [13], the signal transducer and activator of transcription 5 (STAT5) usually refers to STAT5A and STAT5B proteins [14], thus activates phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) [15, 16]. EPOR was originally discovered and described in erythroid progenitor cells, but it is also present in non-hematopoietic cells (tissues, organs) such as adipose tissues [17], bone progenitor cells [18], neurons [19], endothelial cells [20] and intestinal tract [21]. It is also widely present in various cancer cells and tumor tissues, such as head and neck squamous cell carcinoma [22], diffuse large B-cell lymphoma [23], rhabdomyosarcoma [24], breast cancer [25], hepatocellular carcinoma [26] and laryngeal malignancy [27]. Studies have shown that it is upregulated in gastric adenocarcinoma [28], liver cancer [29], prostate cancer [30], glioma [31] and other cancers. EPOR expression was also detected in non-small cell lung cancer cell lines [32, 33], however, tumor immunohistochemistry (IHC) data based on anti-EPOR antibodies have been scarce, and Brown et al. [34] thought that inappropriate use of EPOR antibodies could result in false-positive results. In locally advanced squamous cell carcinoma of the head and neck, EPOR expression was an independent prognostic factor for OS, and improved OS was significantly associated with the absence of EPOR expression [35]. There was no significant difference in overall survival rate and recurrence-free survival rate among patients with different EPOR expression in cervical cancer [36]. Våtsveen et al. [37] found high levels of EPOR in myeloma cells to be associated with a better survival prognosis and suggested that EPOR expression may be a novel prognostic marker in primary myeloma. Thus, it was evident that the prognosis of EPOR expression is very different in various cancers. In NSCLC, many studies have concluded that EPO/EPOR co-expression or co-overexpression is associated with poor prognosis [38–40], however, there is a lack of evidence regarding the prognostic impact of EPOR alone on patients. Given that EPOR is expressed in NSCLC, including LUAD, but evidence at the tissue level is limited, whether EPOR is upregulated or downregulated in LUAD tumor tissue relative to normal tissue needs to be further explored, and an accurate correlation between EPOR signaling and prognosis in LUAD has not been established. Traditional clinical analyses, such as TNM staging and assessment of pathological parameters, do not accurately or dynamically reflect the progression of LUAD, and the continuous discovery of new prognostic markers would be very beneficial in predicting patient prognosis and could contribute to improving the survival rate of patients. The aim of this study was to explore the expression of EPOR in LUAD and related issues such as prognosis by combining various bioinformatics methods, immunohistochemical method, and clinicopathological data of the patients.

Materials And Methods

(1) Tumor Immune Estimation Resource (TIMER)

TIMER (<https://cistrome.shinyapps.io/timer/>) is a web server for comprehensive analysis of tumor-infiltrating immune cells. In this study, the expression of EPOR in a variety of cancers was assessed by the "Diff Exp" module. The "Gene" module can study the relationship between EPOR expression in LUAD and the levels of immune cell infiltration (B cells, CD8+ T cells, CD4+ T cells, neutrophils, macrophages and dendritic cells) by using TCGA database. Using the "Correlation" module, the relationship between

EPOR expression and different gene marker sets in immune cells was investigated. The correlation between EPOR expression and immune infiltration could be evaluated together with Spearman's ρ value and statistical significance after adjusting for tumor purity. The "SCNA" module can compare the level of immune infiltration with different somatic copy number alternations of EPOR gene in LUAD, including "deep deletion", "arm-level deletion", "diploid/normal", "arm-level gain" and "high amplification". Infiltration level for each SCNA category was compared with normal level using a two-sided Wilcoxon rank sum test.

(2) **Oncomine**

Oncomine (<https://www.oncomine.org/resource/login.html>) is currently the world's largest oncogene microarray database and integrated data mining platform designed to help data mine the transcriptional expression of genes in various cancers. The p-value was set to 0.05, fold change was set to 1.5, and gene rank was set to all.

(3) **UALCAN**

UALCAN (<http://ualcan.path.uab.edu/>) is a comprehensive, user-friendly and interactive web resource for analyzing cancer OMICS data, providing easy access to publicly available cancer OMICS data (TCGA, MET500 and CPTAC), expression profiles of coding proteins and evaluating epigenetic regulation of gene expression by promoter methylation and so on. The "TCGA" module can explore the expression of EPOR in LUAD.

(4) **Gene Expression Profiling Interactive Analysis (GEPIA)**

GEPIA (<http://gepia.cancer-pku.cn/index.html>) is a interactive web server that integrates both TCGA and GTEx data for gene expression analysis. In this study, EPOR expression analysis was evaluated using the TCGA-LUAD dataset. In the "Expression DIY" module, we used TCGA Normal and GTEx data matching method and $\log_2(\text{TPM}+1)$ for log-scale to study the expression of EPOR in LUAD and normal lung tissue specimens. Co-expressed genes were screened in the "Similar Genes" module.

(5) **MuTarget**

The muTarget (<https://www.mutarget.com/>) is an online tool for linking mutation status to gene expression changes in solid tumors. "All somatic mutations" was selected as the somatic mutation type and the mutation prevalence was set at least 2%.

(6) **Analysis of EPOR-interacting genes and proteins**

The GeneMANIA database (<https://genemania.org/>) was applied to construct the EPOR gene-gene interaction network. The STRING online database (<https://string-db.org/>) was applied to construct the EPOR protein-protein interaction (PPI) network.

(7) **Gene Ontology (GO) term, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and gene set enrichment analysis (GSEA)**

RNAseq data for EPOR in LUAD were obtained from The Cancer Genome Atlas (TCGA; <https://tcga-data.nci.nih.gov/tcga/>), and analysis of differentially expressed genes (DEGs) for EPOR was performed by the R package DESeq2. GO and KEGG analyses were applied to explore the potential biological functions of DEGs in LUAD, complemented by GSEA. GO enrichment analysis contains three levels: molecular function (MF), cellular component (CC) and biological process (BP). GO, KEGG and GSEA were performed by the R package ClusterProfiler. Normalized enrichment score (NES) >1.5, false discovery rate (FDR) <0.25 and $p_{\text{adjust}} < 0.05$ were considered to be of significant significance in GSEA.

(8) **Kaplan-Meier Plotter**

KM Plotter (<http://kmplot.com/analysis/>), containing gene expression data and survival information in GEO, EGA and TCGA databases. The samples of LUAD patients were divided into two groups (high and low expression) according to the best cutoff and different Affymetrix IDs (37986_at, 216999_at, 209962_at, 209963_s_at, 396_f_at) were selected to explore the prognostic value of EPOR in LUAD. The relationship of overall survival (OS) and progression-free survival (PFS) with hazard ratio (HR) was analyzed using 95% confidence interval (95% CI) and the log-rank test p value.

(9) **PrognoScan**

PrognoScan (<http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html>), a new database for meta-analysis of prognostic value of genes. PrognoScan employs the minimum P-value approach for grouping patients and searches the biological relationship between gene expression and patient prognosis across a large collection of publicly available cancer microarray datasets with clinical annotation. To explore the relationship between EPOR expression and patient prognosis in LUAD, the screening parameters of the datasets were set as follows: "cancer type" was lung cancer, and "subtype" was "adenocarcinoma". Five datasets were finally obtained, including GSE13213, GSE31210, jacob-00182-CANDF and so on, in which the GSE13213 dataset contained 117 patients with lung adenocarcinoma with high probability of recurrence and the GSE31210 dataset contained 204 patients with pathological stage I-II lung adenocarcinoma. HR (95% CI) was calculated, and the threshold was adjusted to corrected P-value < 0.05.

(10) **cBioPortal**

The cBioPortal (<https://www.cbioportal.org/>) contains a large-scale cancer genome dataset with visualization, download, analysis and other functions. We selected three lung cancer datasets with 855 cases and three LUAD datasets with 718 cases, respectively, for further analysis using cBioPortal. The type and frequency of EPOR gene alterations in LUAD tissues were analyzed by the "OncoPrint" module and the "Cancer Types Summary" module. The OS of EPOR genes in the altered and non-altered groups was analyzed by the "Comparison/Survival" module.

(11) **Immunohistochemistry (IHC)**

Lung adenocarcinoma 180 point tissue microarrays (HLugA180Su04, XT16-032, Outdo Biotech, Shanghai, China) were placed in an oven and baked at 63 °C for 1 h (including 88 pairs of lung adenocarcinoma and adjacent normal lung tissue specimens, and tumor tissues had 4 extra points). Dewaxing was followed by antigen repair and subsequently placed in distilled water at room temperature and allowed to cool naturally for at least 10 min. Rinsed by PBS buffer, adding anti-EPOR primary antibody working solution (1:1000, bs-1424R, Bioss, Beijing, China), and at 4 °C overnight. Then it was incubated with biotin-labeled secondary antibodies for 45min at room temperature and cleaned with PBS. The slides were placed into an automatic immunohistochemistry instrument (Dako, United States), and the blocking, secondary antibody binding, and 3,3'-diaminobenzidine (DAB) color development programs were performed according to the "Autostainer Link 48 User's Guide". Hematoxylin staining for 1 min, submerged in 0.25% hydrochloric alcohol (400 ml 70% alcohol + 1 ml concentrated hydrochloric acid) for about 10 s, and rinsed with tap water for 5 min. Dried at room temperature and sealed the film with neutral resin. IHC staining score can be divided into two parts: staining intensity and percentage of positive tumor cells. Staining intensity was classified as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Percentage of positive tumor cells was classified as 1 ($\leq 25\%$), 2 (26% – 50%), 3 (51% – 75%) and 4 ($> 75\%$). The final expression scores were calculated by multiplying the two variables together, with a maximum score of 12 [41, 42].

(12) **Validation of EPOR prognostic value based on clinicopathological data of patients**

To verify the prognostic value of EPOR expression in LUAD patients, we performed survival analysis using detailed clinicopathological data from 92 lung adenocarcinoma tissue microarrays (HLugA180Su04) with a follow-up period of 3 - 8.5 years (operation time: January, 2008 - July, 2013, follow-up time: June, 2016), which contained the fluorescence in situ hybridization (FISH) data of anaplastic lymphoma kinase (ALK) and epidermal growth factor receptor (EGFR) and immunohistochemical data of programmed death ligand 1 (PD-L1). After excluding 14 cases of lost follow-up and 7 cases of undetectable EPOR (no cancer/no point), of which 1 overlapped, the 72 cases of lung adenocarcinoma were finally divided into

two groups using cut-off value of EPOR immunohistochemical staining score (≥ 5 : high expression; < 5 : low expression), and 95% CI and log-rank P value were used to explore the effect of EPOR on OS of LUAD patients. After deleting the pathological data of 14 patients with missing clinical stages, the 58 cases were finally used to perform univariate Cox analysis on Gender, Age, AJCC stage, T/N/M stage, EPOR, ALK, EGFR and PD-L1 characteristics, followed by multivariate Cox analysis to explore the risk/protective factors for survival in LUAD patients. Finally, nomogram was created based on the results of multivariate Cox analysis to construct a scoring system capable of assessing patients' 5-year OS, and calibration curve based on the Hosmer-Lemeshow test were used to assess the consistency of the actual results with the predicted results of the nomogram. The above was performed by R package rms, survminer and survival.

(13) **Statistical Analysis**

The results generated in Oncomine were displayed as p-values, fold changes and gene ranks. Kaplan-Meier Plotter and PrognoScan results were shown by log-rank test for HRs (95% CIs) and p-values. EPOR expression levels of ALK, EGFR and PD-L1 high and low groups (grouped by median expression) were compared by RNAseq data of LUAD in TCGA database. Paired-samples T-test or Wilcoxon rank sum test were used for comparison between groups. Correlation heat map of interacting proteins in the STRING database was displayed using Spearman's correlation and the R package ggcorrplot and corrplot. Single gene co-expression heat map was constructed with the R package ggplot2. Forest maps of the value of EPOR prognostic were plotted using the R package forestplot. The R package estimate was used to calculate the relationship between EPOR expression in LUAD and the tumor microenvironment (TME), and gene expression was considered significantly correlated when $P < 0.5$ and $|R| > 0.20$ were both satisfied [43]. Each P value < 0.05 was considered statistically significant.

Results

(1) EPOR expression in LUAD patients

The mRNA expression of EPOR in LUAD tissues was analyzed by TIMER database for the first time. EPOR expression was higher in bladder urothelial carcinoma (BLCA), cholangiocarcinoma (CHOL), head and neck squamous cell carcinoma (HNSC), renal clear cell carcinoma (KIRC), gastric adenocarcinoma (STAD) and thyroid carcinoma (THCA) tissues than in the adjacent normal tissues, and lower in breast invasive carcinoma (BRCA) and lung squamous cell carcinoma (LUSC) tissues than in normal tissues (Figure 1a). In the UALCAN database, EPOR expression was not significantly different in LUAD primary tumor tissues from normal lung tissues (Figure 1b). However, in GEPIA, EPOR expression was lower in LUAD tissues than in normal lung tissues (Figure 1c). Subsequently, EPOR expression in LUAD tissues and in adjacent normal tissues was analyzed using data obtained directly from TCGA. The expression of EPOR in LUAD was significantly decreased (Figure 1d). After sample matching, 57 paired LUAD tumor specimens and adjacent normal tissue specimens were obtained, and EPOR expression was significantly

downregulated in LUAD tissues (Figure 1e). In addition, the EPOR mRNA expression was further detected by Oncomine database, and the expression of EPOR in LUAD tissues of six cohorts was downregulated compared to normal lung tissues, including "Bhattacharjee Lung" and "Stearman Lung" and so on (Supplementary Figure 1). These findings were based on mRNA levels, but protein levels seems closer to the real situation. Therefore, we further studied the expression of EPOR protein in LUAD tissues and found that the protein levels of EPOR in LUAD tissues was higher than that in paired normal lung tissues ($t = -10.184$, $P < 0.001$) (Figure 2a, b).

(2) Gene mutations affecting EPOR expression and the correlation of EPOR expression with ALK, EGFR and PD-L1 in LUAD

We were curious about the gene mutations affecting EPOR expression in LUAD, and to this end, explored which gene mutations in LUAD lead to downregulation/upregulation of EPOR expression. The muTarget database showed that mutations of DDX60L, LGR6, POTE3, RIF1 and SOX5 genes resulted in the downregulation of EPOR expression (Figure 3a), and mutations of C1orf168, DBX2, EIF5B, FNDC1, KIAA0430, LRRC16A, MGAT3, PTPRM, TPH2 and UNC80 genes resulted in the upregulation of EPOR expression (Figure 3b). The presence of ALK gene rearrangements and EGFR mutations in LUAD, both of which are tumor driver genes with targeted drug therapy and are of great significance to LUAD patients. PD-L1 is often closely associated with immune escape in cancer, and PD-L1 is also one of the common immunotherapy targets in lung cancer. Based on our clinicopathological data of tissue microarrays containing FISH data of ALK and EGFR and IHC data of PD-L1, we first compared the expression of EPOR in the ALK/EGFR/PD-L1 groups with high and low expression using the TCGA database. The results showed that the expression level of EPOR was significantly higher in the ALK high expression group than in the ALK low expression group, and there was no significant difference in EGFR and PD-L1 groups between high and low expression. In our pathological data, no difference was found in the expression of EPOR between ALK/EGFR negative and positive groups and between PD-L1 high and low expression groups (Figure 2c).

(3) EPOR-interacting genes and proteins, EPOR co-expressed genes and genetic alterations

The PPI network of EPOR was generated using the STRING database. There were 46 edges and 11 nodes, including EPO, JAK2 and STAT5B, etc (Figure 4a). We also constructed correlations between EPOR genes and genes expressing these proteins. EPOR genes were more correlated with STAT5B and PTPN6 genes ($R > 0.2$), and was correlated with EPO, KIT and GRB2 (Figure 4c). The EPOR-interacting genes network was constructed by GeneMANIA. EPOR interacted physically with EPO, CCDC150 and CFAP161, with the strongest interactions with EPO, and had weak genetic interaction with PCNA (Figure 4b). The top 40

EPOR co-expressed genes were screened by GEPIA database and a single gene co-expression heat map was constructed (Figure 4d). The three datasets, LUAD (TCGA, Nature 2014), LUSC (TCGA, Nature 2012) and NSCLC (TRACERx, NEJM&Nature 2017), were first analyzed in the cBioPortal database, and the results showed that the alteration frequencies of EPOR in LUSC and NSCLC were more than 2% and less than 1% in LUAD (Supplementary Figure 2a). The EPOR gene alterations in the three LUAD datasets were further analyzed, and the average alteration frequency was only 0.7%, with "Deep Deletion" being the more common type (Supplementary Figure 2b, d). Due to the lack of alteration data, the Kaplan-Meier plotter and log-rank test could only obtain the correlation between OS and EPOR altered/unaltered groups, and the result showed that the difference is not statistically significant (Supplementary Figure 2c).

(4) Correlation of EPOR expression with tumor purity, immune cells, SCNA and tumor microenvironment

In the TIMER database, we analyzed the correlation of EPOR expression with tumor purity and six infiltrating immune cells including B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells (DCs). The results showed that EPOR expression levels were not significantly correlated with tumor purity and infiltration of macrophages, neutrophils, DCs, negatively correlated with infiltration of CD8+ T cells, and positively correlated with infiltration of B cells and CD4+ T cells, where the correlation coefficient with CD4+ T cells was close to 0.3 (Figure 5a). We further analyzed the correlation of EPOR expression with gene markers of immune cells in the TIMER database. The results after adjusting for tumor purity showed that EPOR expression correlated with some gene markers of B cells, M1 macrophages, neutrophils, natural killer (NK) cells, DCs, type 1 T - helper (Th1) cells, type 2 T - helper (Th2) cells, type 17 T - helper (Th17) cells, follicular helper T (Tfh) cells, regulatory T (Treg) cells, effector memory T (Tem) cells and natural killer T (NKT) cells. Among them, the correlation coefficient with CD19 (B cell), CD11c (DC), STAT6 (Th2), BCL6 (Tfh) and TGFB1 (Treg) was more than 0.2, but the overall showed a weak correlation between EPOR expression and immune cell markers in LUAD (Table 1). The results of the SCNA module showed that significant differences in infiltration levels of B cells, CD4+ T cells, macrophages, neutrophils and DCs at the "Arm-level Deletion" somatic copy number state of EPOR in LUAD compared to normal levels, in addition, the infiltration levels of macrophages in the "Arm-level Gain" somatic copy number state of EPOR was significantly different compared to the normal level (Figure 5b). In addition, we further investigated the correlation between EPOR expression and tumor microenvironment (TME) in LUAD. TME can be evaluated by ImmuneScore, StromalScore and ESTIMATEScore, which indicate tumor immune cell infiltration, presence of tumor tissue mesenchyme and tumor purity, respectively. The results showed no significant correlation between EPOR mRNA expression levels and the three scores, with $|R| \leq 0.2$ (Figure 5c).

Table 1

Correlation analysis between EPOR and gene markers of immune cells in TIMER

Description	Gene markers	LUAD			
		None		Purity	
		Cor	P	Cor	P
B cell	CD19	0.138	**	0.218	***
	CD79A	0.046	0.299	0.107	*
T cell (general)	CD3D	-0.079	0.075	-0.043	0.346
	CD3E	-0.012	0.784	0.045	0.322
	CD2	-0.025	0.568	0.026	0.569
CD8+ T cell	CD8A	-0.077	0.079	-0.046	0.311
	CD8B	-0.094	*	-0.073	0.105
Monocyte	CD86	-0.087	*	-0.058	0.200
	CD115(CSF1R)	0.024	0.586	0.069	0.126
TAM	CCL2	-0.088	*	-0.067	0.140
	CD68	-0.085	0.051	-0.057	0.203
	IL10	-0.05	0.254	-0.02	0.652
M1	IRF5	0.153	***	0.185	***
	COX2(PTGS2)	0.041	0.359	0.033	0.463
	INOS(NOS2)	0.061	0.169	0.063	0.161
M2	CD163	-0.06	0.174	-0.03	0.500
	VSIG4	-0.078	0.078	-0.059	0.190
	MS4A4A	-0.084	0.056	-0.056	0.218
Neutrophil	CC16b(CEACAMB)	0.165	***	0.173	***
	CC11b(ITGAM)	0.07	0.110	0.11	*
	CCR7	0.104	*	0.184	***
NK	KIR2DL1	-0.019	0.664	-0.011	0.804
	KIR2DL3	-0.09	*	-0.076	0.090
	KIR2DL4	-0.142	**	-0.138	**
	KIR3DL1	0.023	0.595	0.039	0.381
	KIR3DL2	-0.068	0.123	-0.047	0.302
	KIR3DL3	-0.047	0.288	-0.035	0.435

DC	KIR2DS4	-0.006	0.899	0.002	0.965
	HLA-DPB1	0.037	0.398	0.086	0.056
	HLA-DQB1	0.063	0.156	0.097	*
	HLA-DRA	-0.062	0.163	-0.026	0.566
	HLA-DPA1	-0.015	0.735	0.028	0.529
	BDCA-1(CD1C)	0.094	*	0.132	**
	BDCA-4(NRP1)	0.093	*	0.099	*
	CD11c(ITGAX)	0.228	***	0.3	***
Th1	TBX21	0.047	0.286	0.091	*
	STAT1	-0.105	*	-0.099	*
	STAT4	0.075	0.088	0.113	*
	IFN- γ (IFNG)	-0.091	*	-0.072	0.109
	TNF- α (TNF)	0.082	0.064	0.137	**
Th2	STAT5A	0.075	0.090	0.129	**
	STAT6	0.289	***	0.295	***
	GATA3	-0.022	0.626	0.012	0.783
	IL13	0.116	**	0.136	**
Th17	STAT3	0.107	*	0.101	*
	IL17A	-0.019	0.664	0.001	0.977
Tfh	BCL6	0.263	***	0.276	***
	IL21	-0.03	0.491	-0.012	0.791
Treg	FOXP3	0.042	0.347	0.091	*
	CCR8	-0.025	0.571	0.014	0.752
	TGFB1	0.178	***	0.217	***
Tem	DUSP4	-0.07	0.111	-0.066	0.146
	GZMK	-0.017	0.692	0.038	0.398
	GZMA	-0.121	**	-0.1	*
NKT	ZBTB16	0.121	**	0.134	**
	KLRB1	-0.086	0.052	-0.046	0.312

*P < 0.05, **P < 0.01, ***P < 0.001

(5) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis and gene set enrichment analysis (GSEA) of DEGs

Data mining techniques in the TCGA database were used to screen the top 300 EPOR differentially expressed genes (DEGs) in LUAD. To explore the potential biological functions of DEGs, GO and KEGG enrichment analyses were performed. The results showed that MFs of DEGs were significantly enriched in passive transmembrane transporter activity, channel activity, substrate-specific channel activity, ion channel activity and sodium ion transmembrane transporter activity. In CCs, DEGs mainly distributed in neuronal cell body, presynapse, collagen-containing extracellular matrix, neuron projection terminus and axon terminus. In BPs, signal release, response to metal ion, neurotransmitter transport, hormone metabolic process and amine transport dominated (Figure 6a). And DEGs were enriched in neuroactive ligand-receptor interaction pathway and protein digestion and adsorption pathway (Figure 6b). To make the enrichment pathways more comprehensive, we further performed gene set enrichment analysis (GSEA) on all DEGs. The results showed that they were significantly enriched in KEGG pathways such as calcium signaling pathway, pathways in cancer, cytokine-cytokine receptor interaction, MAPK signaling pathway and neuroactive ligand-receptor interaction (Figure 6c) and significantly enriched in Wiki pathways such as focal adhesion PI3K/Akt/mTOR signaling pathway, sudden infant death syndrome-sids susceptibility pathways, MAPK signaling pathway, PI3K/Akt signaling pathway and nuclear receptors meta pathway (Figure 6d).

(6) Prognostic analysis of EPOR in LUAD patients

We first analyzed the prognosis of EPOR in LUAD patients in KM Plotter database. In OS, 37986_at and 216999_at probes showed that patients with high EPOR mRNA expression in LUAD had a better prognosis, and 209962_at, 209963_s_at and 396_f_at probes showed that patients with high EPOR expression in LUAD had a worse prognosis (Figure 7a). In PFS, the 37986_at, 209962_at and 209963_s_at probes showed that patients with high EPOR mRNA expression in LUAD had a better prognosis, and the 396_f_at probe showed that patients with high EPOR expression in LUAD had a worse prognosis (Figure 7b). We then performed a further prognostic analysis in the PrognScan database, which showed that high EPOR expression was associated with better OS in the GSE13213, GSE31210, jacob-00182-MSK and jacob-00182-UM LUAD datasets, and in the jacob-00182-CANDF dataset, high EPOR expression was associated with poorer OS (Figure 7c).

(7) Validation of EPOR prognostic value based on clinicopathological data of patients

To validate the prognostic value of EPOR expression in LUAD patients, we performed a prognostic analysis using detailed clinicopathological data from our 92 cases of lung adenocarcinoma tissue microarrays (HLugA180Su04). KM analysis showed poorer OS in the EPOR high expression group (29.5 vs 46 months) (Figure 8a). To test the independence of EPOR as a prognostic factor, we performed the Cox risk regression analysis. Multivariate Cox risk regression analysis showed that T3-T4 stage, AJCC stage III/IV, high expression of EPOR and ALK (+) were associated with OS (Table 2). KM analysis and multivariate Cox analysis demonstrated better agreement on EPOR being a risk factor for OS in LUAD. Subsequently, based on the results of multivariate Cox risk regression analysis, we constructed a prediction model, and the nomogram more visually demonstrated the effect of EPOR on 5-year survival rate (C-index = 0.711) (Figure 8b). The calibration curve showed good agreement between our results and predicted values in the 5-year survival rate (Figure 8c).

Table 2

Univariate and multivariate analyses of prognostic factors for OS

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
Gender	58				
Male	34	Reference			
Female	24	0.733 (0.384-1.398)	0.345		
Age	58				
<65	34	Reference			
≥65	24	1.773 (0.944-3.328)	0.075	1.984 (0.977-4.028)	0.058
T stage	58				
T1-T2	41	Reference			
T3-T4	17	1.985 (1.019-3.866)	0.044	0.256 (0.085-0.770)	0.015
N stage	58				
N0	27	Reference			
N1	13	1.470 (0.635-3.402)	0.369	0.390 (0.087-1.760)	0.221
N2	17	3.281 (1.542-6.978)	0.002	0.189 (0.028-1.262)	0.085
N3	1	4.357 (0.550-34.494)	0.163	0.425 (0.028-6.358)	0.535
M stage	58				
M0	56	Reference			
M1	2	1.742 (0.414-7.338)	0.449		
AJCC stage	58				
	21	Reference			
	16	1.440 (0.598-3.467)	0.416	4.100 (0.869-19.342)	0.075
	19	4.283 (1.898-9.669)	<0.001	59.422 (5.978-590.716)	<0.001
	2	3.347 (0.711-15.765)	0.127	13.110 (2.149-79.995)	0.005
EPOR	58				
Low	24	Reference			
High	34	2.108 (1.076-4.128)	0.030	2.857 (1.202-6.792)	0.017

ALK	58				
-	51	Reference			
+	7	2.640 (1.092-6.384)	0.031	3.937 (1.372-11.299)	0.011
EGFR	58				
-	43	Reference			
+	15	1.229 (0.622-2.428)	0.553		
PD-L1	58				
Low	30	Reference			
High	28	1.121 (0.598-2.102)	0.721		

Bold text indicated P < 0.05

Discussion

Among cancers, lung cancer has a high incidence and mortality rate [1], among which the survival rate of lung adenocarcinoma is not ideal due to the heterogeneity of tumor and the lack of prognostic markers [4–6]. Meanwhile, because the expression of EPOR and its correlation with patient prognosis in LUAD are not well defined, we explored the expression of EPOR in LUAD and the possibility of EPOR as a prognostic marker by combining various bioinformatics methods, immunohistochemical methods, and patients clinicopathological data. In this study, using TIMER, UALCAN, GEPIA, Oncomine and TCGA bioinformatics databases and immunohistochemistry we found that the EPOR mRNA expression in LUAD tissues was possibly downregulated compared with that in normal lung tissues, but the EPOR protein expression in LUAD tissues was higher than that in paired normal lung tissues (Fig. 1, 2; Supplementary Fig. 1). This showed that the mRNA and protein levels of EPOR may be inconsistent, complementing the EPOR expression data in LUAD. To this end, we explored the muTarget database and found that mutations in five genes in LUAD, DDX60L, LGR6, POTE3, RIF1, and SOX5, cause downregulation of EPOR expression, and mutations in 10 genes including C1orf168, DBX2 and EIF5B lead to upregulation of EPOR expression (Fig. 3). The expression of ALK genes may also affect EPOR expression (Fig. 2). We also found that EPOR-interacting proteins include EPO, JAK2, STAT5 and so on (Fig. 4), this fully illustrated that EPOR interacts with EPO and is closely associated with the activation of JAK2 and STAT5 [12, 13]. Based on patient samples in the cBioPortal database, approximately 0.7% of LUAD patients had EPOR genetic alterations, with “Deep Deletion” being the more common type and EPOR alteration frequency in NSCLC was also only 2% (Supplementary Fig. 2). This was consistent with previous reports, which EPOR mutations were very rare [44]. The TIMER database showed that EPOR expression in LUAD was negatively correlated with infiltration of CD8 + T cells and positively correlated with infiltration of B cells and CD4 + T cells (Fig. 5a). EPOR expression correlated with CD19 (B cell), CD11c (DC), STAT6 (Th2), BCL6 (Tfh) and TGFB1 (Treg) gene markers with coefficients above 0.2, but overall there were weak correlation with immune cell markers (Table 1). The infiltration levels of B cells, CD4 + T cells,

macrophages, neutrophils and DCs in the “Arm-level Deletion” somatic copy number state of EPOR were significantly different compared to normal levels (Fig. 5b). In addition, EPOR expression in LUAD was negatively correlated with ImmuneScore, StromalScore and ESTIMATEScore ($P < 0.05$), but not significantly ($|r| \leq 0.2$) (Fig. 5c). These results illustrated that EPOR expression in LUAD correlated weakly with immune cell infiltration. In the DEGs enrichment analyses, we enriched the information related to the nervous system, which validated the expression of EPOR on neurons [19]. And we also found that EPOR was closely associated with pathways such as MAPK signaling pathway and PI3K/Akt signaling pathway, which confirmed that activation of MAPK, PI3K and Akt cannot be achieved without the expression of EPOR [15, 16]. (Fig. 6) Finally, the KM Plotter database showed that different prognostic results may be obtained when using different probes to detect EPOR, and the PrognScan database also showed different prognostic results in different LUAD datasets, but consistently concluded that EPOR was associated with prognosis in LUAD patients (Fig. 7). Our clinicopathological data showed that high EPOR expression was associated with poorer OS, and that high EPOR expression was an independent risk factor for prognosis in LUAD patients and had a good predictive power for 5-year survival (Fig. 8; Table 2). These evidences suggested that EPOR may be an independent and novel prognostic marker for LUAD.

EPOR is present not only in hematopoietic cells but also in non-hematopoietic cells such as neurons [19], endothelial cells [20], skeletal muscle cells [45] and in various tumors such as breast cancer [46] and head and neck cancer [47]. EPOR expression was also detected in lung cancer cell lines such as H838 [48]. Regarding EPOR expression in cancers, on the one hand, it is believed that EPOR expression is upregulated, such as in gastric adenocarcinoma [28], hepatocellular carcinoma [29], prostate cancer [30], and glioma [31]; on the other hand, no significant EPOR expression was detected in tumor cell lines and solid tumor specimens, and Swift et al. [49] suggested that low levels of EPOR expression are common in tumor cell lines, meanwhile, Elliott et al. [50] questioned the assumption that most tumors express high levels of functional EPOR proteins, and Patterson et al. [51] did not detect the utilization of the functional EPOR pathway in primary tumor cells isolated from tumor tissues such as human breast cancer. Our study yielded the conclusion that EPOR expression in LUAD may be downregulated at the mRNA levels and significantly upregulated at the protein levels, showing that mRNA and protein levels are inconsistent, which added new evidence for EPOR expression in tumors.

EPO plays a role in promoting proliferation in hematopoietic progenitor cells and may also promote the growth of tumor cells, thus promoting tumor progression and metastasis, leading to poor prognosis of patients. And as the receptor of EPO, EPOR's effect on prognosis of cancer patients has been also discussed. On the one hand, high EPOR expression in locally advanced squamous cell carcinoma of the head and neck was considered to be an independent prognostic factor for OS and was associated with poorer OS [35], and activation of EPOR in melanoma was thought to promote tumor progression and contributed to survival of tumor cells [52], meanwhile, inhibition of EPOR gene expression in NSCLC reduced the growth of NSCLC cells under hypoxia [53]; on the one hand, there was no significant difference in survival rates between patients with different EPOR expression in gastric and cervical adenocarcinoma [28, 36]; on the other hand, recurrence-free survival was significantly improved in

ER+/EPOR + breast cancer patients with untreated tamoxifen in breast cancer [54], and in the breast cancer cell lines, RAMA 37 cells (low EPOR expression) had a stronger proliferation ability than RAMA 37 – 28 cells (high EPOR expression), suggesting that high EPOR expression can reduce the ability of cells to divide [55], in addition, high levels of EPOR mRNA in myeloma were associated with better survival [37]. In some data, EPO/EPOR co-expression or co-overexpression in NSCLC was associated with poor prognosis [38–40], but independent evidence on the prognostic impact of EPOR on patients was lacking. Our results showed that high EPOR expression was associated with poorer prognosis, which may contradict the conclusions of Rozsas et al.[56].

This study improves our understanding of the relationship between EPOR and LUAD, but there are still some limitations. First, this study was based on bioinformatics analyses and combined with immunohistochemistry and some clinicopathological data, but lacks experimental depth. Second, most of our analyses were based on the mRNA levels of EPOR, and the inclusion of more evidence at the protein levels would be closer to the truth and more convincing. Third, due to our limited funds, we could only rely on bioinformatics databases for the detection of EPOR mRNA expression levels, which could not be verified by RT-PCR assays. Fourth, the prognostic results in the bioinformatics analysis were not very uniform, which may be related to factors such as the study subjects; for example, the GSE13213 dataset included only patients with adenocarcinoma with a high probability of recurrence, and the GSE31210 dataset included only patients with stage II lung adenocarcinoma. Although our clinicopathological data validation results showed that high EPOR expression was associated with poorer prognosis, prospective trials with large clinical samples are still needed for validation.

Conclusions

EPOR expression may be downregulated at the mRNA levels and significantly upregulated at the protein levels in LUAD. The high expression of EPOR is associated with poor prognosis and may be a potential new prognostic marker for LUAD. Overall, our study will provide a solution ideas to the hot issue of poor survival rate that has been a problem in LUAD and provide new evidence for the study of EPOR in cancer. At the same time, our findings agree on the need to further explore the prognosis of EPOR in LUAD using a multicenter, large-sample clinical study.

Abbreviations

EPO: Erythropoietin; EPOR: Erythropoietin receptor; LUAD: Lung adenocarcinoma; NSCLC: Non-small cell lung cancer; JAK2: Janus kinase 2; STAT5: Signal transducer and activator of transcription 5; PI3K: Phosphatidylinositol 3-kinase; AKT: Protein kinase B; MAPK: Mitogen-activated protein kinase; ERK1/2: Extracellular signal-regulated kinase 1/2; ALK: Anaplastic lymphoma kinase; EGFR: Epidermal growth factor receptor; PD-L1: programmed death ligand 1; TME: Tumor microenvironment; IHC: Immunohistochemistry; FISH: Fluorescence in situ hybridization; TCGA: The Cancer Genome Atlas; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: Gene set enrichment analysis; DEGs: Differentially expressed genes; MF: Molecular function; CC: Cellular component; BP: biological

process; NES: Normalized enrichment score; FDR: False discovery rate; OS: Overall survival; PFS: Progression-free survival; HR: Hazard ratio; 95% CI: 95% confidence interval.

Declarations

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Authors' contributions

YJZ proposed the research idea, and designed the study with SYW. YJZ, SYW and STH collected the data, performed the experiments. YYH interpreted the data. YJZ drafted the manuscript. YCF critically revised the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the first author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Academic Committee of The Third Clinical Medical College of Xinjiang Medical University (affiliated Tumor Hospital) and was carried out under the rules put forward in the Declaration of Helsinki. The need for obtaining informed consent was waived owing to the retrospective nature of the study. This study had the relevant exemption certificate of informed consent issued by the Academic Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

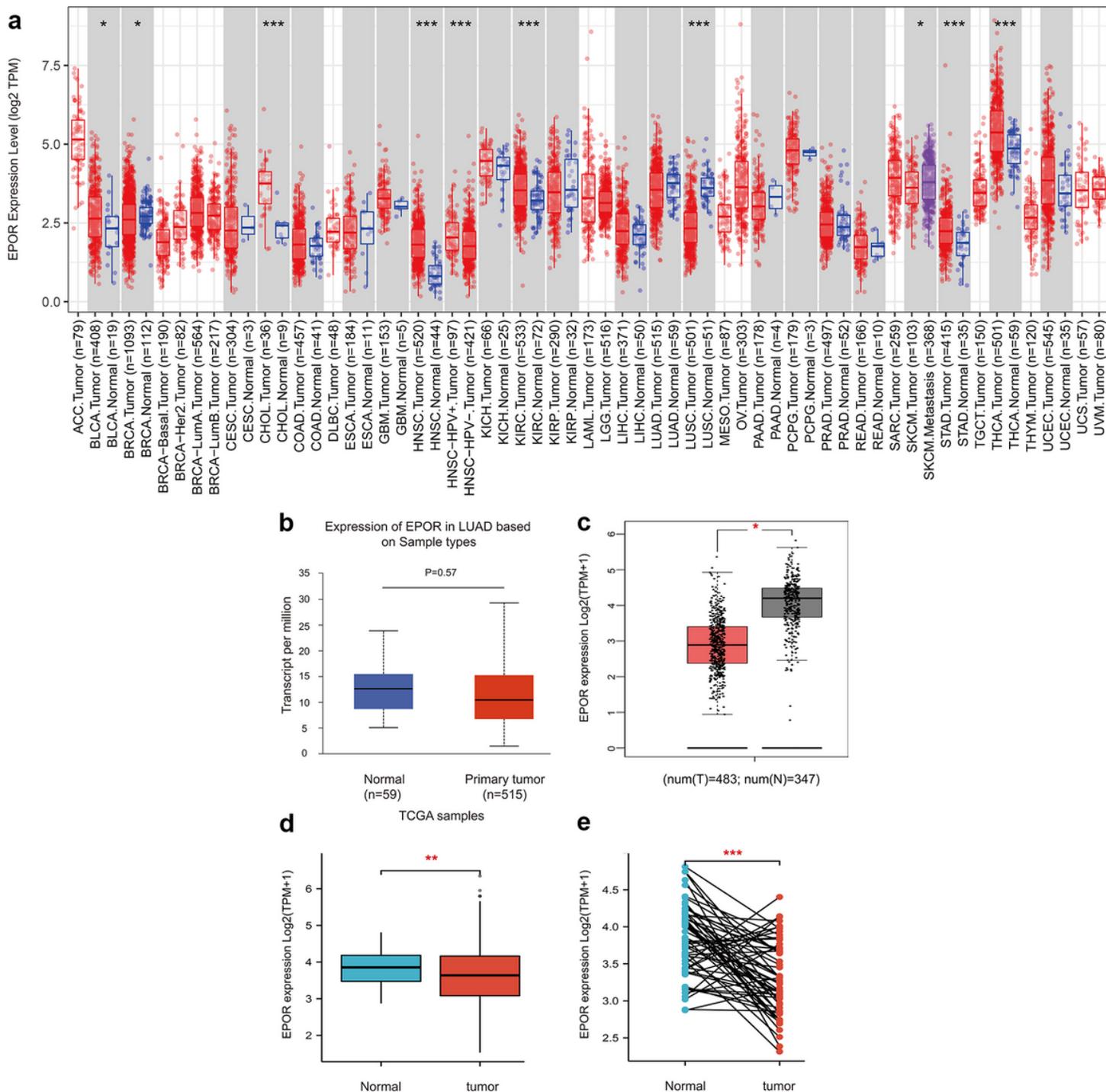


Figure 1

EPOR mRNA expression in LUAD. (a) EPOR expression in different types of cancer was investigated with the TIMER database. (b) EPOR expression in LUAD was examined by using the

UALCAN database. (c) EPOR expression in LUAD was examined by using theGEPIA database. (d)

Analysis of EPOR expression in LUAD and adjacent normal lung tissues in the TCGA database. (e)

Analysis of EPOR expression in 57 pairs of LUAD tissues and adjacent normal lung tissues in the TCGA database. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

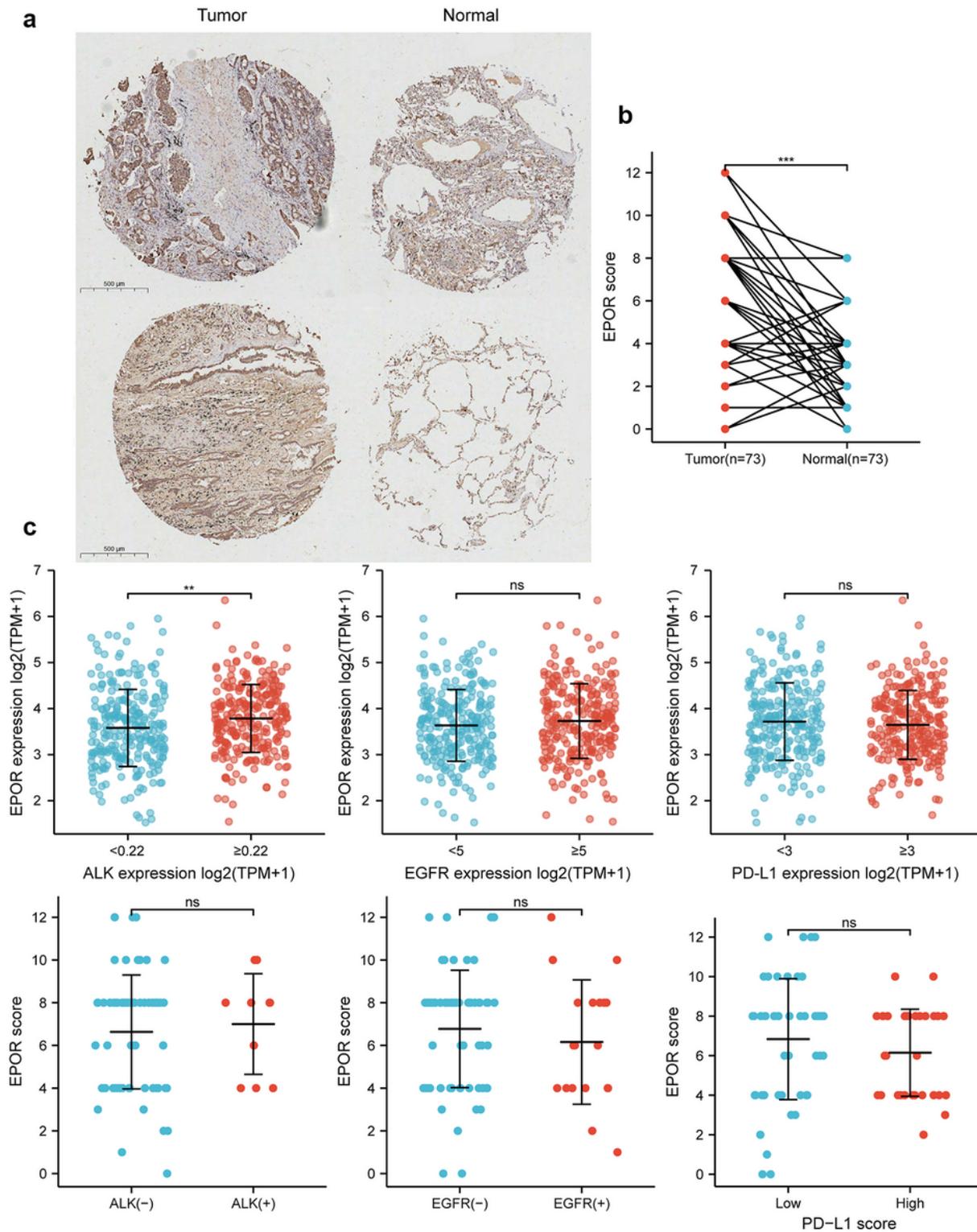


Figure 2

EPOR protein expression in LUAD patients. (a) Immunohistochemical staining of EPOR was performed in LUAD and adjacent normal lung tissues. Representative images were shown. Scale bars, 500 μm . (b) EPOR expression scores were calculated by multiplying staining intensity scores and positive rate scores.

The paired plot depicts EPOR expression in 73 pairs of LUAD tissues and adjacent normal lung tissues. (c) The dot plots in the first line describe the EPOR expression between the high and low expression groups of ALK, EGFR and PD-L1 in TCGA database (grouped by median expression). The dot plots in the second line are based on the FISH data of ALK and EGFR and the immunohistochemical data of PD-L1 from the Lung pathological data of tissue microarrays. EPOR expression score was used as ordinate to describe the expression of EPOR in each group. ns: $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$.

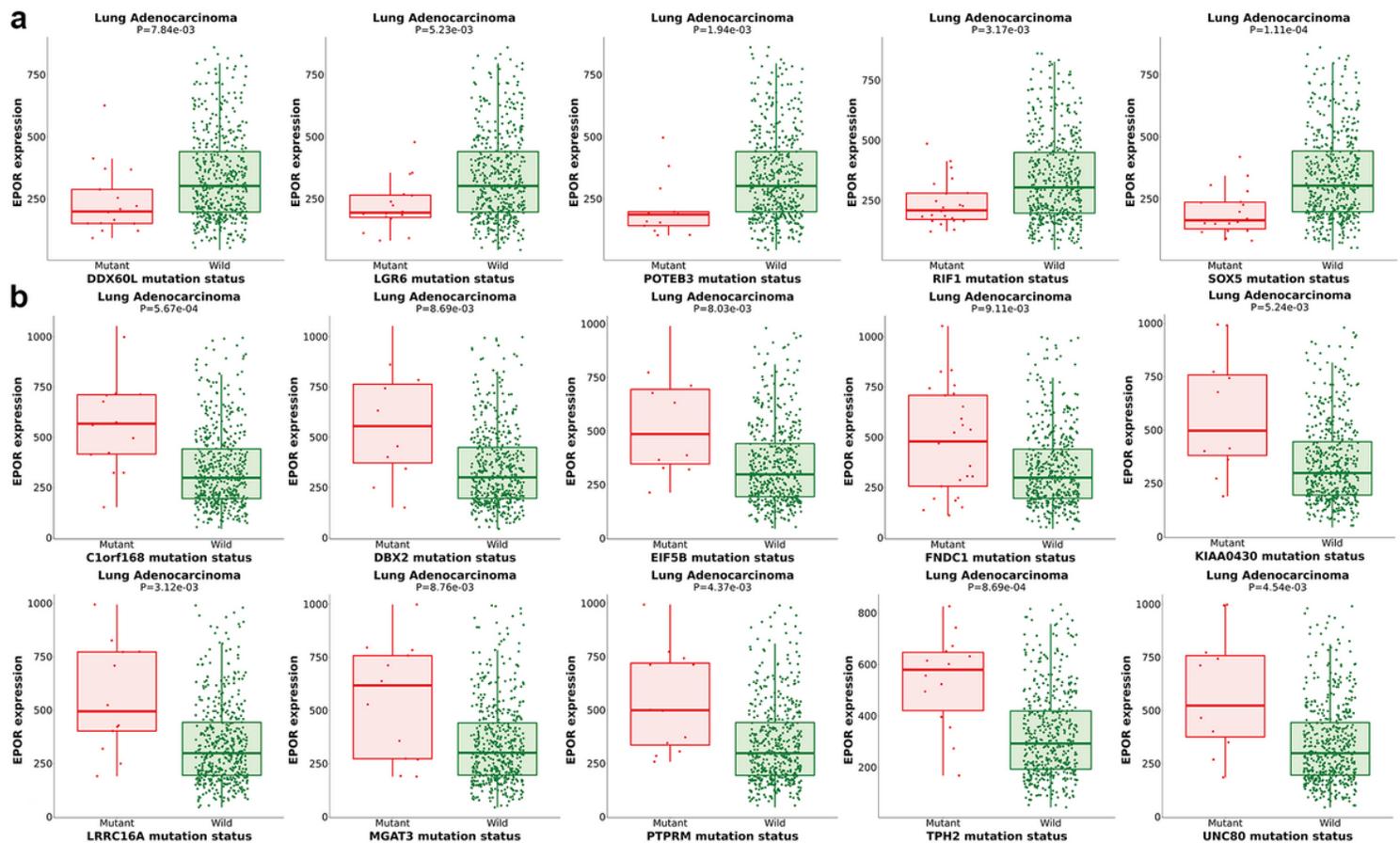


Figure 3

Gene mutations that affect EPOR expression. (a) Gene mutations affecting EPOR expression downregulation in the muTarget database. (b) Gene mutations affecting EPOR expression upregulation in the muTarget database.

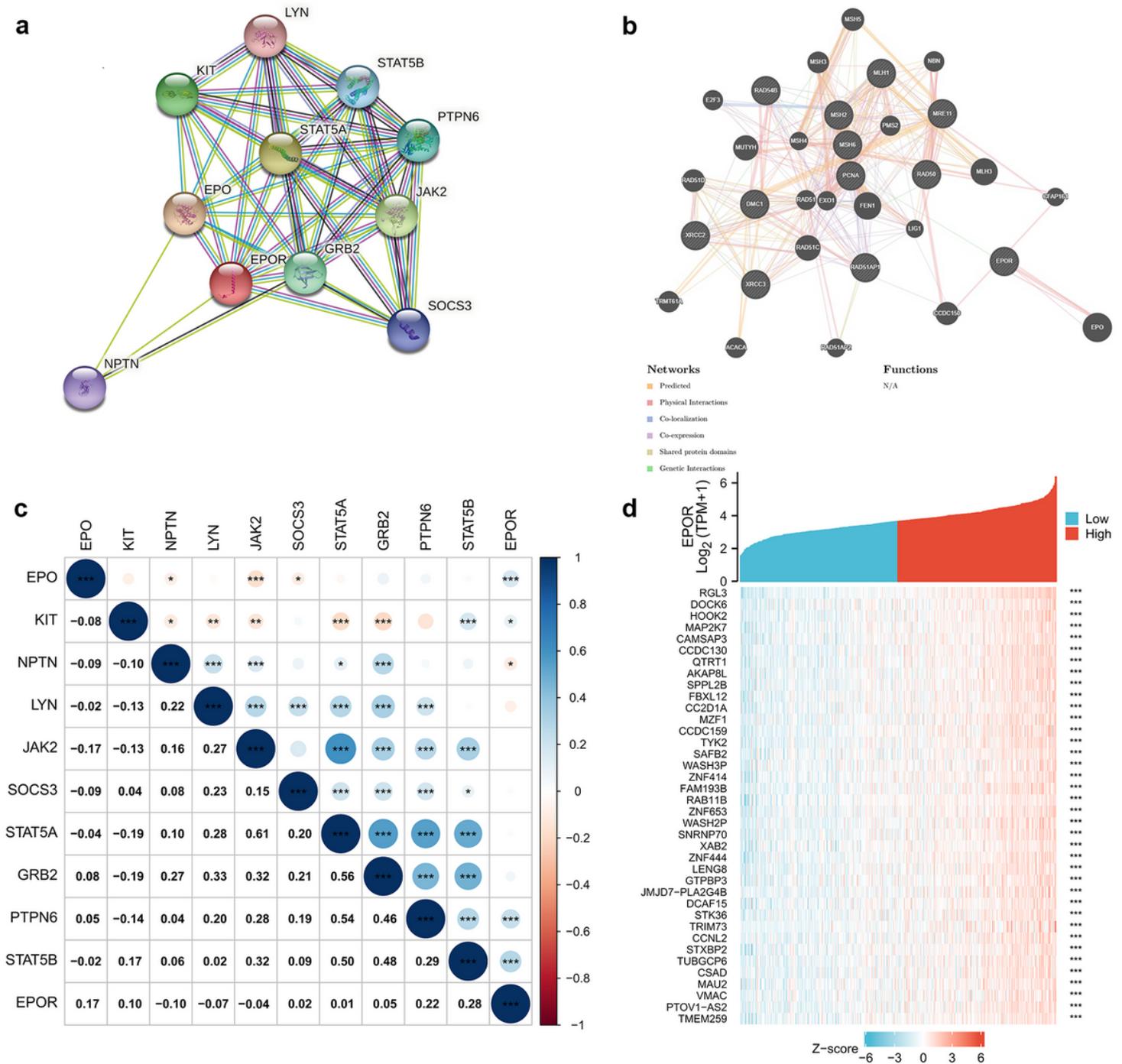


Figure 4

EPOR-interacting genes and proteins and EPOR-coexpressed genes. (a) The PPI network of EPOR was generated using STRING. (b) The gene-gene interaction network of EPOR was constructed using GeneMANIA. (c) The heat map shows the correlation between EPOR genes and genes expressing EPOR interacting proteins. (d) The single gene co-expression heat map shows top 40 EPOR-coexpressed genes in GEPIA database. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

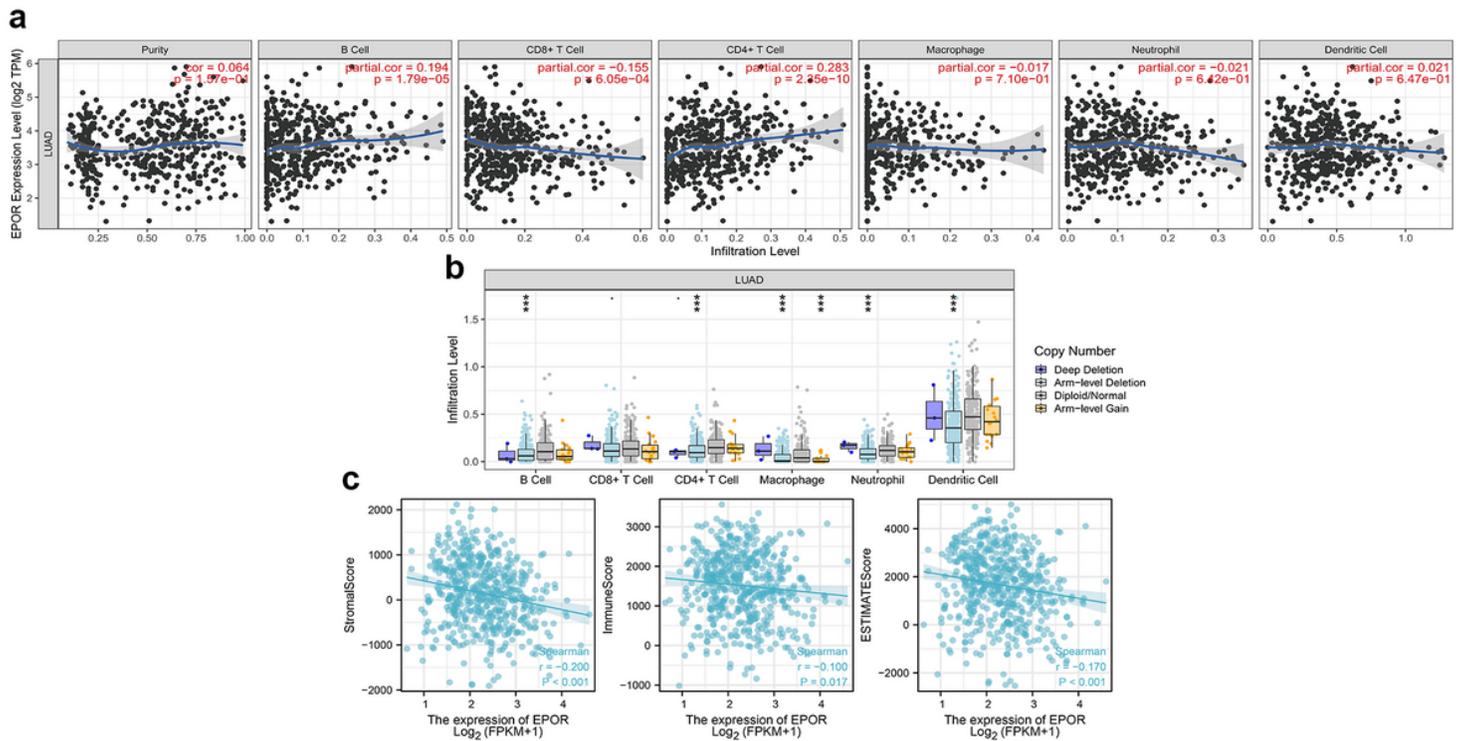


Figure 5

Correlation of EPOR expression with tumor purity, immune cells, SCNA and tumor microenvironment in LUAD. (a) Correlation of EPOR expression with the infiltration of different immune cells using the TIMER database. (b) Comparing the level of immune infiltration with different somatic copy number alternations of EPOR gene in the TIMER database. (c) Correlation of EPOR expression with tumor microenvironment (ImmuneScore, StromalScore and ESTIMATEScore).

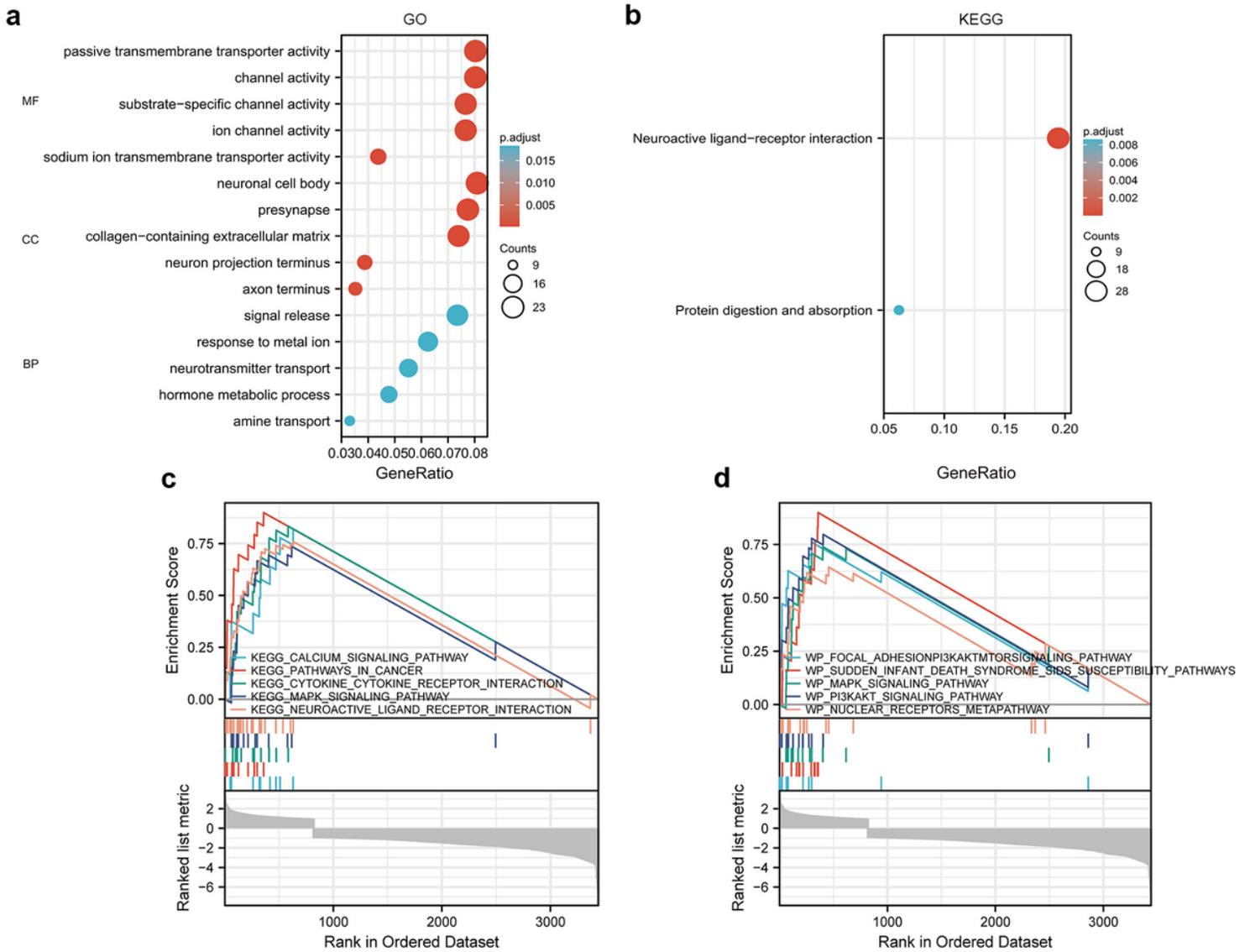


Figure 6

GO and KEGG enrichment analysis and gene set enrichment analysis for EPOR. (a) Top 5 terms in MF/CC/BP categories from GO enrichment analysis in LUAD. (b) KEGG enrichment pathways which were statistically significant in LUAD. (c) Top 5 KEGG pathways from GSEA in LUAD. (d) Top 5 Wiki pathways from GSEA in LUAD.

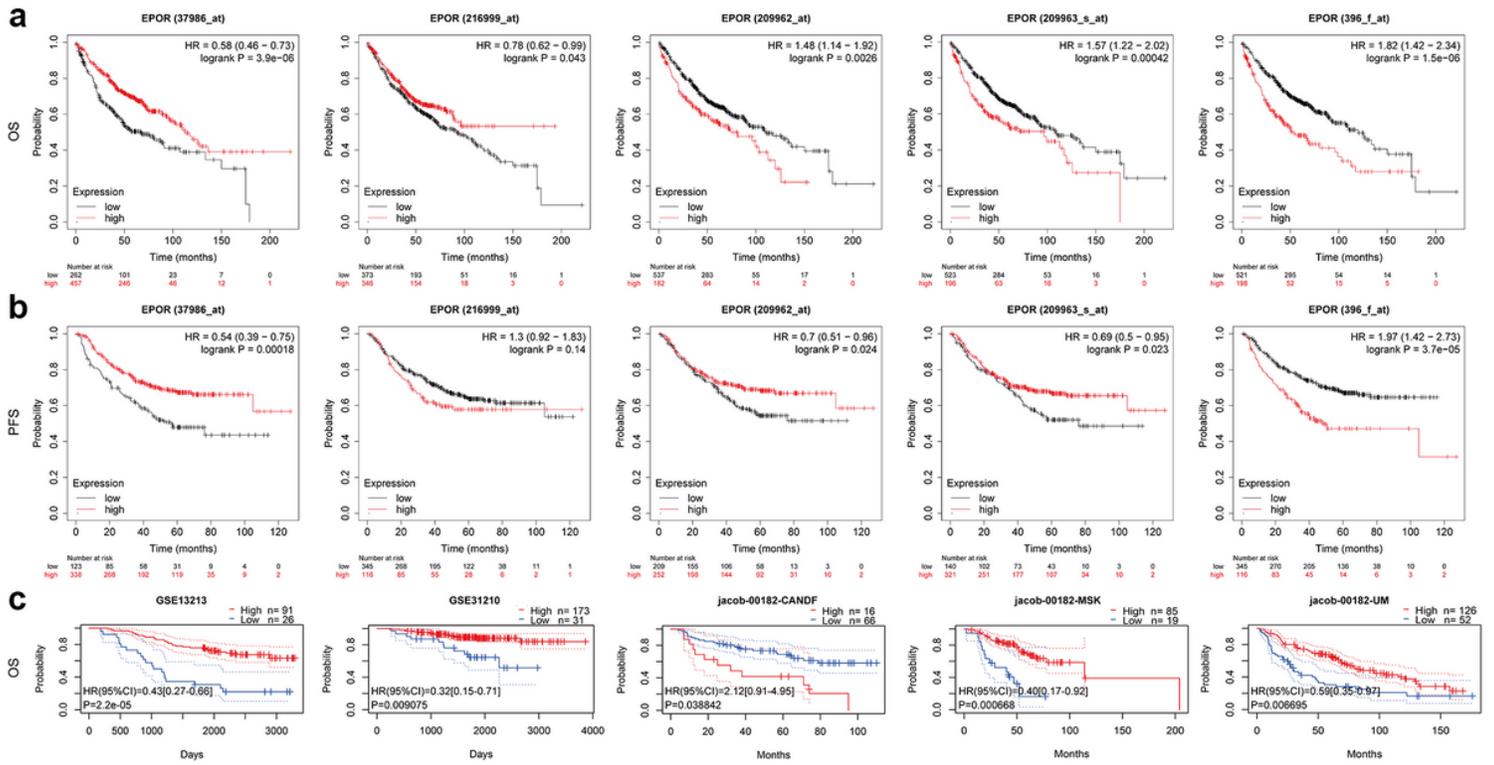


Figure 7

Survival curve evaluating the prognostic value of EPOR in LUAD. (a) Prognostic analysis of EPOR in the KM Plotter database for OS. (b) Prognostic analysis of EPOR in the KM Plotter database for PFS. (c) Prognostic analysis of EPOR in the Prognoscan database for OS.

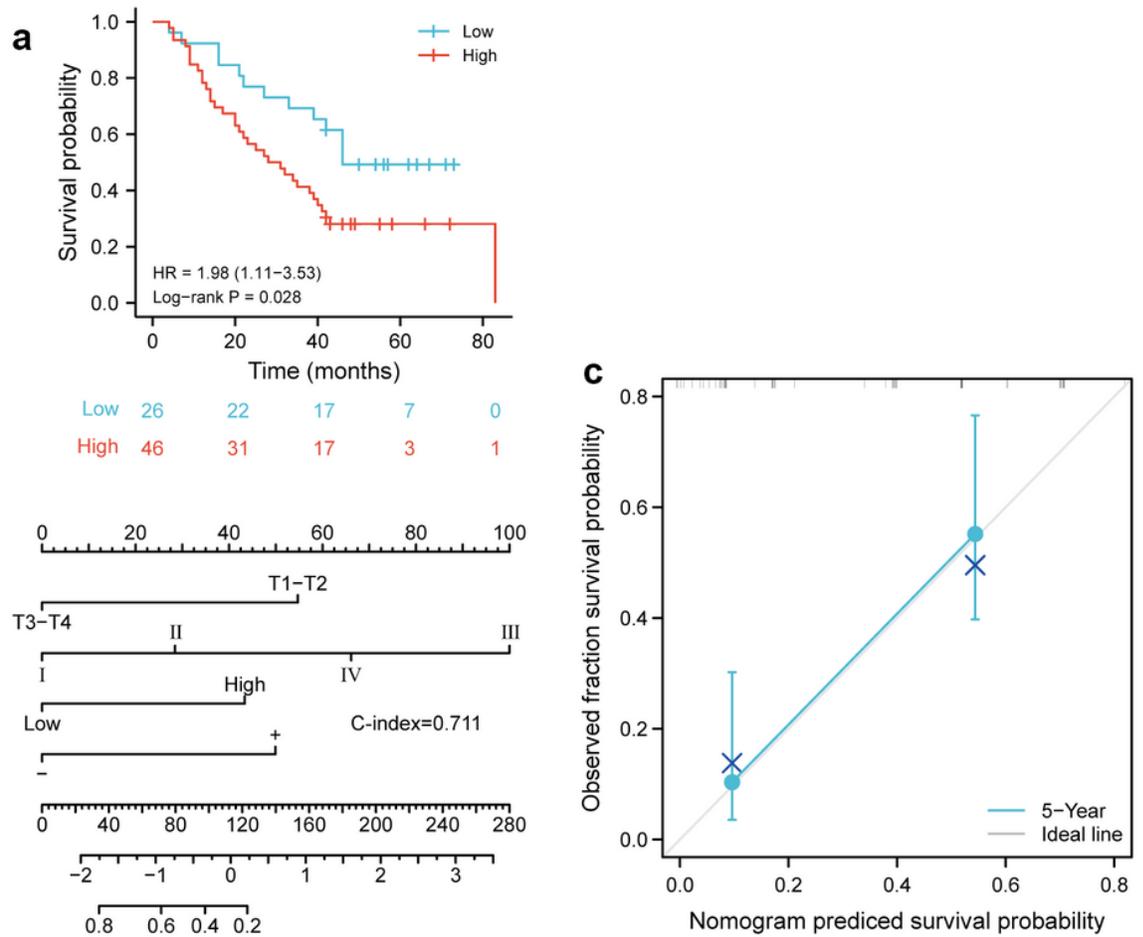


Figure 8

Validation of EPOR prognostic value based on clinicopathological data of patients. (a) Survival curve evaluating the prognostic value of EPOR in LUAD. (b) Nomogram model combined with AJCC stage, T stage, EPOR and ALK. (c) Calibration curve examining predictive accuracy for 5-year overall survival.

Supplementary Files

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