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ORIGINAL RESEARCH

Identification and Validation of Epithelial Cell Centre Regulatory Transcription Factors in the Gastric Cancer Microenvironment

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Purpose: To identify the epithelial cell centre regulatory transcription factors in the gastric cancer (GC) microenvironment and provide a new strategy for the diagnosis and treatment of GC.

Methods: The GC single-cell dataset was downloaded from the Gene Expression Omnibus (GEO) database. The regulatory mechanisms of transcription factors in both pan-cancer and GC microenvironments were analysed using the Cancer Genome Atlas (TGCA) database. Real-time quantitative PCR (RT-qPCR) was used to determine the mRNA expression levels of Prospero homeobox gene 1 (PROX1) and Endothelial PAS domain-containing protein 1 (EPAS1) in the human gastric mucosal normal epithelial cell line (GES-1) and the GC cell line (AGS). Immunohistochemistry (IHC) was used to determine the amounts of PROX1 and EPAS1 protein expression in GC and adjacent tissues. GC patients' overall survival (OS) was tracked through outpatient, Inpatient case inquiry, or phone follow-up.

Results: The single-cell data from GSE184198 was re-annotated, resulting in nine cell subsets: T cells (13364), NK cells (606), B cells (2525), Epithelial cells (2497), DC cells (1167), Fibroblast cells (372), Endothelial cells (271), Neutrophils cells (246) and Macrophage cells (420). Analysis of cell subgroup signalling pathways revealed that communication intensity between epithelial cells and smooth muscle cells was highest. Transcription factors *PROX1* and *EPAS1* were notably active in epithelial cells. Cell communication analysis indicated that IFNG may interact with IFNGR1/2 and LIF with IL6ST and LIFR to regulate the downstream *PROX1* and *EPAS1*. *PROX1* and *EPAS1* were upregulated and negatively correlated with tumour mutation burden (TMB). *They* also exhibited high positive correlations with immune checkpoints CTLA4 and PDCD1LG2, as well as with chemokines CCL24 and CXCL12 and their receptors CCR3 and CCR4. Additionally, *PROX1* and *EPAS1* were positively correlated with immunosuppressive factors ADORA2A, CD160, IL10, TGFBR1, KDR and CSF1R, as well as with immunostimulators CD276, PVR, TNFRSF25, ULBP1, CXCL12 and ENTPD1. In GC tissues and AGS, PROX1 and EPAS1 were both substantially expressed. In the meantime, they showed a positive correlation with clinicopathological features such TNM stage and degree of differentiation. In GC patients, the up-regulated group's PROX1 and EPAS1 prognosis was noticeably poorer than the down-regulated group's.

Conclusion: *PROX1* and *EPAS1* are likely central regulatory transcription factors in the epithelial cells of the GC environment, regulated by IFNG and LIF. They may contribute to GC progression by modulating the tumour's immune microenvironment.

Keywords: *PROX1*, *EPAS1*, GC, tumour microenvironment, transcription factor, cell communication

Introduction

Gastric cancer (GC) is a prevalent malignancy worldwide, ranking among the top five in terms of incidence and mortality.[1] GC primarily arises from the gastric mucosal epithelium, with adenocarcinoma being the predominant type. The pathogenesis of GC involves a multifactorial interplay of genetic, environmental and host-related factors. Early-stage diagnosis is rare, with most patients presenting at an advanced stage. As tumour progression continues and

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pharmacotherapy advances, the risk of drug resistance increases.[2,3] Current therapeutic strategies for GC include surgical intervention or endoscopic resection combined with chemotherapy, targeted therapy and immunotherapy. Due to its highly aggressive nature, GC exhibits significant heterogeneity in targets, regulatory mechanisms, cell types, states, and subpopulation distribution within the tumour microenvironment (TME).[4,5] Conventional population-level analyses often fail to capture these variations, highlighting the need for novel detection techniques to precisely identify the benefits of GC cell heterogeneity for accurate diagnosis, potential molecular target identification and prognosis evaluation.

The TME consists of the peritumoral milieu, including adjacent blood vessels, immune cells, stromal cells, various signalling molecules, and the extracellular matrix.[6] Cellular subsets within the TME can be broadly categorised into tumour cells, immune cells and stromal cells.[7] Tumour progression is speculated to result from intricate interactions among these cell populations.[8] The regulation of tumour immune responses, extracellular matrix remodelling and neovascularisation fundamentally influences cancer development and progression.[9,10] Transcription factors are proteins or RNA molecules that bind to DNA and regulate gene transcription. They exhibit diverse structures, functions and regulatory mechanisms, making them crucial in governing gene expression.[11,12] Consequently, transcription factors play a pivotal role in the pathogenesis and progression of numerous diseases, including cancer and metabolic disorders. Previous studies have demonstrated the pivotal role of transcription factors in cellular processes such as differentiation, development and metabolism. Exploring the relationship between transcription factors and tumours offers substantial potential for tumour prevention and treatment.[13,14] Therefore, this study employs single-cell sequencing technology to analyse the GC microenvironment, with the aim of identifying differentially regulated transcription factors within the epithelial core of tumours. This approach facilitates the discovery of potential targets for precise GC treatment and provides a crucial theoretical foundation for unravelling the underlying molecular mechanisms (Experimental design, Figure 1).

Materials and Methods

Public Data Sources and Clinical Data of Patients with GC

The GC single-cell dataset (GSE184198) was downloaded from the GEO database. The regulatory mechanisms of transcription factors in both pan-cancer and GC microenvironments were analysed using the TGCA database. Furthermore, between September 2022 and April 2024, paraffin tissue samples from 48 cancer and paracarcinoma cases were taken from patients having radical GC surgery at Kunming Medical University's First Affiliated Hospital. We adhere to the following inclusion criteria: Prior to surgery, none of the patients had received anticancer therapies such targeted therapy or radiotherapy. The clinical records were thorough and comprehensive. Ultimately, 46 GC tissue instances satisfied the criteria. Then, immunohistochemistry (IHC) was used to determine the levels of PROX1 and EPAS1 protein expression.

Methods for Single-Cell Data Analysis

Single-cell data filtering was performed using the Seurat 4.4.0 package, excluding cells with fewer than 3 or more than 5000 gene expressions and those with fewer than 80 mitochondrial reads. Subsequently, the FindVariableFeatures function identified the top 2000 genes exhibiting the highest intercellular variation coefficients for principal component analysis (PCA). Cell clustering was performed using the FindClusters function and differential gene expression analysis within each cluster was performed with the FindAllMarkers function. Cell annotation was achieved by integrating the SingleR version 2.4.0 package with the CellMarker database (http://bio-bigdata.hrbmu.edu.cn/CellMarker/CellMarkerSearch.jsp).

Other Analysis

Target gene predictions for intercellular ligands were performed using the Nichenetr 2.0.4 package. Cell signalling pathway predictions were analysed with the CommPath 1.0.0 package. Cellular transcription factors were analysed using the Dorothea 1.14.0 package. Using the TCGA plot 4.0.0 package, key transcription factors in pan-cancer were examined, focusing on their expression, Cox risk, correlations with TMB, immune checkpoints, chemokines and their

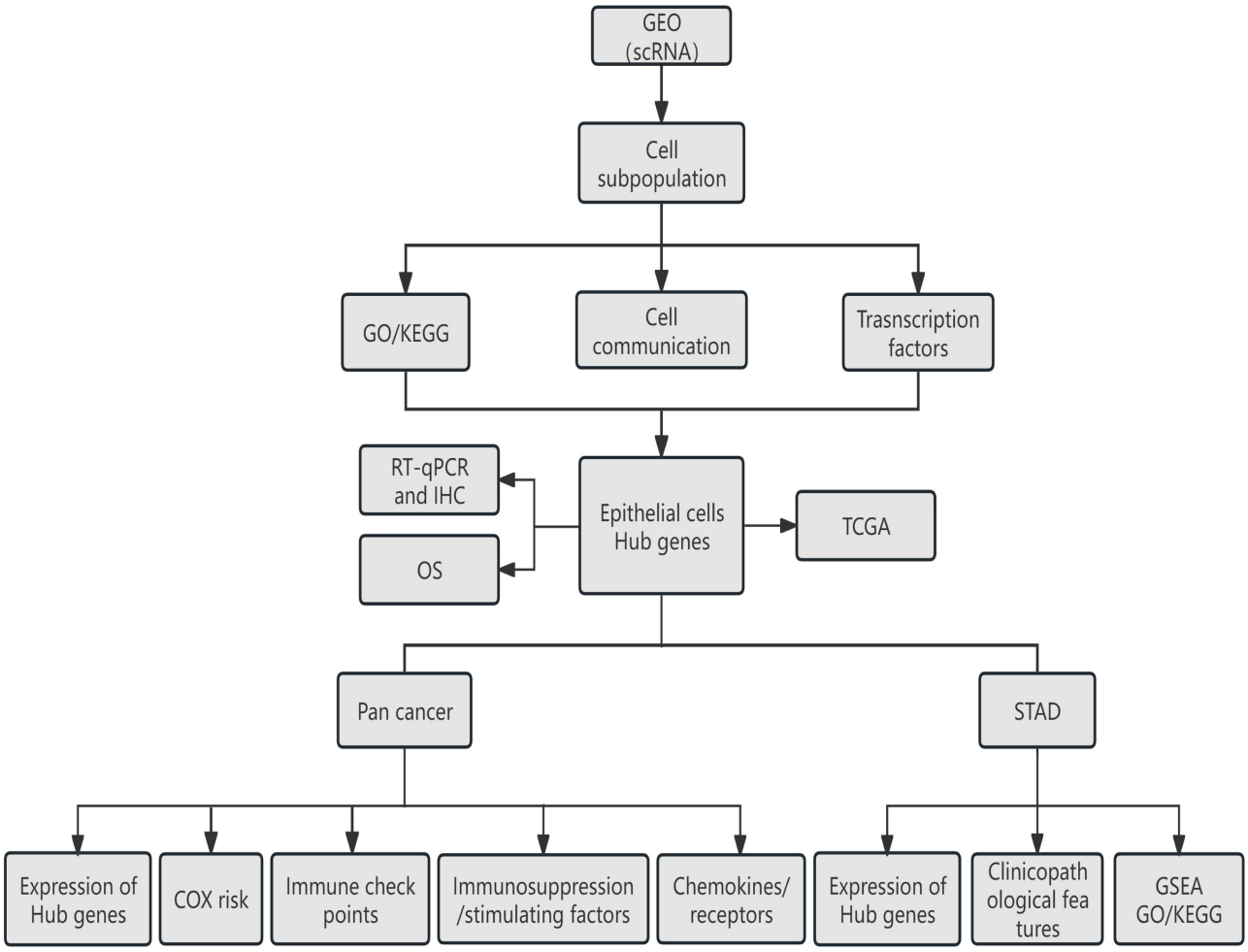

Figure 1 Design of the experiment.

receptors, immunosuppressive factors and immune stimulating factors in the TCGA database. Further analysis included the expression of key transcription factors in relation to grading, age, gender, differentially expressed genes between the high and low groups and co-expressed genes in the TCGA-STAD database. Finally, Gene Set Enrichment Analysis (GSEA) was employed to analyse GO terms and KEGG pathways associated with key transcription factors.

Cell Culture

GES-1 and AGS were grown in RPMI 1640 medium at 37°C, 10% PBS, and 5% CO2. GES1 was sold to Suzhou Hysigen Biotechnology Co., Ltd. AGS purchased Wuhan Procell Life Sciences Co.

RT-qPCR

Trizol reagent (Invitrogen) was used to extract total RNA from the cell line. Following the directions, 10 μL of cDNA were made from 5 μg of total RNA. Table 1 indicated the RT-qPCR primers that were used. The $2^{-\Delta\Delta Ct}$ technique was used to determine the relative expression of the RNA of interest.

IHC Detection

Paraffin-embedded carcinoma and paracarcinoma tissue samples from GC patients were sectioned (3 μm thickness), baked, deparaffinised and heat-repaired with EDTA (pH 9.0). Sections were then incubated overnight at 4°C with primary antibodies PROX1 (Brand: Proteintech, Cat No: 26422-1-AP) and EPAS1 (Brand: RabMAb, Cat No: ab199359).

Table 1 Primer Sequences for RT-qPCR

Genes	Primer Sequences
PROX1	F:5'-ATTGCCTTGTGTGCCTTCCA-3'
	R:5'-CAAACGGCACTGAGCTTGTA-3'
EPAS1	F:5'-CCATGGTAGCCCTCTCCAAC-3'
	R:5'-CCCCTGAGGTTCTTCATCCG-3'
GAPDH	F:5'-TGTTGCCATCAATGACCCCTT-3'
	R:5'-CTCCACGACGTACTCAGCG-3'

Following this, secondary antibodies (Brand: Dako, Cat No: K5007) were applied, followed by hematoxylin counter-staining at 37°C. The staining index was independently scored by two pathologists and calculated by multiplying the intensity score (negative = 0; weak = 1; moderate = 2; strong = 3) with the percentage of positive cells (<25% = 1; 25–50% = 2, 50–75% = 3; ≥75% = 4).

Clinical Follow-Up

Medical data from outpatient, inpatient, or telephone follow-up were used to document the survival of GC patients. The final follow-up deadline was November 24, 2024, and OS was the postoperative to last follow-up or death.

Analysis of Statistics

R program was used for bioinformatics analyses. The *T* test was used to analyze the IHC data. The chi-square test was used to examine the clinicopathological characteristics. The Kaplan-Meier method was used to examine survival time. $p<0.05$ was deemed statistically significant.

Ethical Considerations

We purchased the cell lines we used for our study from commercial vendors. The Ethics Committee of Kunming Medical University's First Affiliated Hospital examined and approved this study, which involved human data, in accordance with the Declaration of Helsinki ((2024) Ethics L No. 136). Every participant provided written informed permission. All participants' personal information was kept private, participation in the study was entirely voluntary, and all data sets were coded and maintained over the entire data gathering and analysis process.

Results

Single-Cell Data Analysis

We downloaded the GSE184198 microarray data from the GEO database and re-annotated the cells from the tumour group (12968 cells) and the normal group (8500 cells). The re-annotation resulted in the following cell subsets: T cells (13364), NK cells (606), B cells (2525), Epithelial cells (2497), DC cells (1167), Fibroblast cells (372), Endothelial cells (271), Neutrophils (246) and Macrophages (420) (Figure 2A). Marker gene expression in these cell subpopulations is shown in Figure 2B. Cellular communication was analysed using the nichnet package for both tumour and normal groups. We found that epithelial and fibroblast cells had the highest communication intensity (Figure 2C). IFNG was more actively expressed in NK cells, while Leukaemia inhibitory factor (LIF) was primarily expressed in DC cells. Both IFNG and LIG have a higher probability of targeting *PROX1* and *EPAS1*, suggesting their involvement in regulating these transcription factors (Figure 2D). Specifically, IFNG may bind to IFNGR1/2, and LIF may interact with IL6ST and LIFR to regulate downstream *PROX1* and *EPAS1* (Figure 3A). Subsequently, we analysed all cell signalling pathways and highlighted the Top15 pathways. The PI3K-AKT pathway was relatively downregulated in epithelial cells and upregulated in fibroblast cells (Figure 3B and C). Upstream signalling regulatory molecules in epithelial cells showed greater regulatory strength on the signalling pathways related to stem cell pluripotency (Figure 3C). We also predicted nine cellular transcription factors and examined their intersections with paired target genes in epithelial cells across all cell types. The most active transcription factors identified were *PROX1* and *EPAS1* (Figure 3D). The expression of these

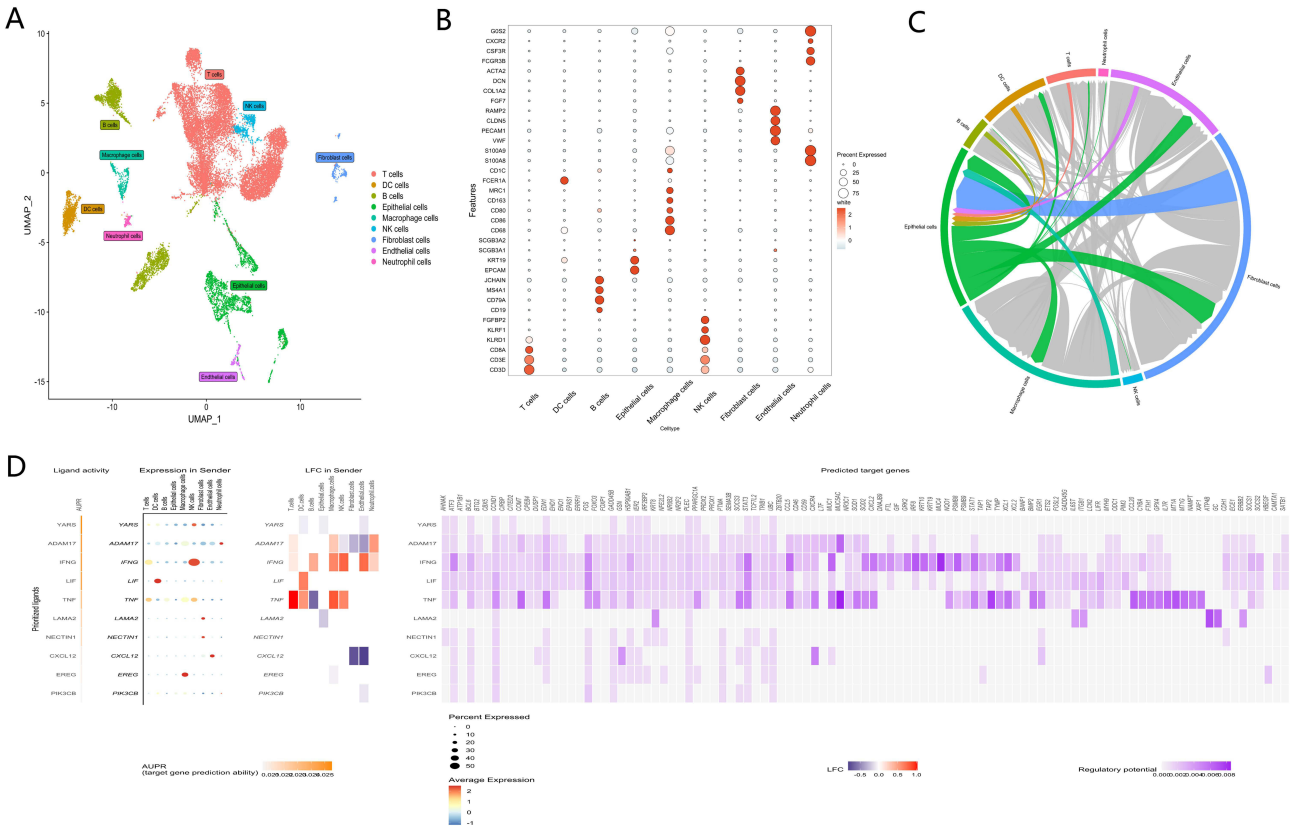

Figure 2 (**A**). GSE184198 USCC reannotation and cellular communication. After applying UMAP dimensionality reduction clustering to the data of the tumour group (12,968 cells) and the normal group (8500 cells), nine distinct cell subpopulations were identified, comprising T cells, NK cells, B cells, epithelial cells, DC cells, fibroblast cells, endothelial cells, neutrophils, and macrophage cells. (**B**). The way that B cell subpopulations' flag genes are expressed. (**C**). All cell subsets are in communication with epithelial cells. Darker in colour and possibly having the strongest communication are epithelial and fibroblast cells. (**D**). To target all cells, epithelial cells act as ligands by supplying paired target genes to cells. The relative up-regulation of LIF and IFNG in NK cells was depicted in the left figure. The logFC changes of IFNG and LIF in each cell subset were displayed in the middle figure. The target genes for IFNG were displayed in the right figure. LIF may be *PROX1 or EPAS1*, and the darker the colour, the greater the likelihood of higher.

transcription factors in cellular subpopulations is illustrated in Figure 3E. Notably, *PROX1* expression was relatively upregulated in epithelial cells, while *EPAS1* expression was upregulated in endothelial cells.

TCGA Database

PROX1 was upregulated in STAD, according to a pan-cancer analysis of the TCGA database (Figure 4A). Only in LAML was PROX1 found to be a risk factor (Figure 4B). In STAD, PROX1 and TMB showed a negative connection (Figure 4C). Furthermore, in STAD, PROX1 exhibited a strong positive connection with the immunological checkpoint CTLA4 (Figure 4D). The chemokines CCL24 and CXCL12 (Figure 5A) and the chemokine receptors CCR3 and CCR4 (Figure 5B) showed positive correlations with PROX1 in STAD. Additionally, PROX1 had robust positive associations with immunosuppressive factors ADORA2A, CD160, IL10, TGFBR1, KDR, and CSF1R (Figure 5C) and immunostimulatory factors CD276, PVR, TNFRSF25, ULBP1, CXCL12, and ENTPD1 (Figure 5D). Analysis of individual tumors showed that PROX1 was substantially expressed in STAD (Figure 6A). There was no discernible difference in PROX1 expression between TNM phases (Figure 6B). Regarding age and sex, PROX1 expression did not significantly change (Figure 6C and D). Co-expression analysis, GSEA GO/KEGG analysis in STAD, and heatmap analysis of differential genes in PROX1 high and low expression groups showed that PROX1-negative co-expressed genes were linked to T cell functions, while PROX1-positive co-expressed genes were primarily associated with the Wnt signaling pathway (Figure 7A and B). It was discovered that PROX1 downregulates pathways involved in Th17 cell development and inhibits T cell proliferation (Figure 7C and D). EPAS1 was increased in STAD, according to a pan-cancer analysis of the

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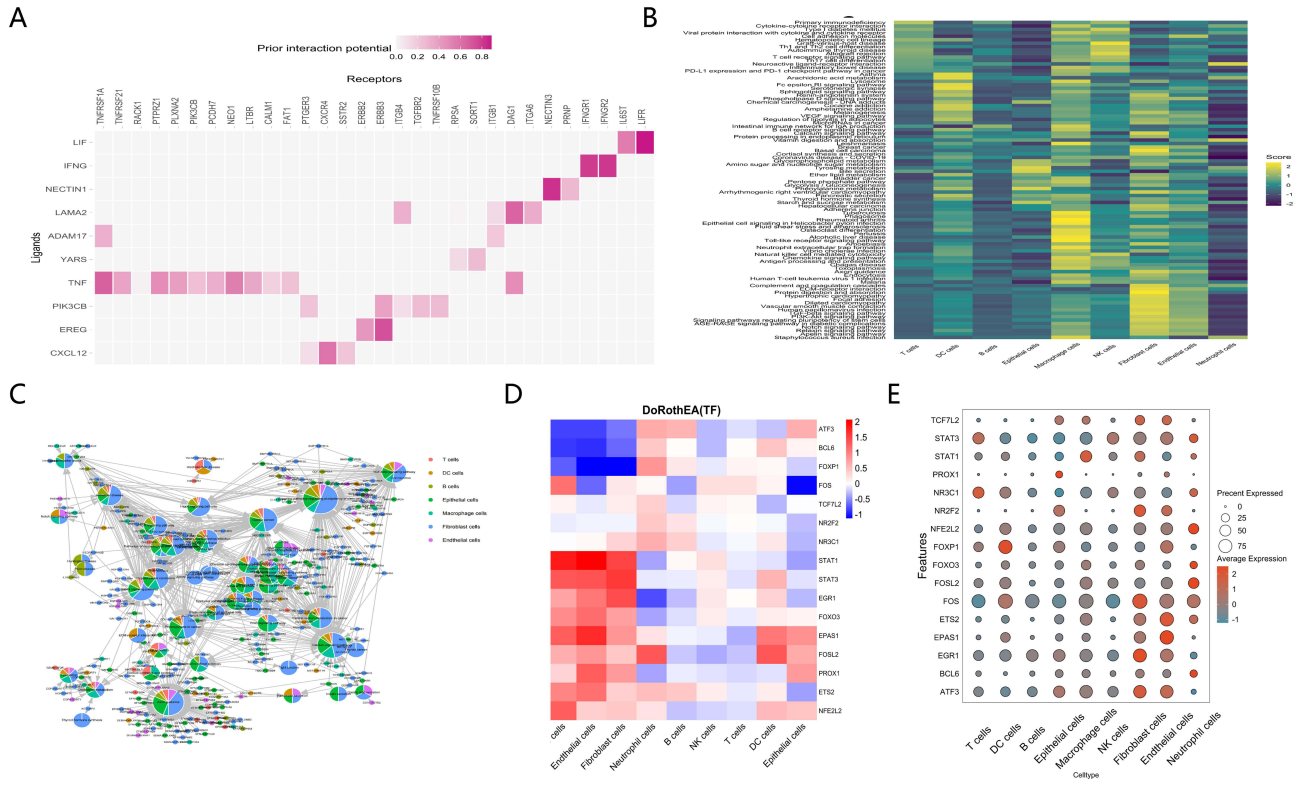

Figure 3 Screened ligands and receptors, upstream active signaling pathways, transcription factors and their expression in single-cell data, and signaling pathways of all cell subsets in the tumour group are examples of cell subset communication. (**A**). Ligand-targeting epithelial cells for all receptor cell pairings. (**B**). PI3K-AKT was comparatively downregulated in epithelial cells and elevated in fibroblast cells in all cellular Top15 signaling pathways. (**C**). Upstream signal transduction molecules The signaling pathways that control stem cell pluripotency were more strongly regulated in epithelial cells. (**D**). It was projected that nine transcription factors, the more active ones being *PROX1* and *EPAS1*, would intersect with the paired target genes of epithelial cells targeting all cells. (**E**). Transcription factor expression within cell subsets. *PROX1* was strongly expressed in epithelial cells, and *EPAS1* was substantially expressed in endothelial cells. The darker circles indicate higher expression, and the size indicates the percentage of expression.

TCGA database (Figure 8A). One risk factor for STAD was found to be EPAS1 (Figure 8B). In STAD, EPAS1 and TMB showed a negative connection (Figure 8C). In STAD, EPAS1 also demonstrated a strong positive association with the immunological checkpoints PDCD1LG2 and CTLA4 (Figure 8D). In STAD, EPAS1 was positively correlated with the chemokines CCL24 and CXCL12 (Figure 9A), and with the chemokine receptors CCR3 and CCR4 (Figure 9B). Additionally, EPAS1 showed robust positive associations with immunosuppressive factors ADORA2A, CD160, IL10, TGFBR1 and KDR and CSF1R (Figure 9C) and immunostimulatory factors CD276, PVR, TNFRSF25, ULBP1, CXCL12 and ENTPD1 (Figure 9D). EPAS1 was shown to be substantially expressed in STAD by individual tumor analysis (Figure 10A). The expression of EPAS1 varied between stages 1 and 3 (Figure 10B). Neither sex nor age significantly affected the expression of EPAS1 (Figure 10C and D). Co-expression analysis, GSEA GO/KEGG analysis, and heatmap analysis of high and low expression groups for EPAS1 showed that EPAS1-negative co-expressed genes were linked to base synthesis, while EPAS1-positive co-expressed genes were mainly linked to endothelial cell differentiation (Figure 11A and B). Additionally, it was discovered that EPAS1 controls the ECM-receptor interaction pathway in STAD (Figure 11C and D).

Analysis of RT-qPCR and IHC Detection Data

PROX1 and EPAS1 mRNA expression levels were substantially greater in AGS compared to GSE-1 (Figure 12A and B). From Kunming Medical University's First Affiliated Hospital, 46 paraffin-embedded tissue samples of GC and surrounding tissues were gathered. IHC detection of the PROX1 and EPAS1 proteins revealed that they were up-regulated in GC tissues (Figure 12C and D). Both the cytoplasm and the nucleus exhibited PROX1 and EPAS1 (Figure 12E and F).

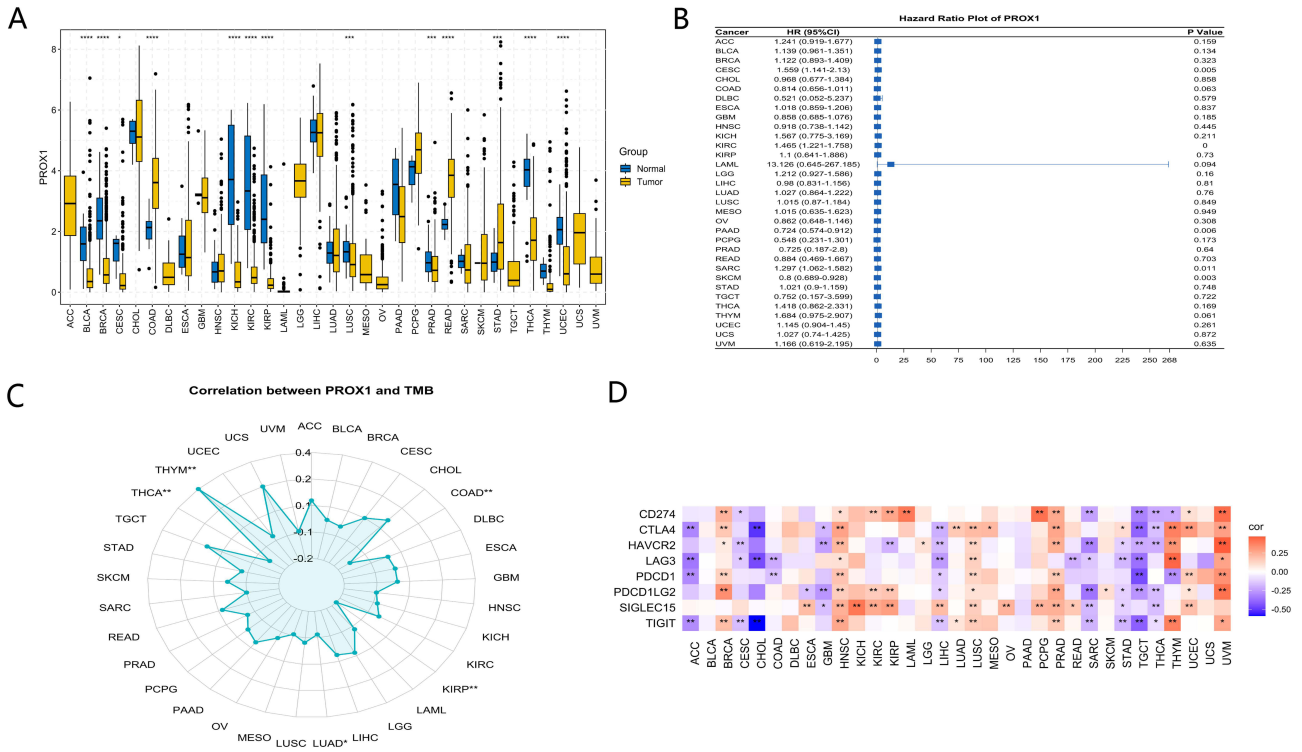

Figure 4 The relationship between *PROX1* expression in tumours and tumour mutation load (TMB), as well as the relationship between immunological checkpoints and COX risk forest graph analysis of risk variables. (**A**). STAD showed upregulation of *PROX1*. (**B**). *PROX1* was unique to LAML as a risk factor. (**C**). In STAD, *PROX1* had a negative correlation with TMB. (**D**). In STAD, there was a positive correlation between *PROX1* and CTLA4. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 2 displays the correlation between the expressions of PROX1 and EPAS1 with the TNM stage and differentiation degree of GC patients ($P < 0.05$).

Clinical Follow-Up

In GC patients, the elevated group of PROX1 and EPAS1 had a noticeably worse prognosis than the downregulated group (Figure 12G and H).

Discussion

GC is currently one of the most common cancers globally, characterised by high mortality rates and poor prognosis, with an average 5-year survival rate of less than 20%.[15] Key risk factors for GC include Helicobacter pylori infection, alcohol consumption, tobacco use, a high-sodium diet, and excessive meat intake. Moreover, GC is often diagnosed at an advanced stage.[16] The pronounced heterogeneity of GC frequently leads to secondary drug resistance during clinical treatment.[17] This heterogeneity largely arises from dynamic alterations within the TME.[18] Consequently, there is an urgent need for novel detection techniques and analytical methods to thoroughly investigate changes in the GC microenvironment, which could facilitate the development of innovative therapeutic strategies.

Currently, traditional sequencing methods in GC research, such as transcriptome sequencing, involve extracting mRNA from organs, tumour tissues or cell populations for subsequent analysis. However, these methods often aggregate data from multiple cell types, failing to accurately capture the heterogeneous gene expression profiles within GC.[19,20] This limitation significantly hampers effective diagnosis, treatment and prognosis. Single-cell sequencing technology offers a comprehensive understanding of GC's mechanisms at the cellular, genetic and molecular levels. Moreover, it has the potential to enhance GC diagnosis, facilitate personalised treatment strategies and improve prognosis evaluation. In this study, single-cell sequencing data were analysed to explore the heterogeneity of GC epithelial cells. *PROX1* and

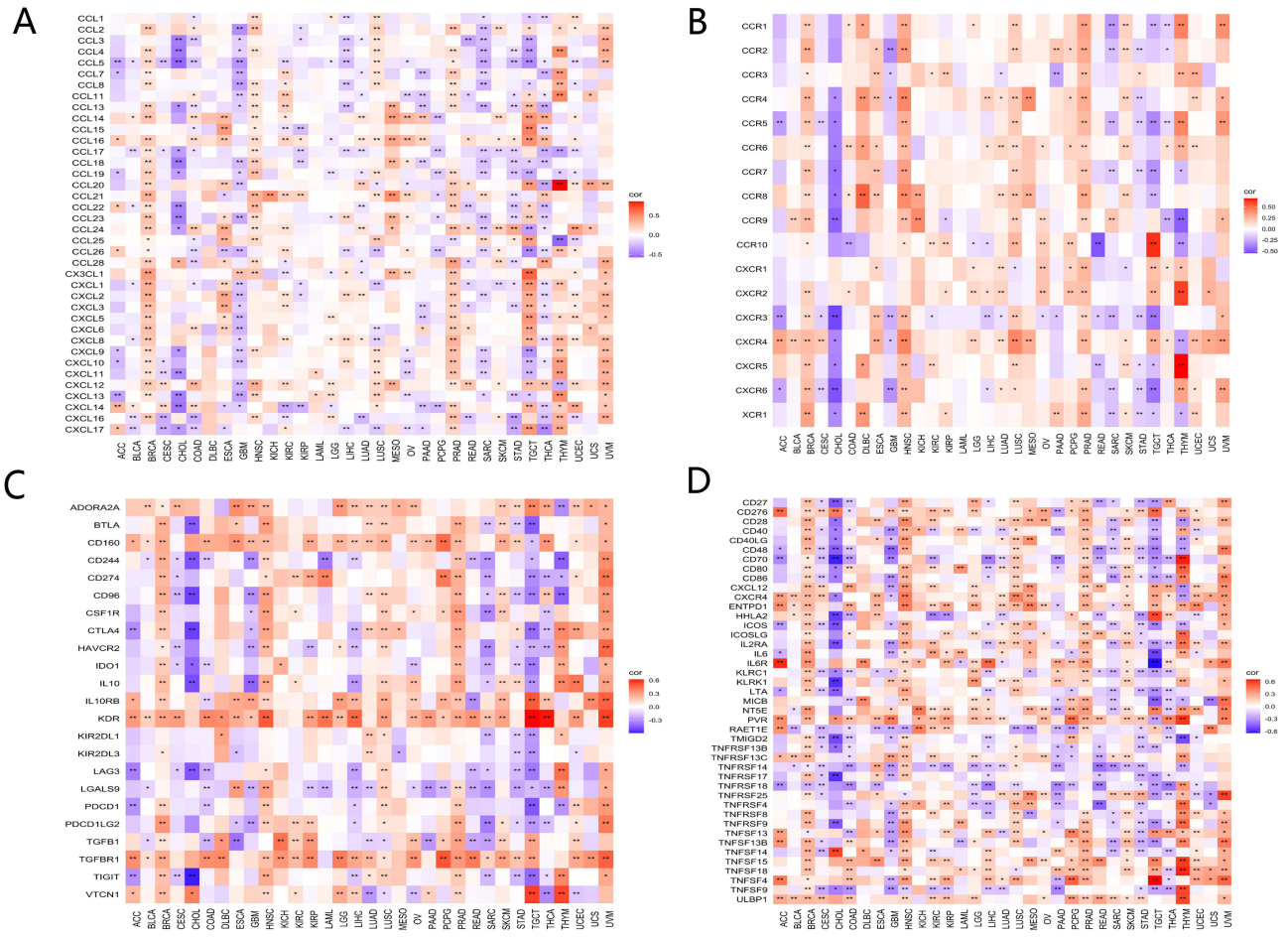

Figure 5 The relationship between *PROX1* and immunosuppressive, immune-stimulating, chemokine receptor, and chemokine in malignancies. (**A**). PORX1 and the chemokine CCL24 exhibited a strong positive connection. (**B**). PORX1 and chemokine CCR3 exhibited a strong positive connection. (**C**). PORX1 shown a strong positive connection with TGFBR1, CD160, IL10, ADORA2A, and other immunosuppressive variables. (**D**). PORX1 and immune-stimulating factors (CD276, PVR, TNFRSF25, and ULB1) exhibited a strong positive connection. * $p < 0.05$; ** $p < 0.01$.

EPAS1 were identified as central regulatory transcription factors crucial to GC epithelial cells. The study also examined the upstream regulatory molecules of *PROX1* and *EPAS1*, immune checkpoints and associated changes in the TME, including immune microenvironment and chemokines. Additionally, potential regulatory signalling pathways that may drive GC development were predicted. These findings provide valuable insights for the early diagnosis, precise treatment and prognosis evaluation of GC.

In our study, we initially re-annotated the single-cell sequencing data for GC from the GEO database, distinguishing between tumour and normal groups. This analysis identified nine cell subpopulations, which were visualised using somatotopic mapping to reveal distinct expression differences. Notably, epithelial cells and fibroblast cells exhibited the highest communication intensity. This finding is consistent with literature indicating that GC primarily originates from epithelial cells and is predominantly adenocarcinomatous. As GC progresses, it infiltrates deeper into the muscular layer,[21] supporting the observed strong communication between epithelial and fibroblast cells. Our analysis also highlighted two transcription factors, *PROX1* and *EPAS1*, which demonstrated high activity in epithelial and endothelial cells, respectively, were upregulated in STAD. *PROX1*, a member of the homeobox transcription factor family, has been implicated in cancer development as both a tumour suppressor and an oncogene. It is associated with various cancers, including gastrointestinal tract, haematological malignancies, breast cancer and brain tumors.[22–24] Given its role in cancer, *PROX1* could serve as a prognostic indicator and a molecular target for GC treatment. *EPAS1*, also known as

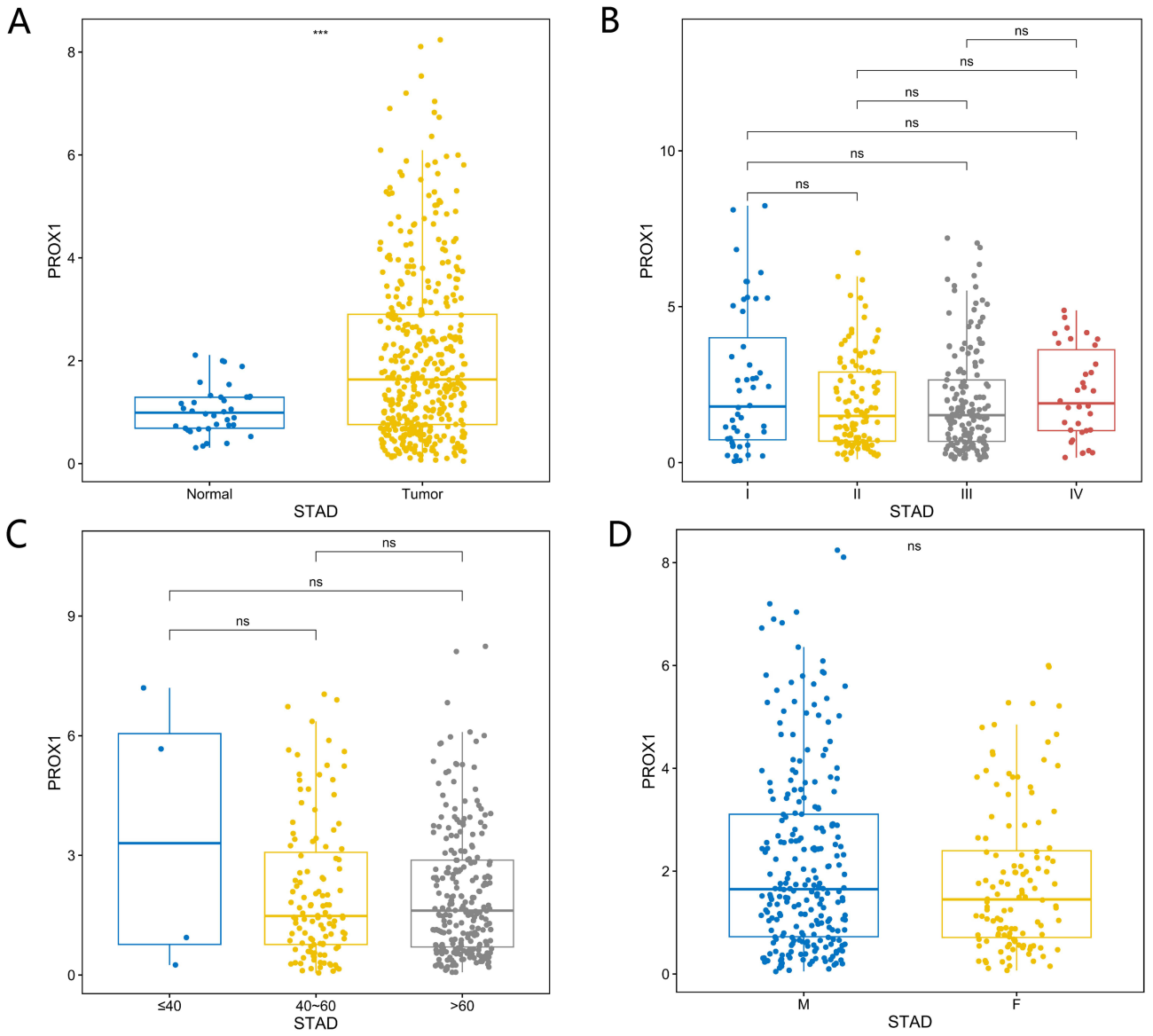

Figure 6 The *PROX1* expression in STAD varies by grade, age, gender, and tumour versus normal group. (**A**). In the tumour group, PORX1 expression was elevated. (**B**). There was no variation in *PROX1* expression between grades. (**C**). There was no variation in *PROX1* expression across the three age groups. (**D**). There was no variation in *PROX1* expression between genders. *** $p < 0.001$.

hypoxia-inducible factor-2 alpha (HIF-2α), is a transcription factor involved in several cellular pathways.[25] Despite its importance, the expression and function of *EPAS1* in GC are not well-studied, and its mechanisms remain poorly defined. Notably, it is commonly found in mammals and plays a central role in the hypoxic response.[26] Several studies have reported that under hypoxic conditions, *EPAS1* can upregulate the transcription of its downstream target genes such as VEGF and glycolytic enzyme genes, thereby promoting tumour angiogenesis and energy metabolism, ultimately driving tumour progression in endometrial cancer, ovarian cancer, breast cancer, bladder cancer, liver cancer, renal cell carcinoma and prostate cancer.[27] However, limited research has been conducted on the expression level and functional significance of *EPAS1* in GC, particularly lacking a comprehensive understanding of the underlying mechanisms involved. In our subsequent cell communication analysis of the GC group and the paraneoplastic control group, we identified that IFNG and LIF may target *PROX1* and *EPAS1*, respectively, potentially influencing GC progression This finding provides a theoretical foundation for further exploration of the specific mechanisms through which *PROX1* and

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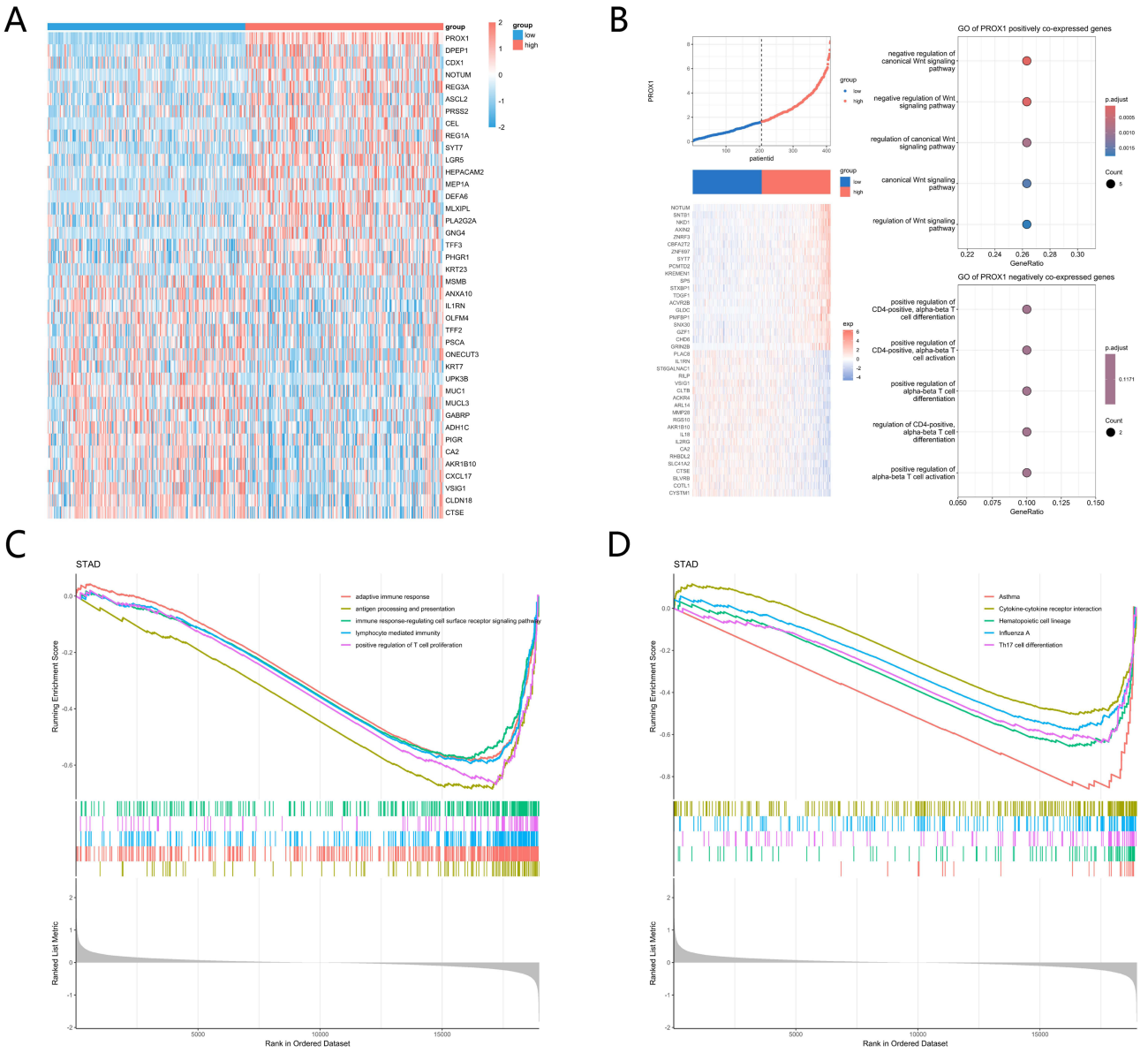

Figure 7 Co-expression analysis, GO/KEGG analysis, and heat map of differentially expressed genes between high and low expression groups of *PROX1* in STAD. (**A**) heat map showing the genes that differ between *PROX1* expression groups with high and low levels in STAD. (**B**). Co-expression module gene analysis in *PROX1* and GO analysis positive and negative expression groups. (**C**). The top 5 GO analysis of GSEA in STAD includes *PROX1*. (**D**). *PROX1*, ranked among the top 5 in STAD's GSEA KEGG analysis.

EPAS1 as regulated by these upstream molecules in GC. The IFNG gene, located on chromosome 12q15, encodes interferon-gamma (IFN-γ), a cytokine crucial for the immune response to viral and bacterial infections.[28] Masuko Katoh et al have shown that IFNG influences cancer stemness and promotes tumorigenesis by modulating the Wnt signalling pathway.[29] Xin Chen et al demonstrated that IFNG can inhibit tumour progression by inducing ferroptosis.[30] Mark Ayers et al highlighted that IFN-γ is a key driver of programmed death ligand-1 (PD-L1) expression in cancer and host cells, playing a significant role in tumour immunotherapy.[31] Pau Morey et al reported that *Helicobacter pylori* infection in GC cells can dimmish IFNG signalling by disrupting lipid rafts, thus reducing inflammatory responses.[32] However, there are no current reports on whether IFNG targets *PROX1* and its effect on GC, necessitating further investigation. LIF is a cytokine with broad biological functions, influencing tumour development, immune evasion and chemotherapy

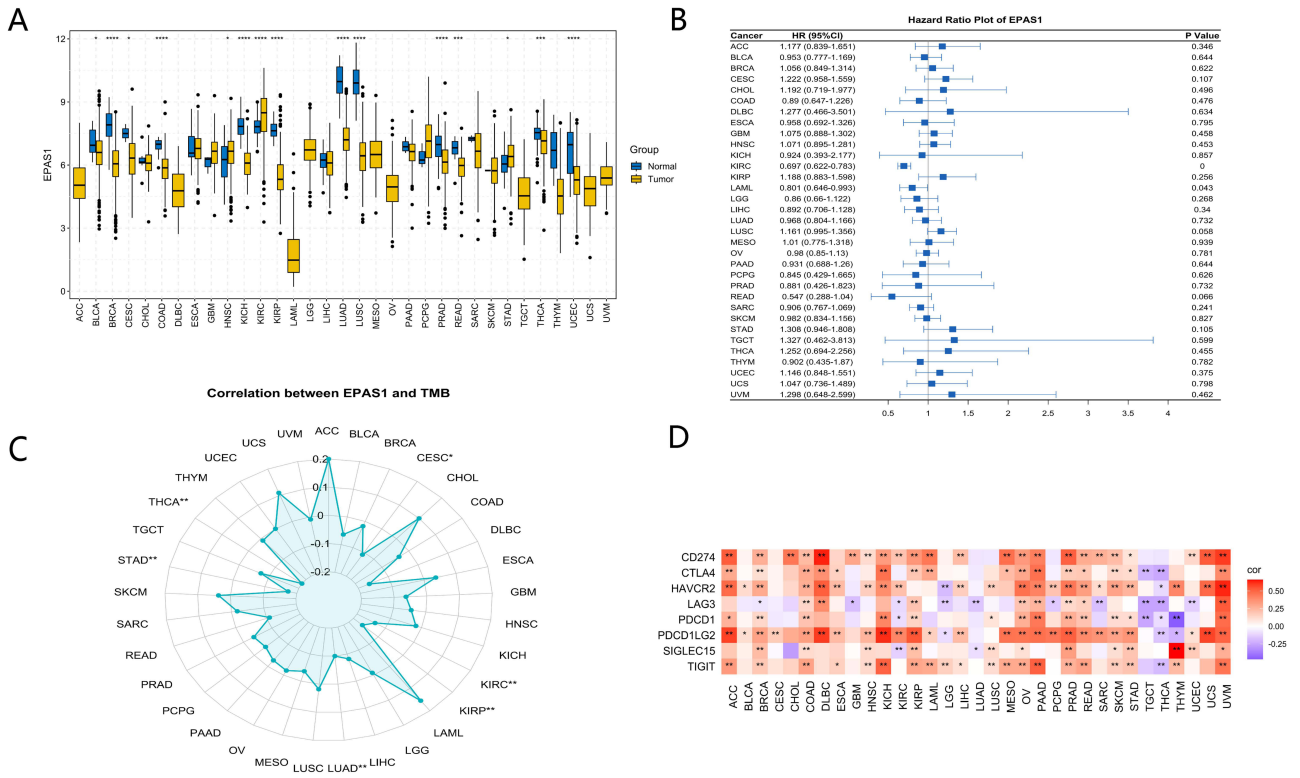

Figure 8 The relationship between TMB, COX risk forest plots, immunological checkpoints, and *EPAS1* expression in cancers. (**A**). STAD has elevated *EPAS1* levels. (**B**). One risk factor for STAD was *EPAS1*. (**C**). TMB and *EPAS1* had a negative correlation in STAD. (**D**). *EPAS1* and PDCD1LG2 in STAD had a good correlation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

resistance.[33–35] Jun Zhang et al demonstrated that LIF promotes the formation of TME and contributes to the occurrence of GC.[36] Cristina Di Giorgio et al reported that LIF regulates fibroblast growth factor receptor 4 transcription in GC, thereby facilitating GC progression.[37] However, whether LIF regulates *EPAS1* and its impact on GC remains unreported and requires further study.

TMB refers to the number and frequency of gene mutations in tumour cells, reflecting genomic instability and tumour evolution. It serves as an indicator of tumour malignancy, development rate and treatment sensitivity.[38] High TMB is generally associated with better responses to immunotherapy or targeted therapy.[39,40] Our analysis revealed that both *PROX1* and *EPAS1* were negatively correlated with TMB in STAD, suggesting that they may play an inhibitory role in the individualised treatment strategies for patients with GC.

TME encompasses the surrounding microenvironment in which tumour cells reside, including adjacent blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, various signalling molecules and the extracellular matrix.[8] The TME is a complex and dynamic environment crucial for tumour survival and progression, with immune cells and their regulatory mechanisms playing a significant role in tumour development. Therefore, understanding the interplay between TME and tumour metastasis and elucidating the molecular mechanisms of different factors within the microenvironment are essential for developing strategies to inhibit tumour metastasis. In this study, we examined the immune microenvironment and changes in chemokines and their receptors in the GC microenvironment. Our analysis revealed that *PROX1* and *EPAS1* were positively correlated with the immune checkpoints CTLA4 and PDCD1LG2, respectively. Additionally, they exhibited a strong positive correlation with various immunosuppressive factors, including ADORA2A, CD160, IL10, TGFBR1, KDR and CSF1R. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), also known as CD152,

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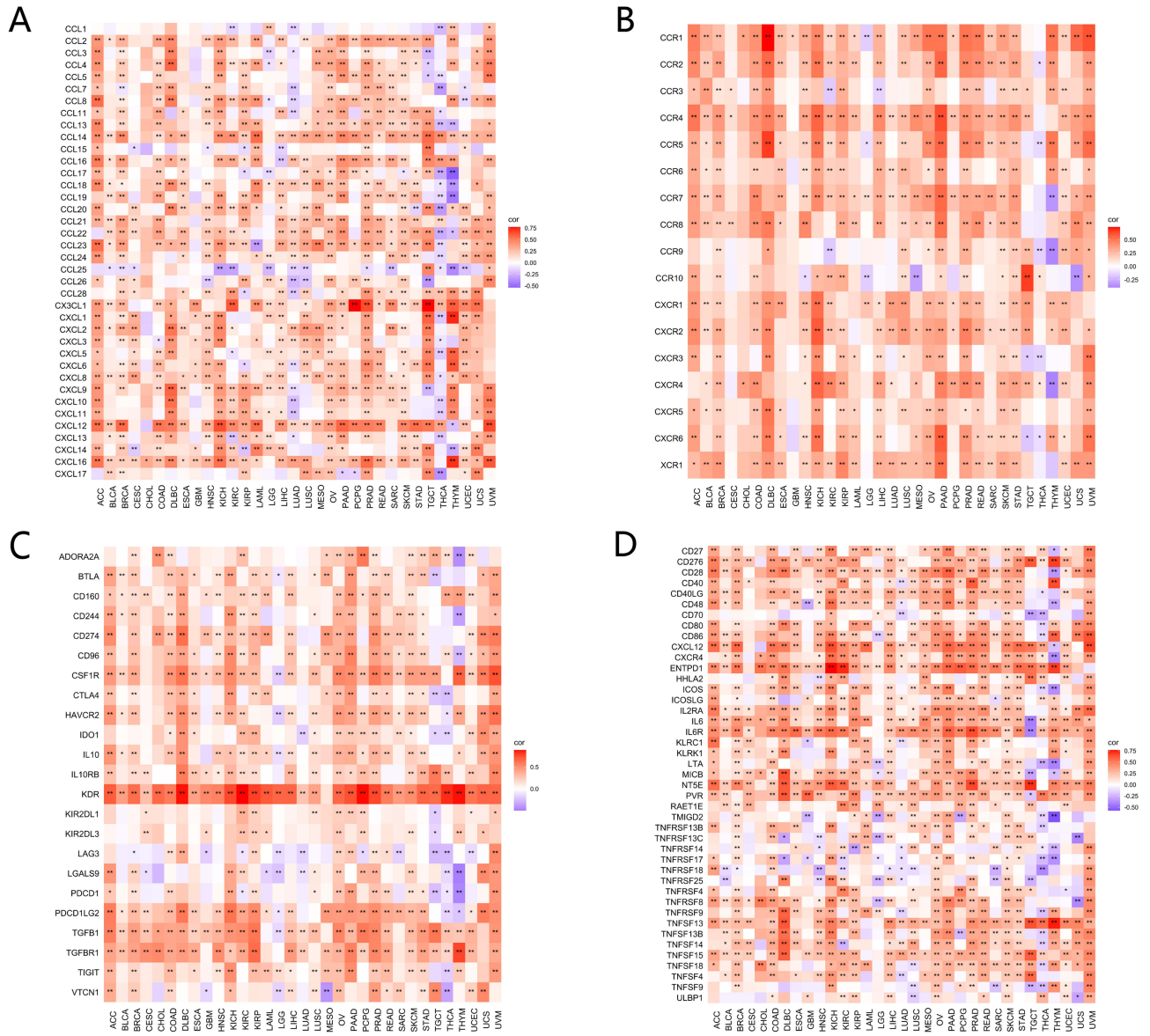

Figure 9 The study examined the relationship between *EPAS1* and immunosuppressive, immune-stimulating, chemokine receptor, and chemokine in malignancies. (**A**). *EPAS1* and the chemokine CXCL12 exhibited a strong positive connection. (**B**). *EPAS1* and the chemokine receptor CCR4 exhibited a strong positive connection. (**C**). KDR and CSF1R showed a favorable correlation with *EPAS1*. (**D**).CXCL12 and ENTPD1 showed a good correlation with *EPAS1*. * $p < 0.05$; ** $p < 0.01$.

is a transmembrane receptor on T cells that binds to the B7 molecule to induce T cell unresponsiveness and negatively regulates immune responses.[41] Programmed cell death 1 ligand 2 (PD-L2), also known as B7-DC or CD273, negatively regulates tumour-adapted immune responses by bindings to PD-1 and inhibiting TCR/BCR-mediated immune cell activation, thereby contributing to immune tolerance and autoimmunity.[42] Our findings indicate that *PROX1* and *EPAS1* are positively correlated with both CTLA-4 and PD-L2, suggesting that these transcription factors may play a role in negatively regulating immune responses in GC, which could impact the effectiveness of immunotherapy for patients with GC. Chemokines are small cytokines or signalling proteins that induce directed chemotaxis in nearby cells,[43] guiding cell migration toward the source of increasing chemokine concentrations. They are crucial for immune surveillance, ontogeny, pathogenic microbial infection, inflammation and tumour treatment.[44,45] We observed that *PROX1* and *EPAS1* were positively correlated with the chemokine receptors CCR3 and CCR4, respectively. CCR3 and CCR4 are proteins associated with the C-C chemokine

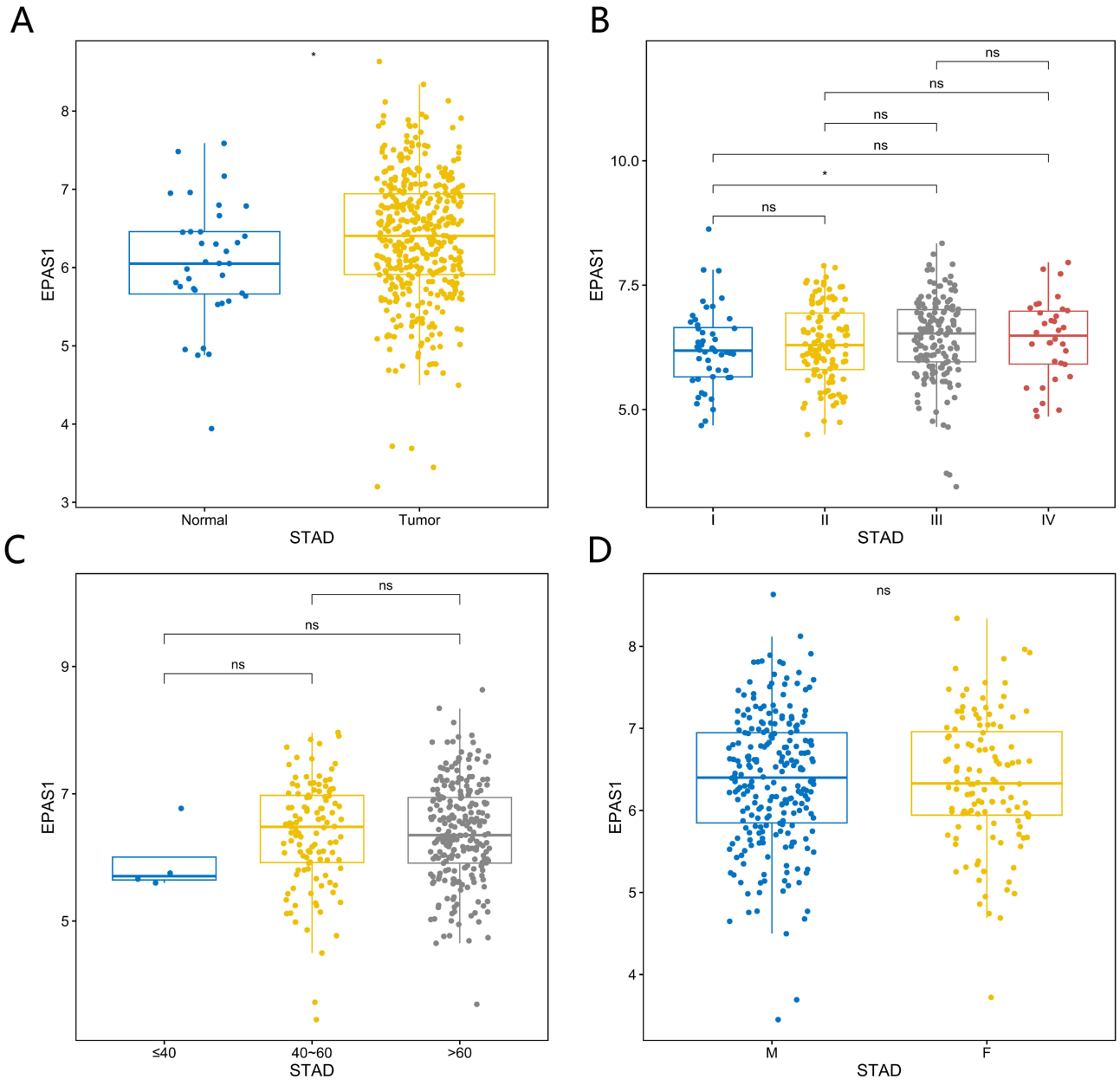

Figure 10 *EPAS1* expression varies by grade, age, gender, and tumour versus normal group in STAD. (**A**). Tumour groups showed up-regulated *EPAS1* expression. (**B**). The way that *EPAS1* was expressed in each grade was the same. (**C**). The three age groups' expressions of *EPAS1* were identical to one another. (**D**). The *EPAS1* expressed in various genders did not differ from one another. * $p < 0.05$.

receptor and play vital roles in cell signalling and immune response. They may all be involved, together with their ligands, in the polarization, activation and regulation of immune cells and immune responses, leading to immune system dysregulation and autoimmune diseases. Moreover, these receptors along with their ligands are implicated in the migration and metastasis of tumour cells.[46–48] Therefore, *PROX1* and *EPAS1* may positively regulate CCR3 and CCR4, thereby potentially inhibiting immune responses and promoting GC progression.

To further investigate the carcinogenic mechanism of *PROX1* and *EPAS1* in GC, we conducted GSEA for GO and KEGG pathways. Our analysis revealed that *PROX1* upregulates the Wnt signalling pathway, while *EPAS1* upregulates

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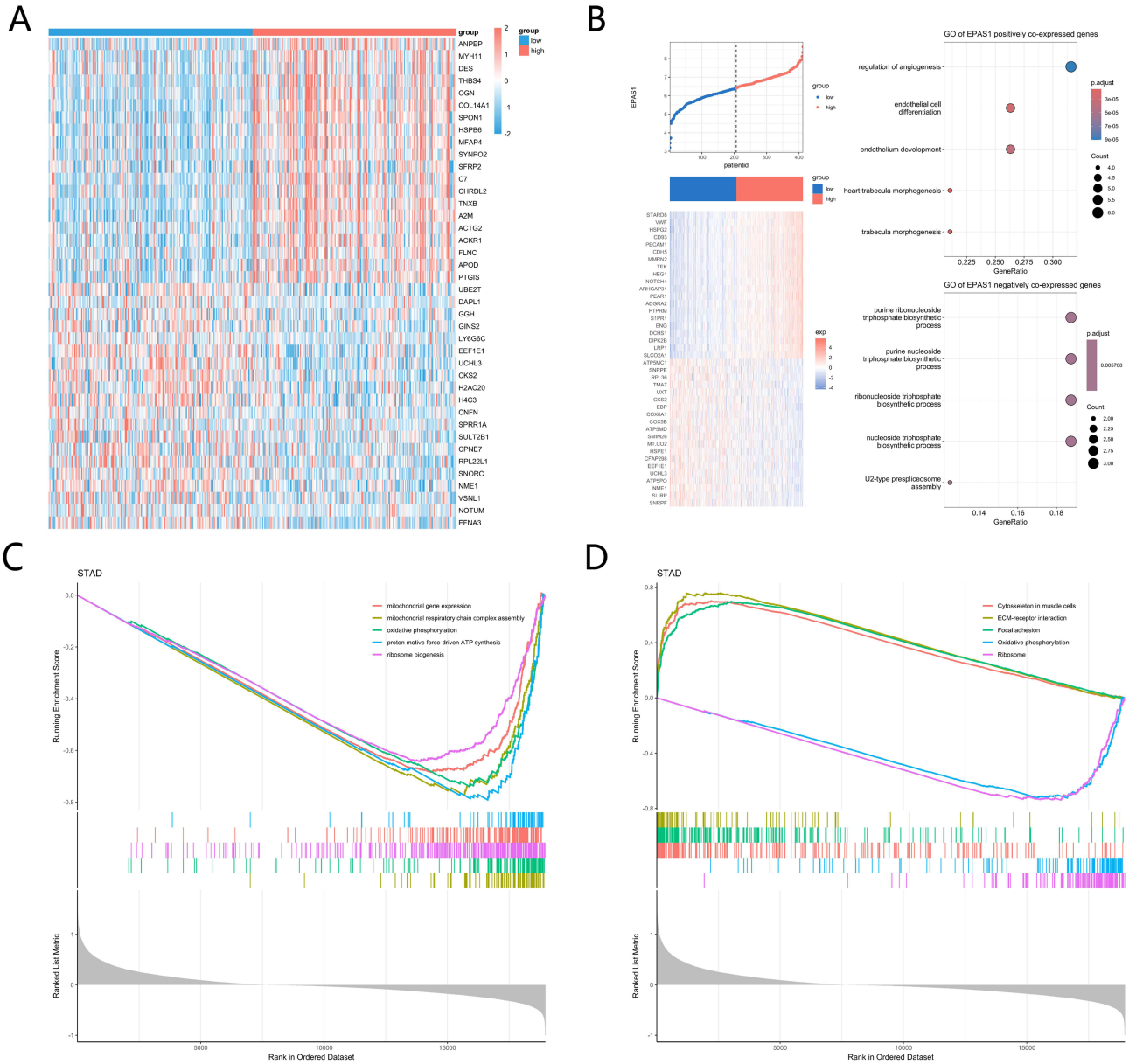

Figure 11 GSEA GO/KEGG analysis in STAD, co-expression analysis, and a differential gene heatmap of *EPAS1* between high and low expression groups. (**A**). The heat map showing the genes that differ in *EPAS1* expression between STAD groups with high and low expression. (**B**). GO analysis and the co-expression module genes of the *EPAS1* positive and negative expression groups. (**C**). STAD contained the top five GO of *EPAS1* that were examined by GSEA. (**D**). STAD contained the top five KEGG of *EPAS1* that were examined by GSEA.

the ECM-receptor interaction pathway. The Wnt signalling pathway is crucial for embryonic development and organogenesis, influencing cell proliferation, differentiation, polarisation, migration and apoptosis.[49] In tumour research, aberrant activation of the Wnt/β-catenin pathway is well-documented in the development of various cancers, such as colon, lung and GCs.[50,51] The ECM-receptor interaction pathway plays a significant role in tumour cell proliferation and metastasis. Increased expression of ECM components in tumour tissues can facilitate EMT, which promotes tumour invasion and metastasis.[52,53] Based on these findings, we hypothesise that *PROX1* and *EPAS1* may contribute to GC

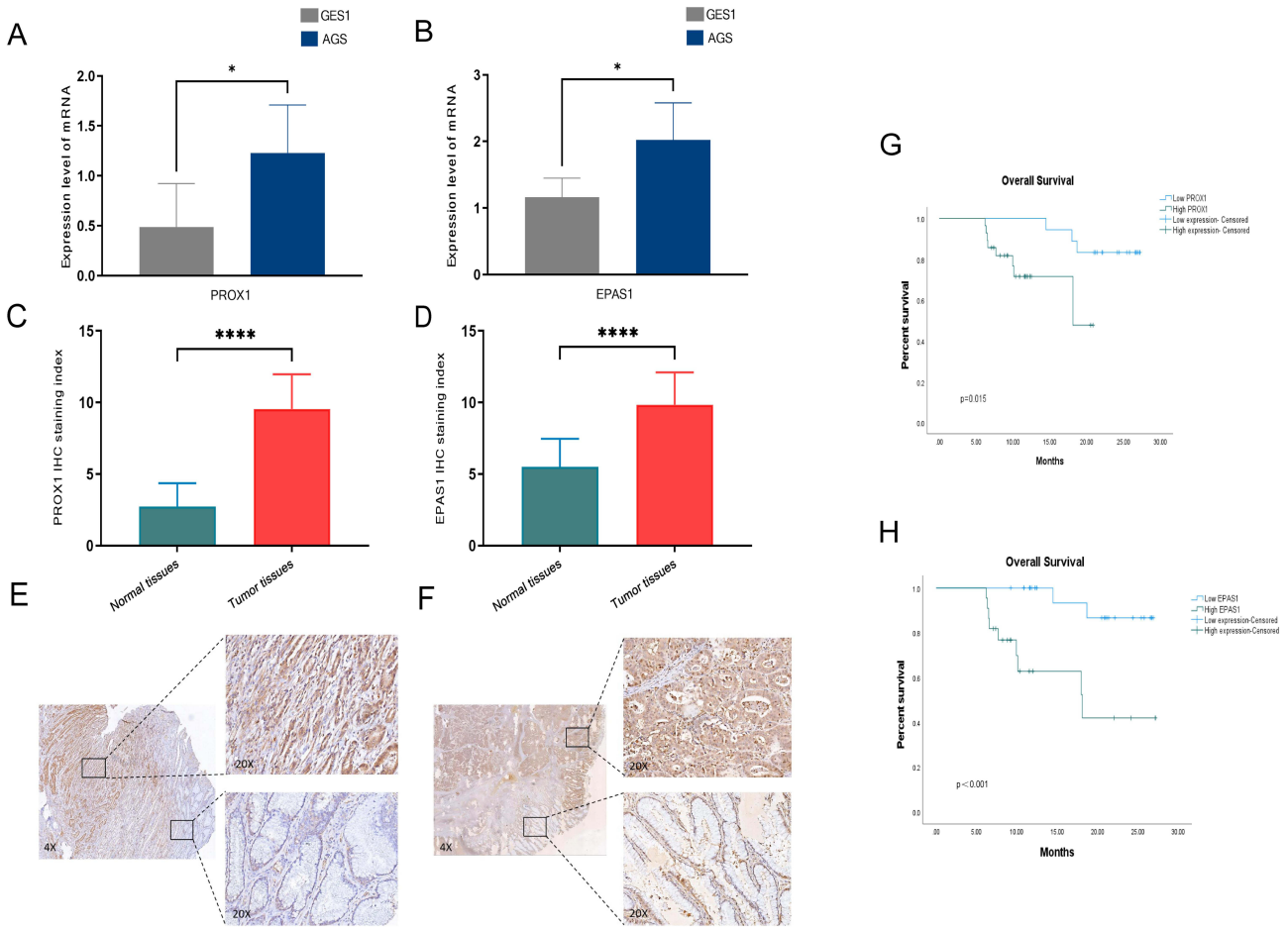

Figure 12 Detection of *PROX1* and *EPAS1* expression levels and prognostic analysis in gastric cancer. (**A**). The mRNA expression of *PROX1* in AGS and GES-1. (**B**). The mRNA expression of *EPAS1* in AGS and GES-1. (**C**). The protein expression of PROX1 in GC and adjacent tissues. (**D**). The protein expression of EPAS1 in GC and adjacent tissues. (**E**). The localization of protein expression of PROX1. (**F**). The localization of protein expression of EPAS1. (**G**).Prognostic analysis of high and low PROX1 expression group in GC. (**H**). Prognostic analysis of high and low *EPAS1* expression group in GC. * $p < 0.05$; **** $p < 0.0001$.

progression by inhibiting the immune response and modifying the TME through the mediation of the Wnt and ECM-receptor interaction signalling pathways, respectively.

Finally, we evaluated the expression levels of *PROX1* and *EPAS1* using RT-qPCR and IHC. Our results demonstrated that both *PROX1* and *EPAS1* were upregulated in GC and positively correlated with clinicopathological characteristics

Table 2 Correlation of Protein Expression Levels of PROX1 and EPAS1 with Clinicopathological Characteristics of GC Patients

Characteristics	N	PROX1		X^2	*P*	EPAS1		X^2	*P*
		Low Expression	High Expression			Low Expression	High Expression		
Gender									
Male	30	9	21	3.019	0.082	14	16	1.048	0.306
Female	16	9	7			10	6		
Age (years)									
≤60	13	4	9	0.532	0.466	8	5	0.637	0.425
>60	33	14	19			16	17		

(*Continued*)

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Table 2 (Continued).

Characteristics	N	PROX1		X^2	*P*	EPAS1		X^2	*P*
		Low Expression	High Expression			Low Expression	High Expression		
Degree of differentiation									
Well & moderately	20	13	7	9.942	0.002	15	5	7.389	0.007
Low	26	5	21			9	17		
Clinical stage									
Stage I & Stage II	19	11	8	4.785	0.029	17	2	18.049	<0.001
Stage III & Stage IV	27	7	20			7	20		
Lymph node metastasis									
No	24	8	16	0.708	0.400	11	13	0.809	0.369
Yes	22	10	12			13	9		

(degree of differentiation and TNM). In addition, our clinical follow-up of GC patients further confirmed that PROX1 and EPAS1 were associated with poor prognosis in GC patients. These findings support our bioinformatics analysis and provide a solid foundation for further exploration of the molecular mechanisms underlying GC.

Limitations

This study did not investigate the molecular mechanisms through in vitro and in vivo experiments. Further research is warranted to explore these aspects.

Conclusions

Our analysis of single-cell sequencing data from the GEO and STAD database identified *PROX1* and *EPAS1* as potential central regulatory transcription factors in the epithelial cells of the GC microenvironment. These factors may be regulated by IFNG and LIF. Additionally, *PROX1* and *EPAS1* appear to influence the immune response and modulate chemokine receptors by mediating the Wnt and ECM-receptor interaction signalling pathways, respectively. This regulation alters the GC microenvironment and may contribute to the progression of GC. These results provide a vital theoretical framework for our later clinical investigation and experimental validation of the underlying biological process.

Ethical Approval

This study involving human data was reviewed and approved by the ethics committee of the First Affiliated Hospital of Kunming Medical University [(2024) Ethics L No. 136] and was performed in compliance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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