Article

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Identification of PROZ as a Cancer-related Gene in Hepatocellular Carcinoma under Hypoxia Condition

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Background: Hepatocellular carcinoma (HCC) is a common human malignancy. In recent years, the study of biomarkers for HCC progression has become a hot topic. This study focused on identifying the key gene protein Z (PROZ) associated with the tumorigenesis of HCC by bioinformatics methods, and exploring its function and regulatory mechanism.
Methods: Differentially expressed genes (DEGs) in HCC cells under hypoxia condition and normoxia condition were analyzed with the data of GSE15366 and GSE41666. The genes associated with the prognosis of HCC patients were analyzed in the Human Protein Atlas (HPA) database and intersected with hypoxia-related DEGs in HCC to obtain key genes. Gene Expression Profiling Interactive Analysis database was used to analyze the relationship between expressions of the above key genes in HCC and the prognosis of patients. The mRNA expression level of PROZ was analyzed by qPCR. Immunohistochemical staining results of PROZ in HCC were obtained from the HPA database. The cell viability and apoptosis were testified by CCK-8 and TUNEL methods. The glucose consumption and lactic acid production of cells were also detected. LinkedOmics database was used to analyze the relevant signaling pathways regulated by PROZ in HCC. Western blot was adopted to detect expressions of Hexokinase 2 (HK2) protein, cyclin D1 (cyclin B1) and Notch signaling pathway-related proteins including notch receptor 1 (Notch1) and Hes family BHLH transcription factor 1 (Hes1).
Results: PROZ was greatly down-regulated in HCC cells (HepG2 and Hep3B) in hypoxia condition. PROZ overexpression in HepG2 cell lines inhibited cell proliferation and glycolysis, and promoted apoptosis; PROZ knockdown worked oppositely. In terms of mechanism, PROZ inhibited the expression levels of cell cycle-related proteins, Notch1 and Hes1.
Conclusions: PROZ inhibits HCC cell proliferation and glycolysis, and promotes apoptosis by inhibiting cell cycle and Notch signaling pathways. PROZ could be a potential biomarker/therapy target for HCC.

Keywords: PROZ; hepatocellular carcinoma; notch

Introduction

Hepatocellular carcinoma (HCC) is a common type of primary liver cancer with high mortality, accounting for approximately 90% of liver cancer cases [1]. HCC ranks the third most common cause of cancer-related death worldwide, and epidemiological surveys show that the morbidity and the mortality of HCC have continued to rise over the last decades [2]. Hypoxia is a vital feature of the tumor microenvironment, which is resulted from excessive oxygen consumption and insufficient vascular supply due to rapid tumor growth [3]. Under hypoxic conditions, the expression of hypoxia-inducible factor 1 subunit alpha (HIF1A) is elevated. This facilitates the progression of HCC and increases the risk of metastasis and death [4]. Therefore, it is pivotal to explore new molecular mechanisms that mediate the progression of HCC under hypoxic microenvironment.

Microarray-based gene expression analysis is a common and powerful method that helps researchers evaluate genes' expression change with high-throughput approach [5]. It is a feasible and valuable method for screening out differentially expressed genes (DEGs) [6,7]. For example, a previous study analyzes hypoxia-related genes in HCC with the data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases, and constructs a prediction model which is associated HCC prognosis, and identifies three hypoxia-related genes (PDSS1, CDCA8 and SLC7A11) [8]. Protein Z (PROZ) is a vitamin K-dependent glycoprotein that participates in regulating anticoagulant process [9]. Many studies have reported that PROZ is associated with the pathogenesis of human diseases including malignancies, and may become their prognostic biomarkers, such as lung adenocarcinoma [10], ovarian cancer [11], colon cancer [12]. However, the biological function of PROZ and the underlying mechanism in HCC cells are not clear.

In this work, bioinformatics analysis highlighted that PROZ expression level was raised in hypoxia-induced HCC cells, and high PROZ expression predicted poor prognosis of HCC patients. In addition, cell cycle pathway and

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Notch signaling pathway are commonly dysregulated in tumorigenesis. The deregulation of cell cycle pathway is the main cause of unlimited proliferation of cancer cells [13]; Notch signaling pathway is an evolutionarily conservative signaling pathway, and its abnormal activation is intimately linked with tumor cell viability, differentiation, and death [14]. We also found, through bioinformatics analysis, high expression of PROZ was associated with the inhibition of cell cycle pathway and Notch signaling pathway. Additionally, we also investigated the effect of PROZ on the proliferation and apoptosis of hypoxia-caused HCC cells.

Materials and Methods

Bioinformatics Analysis

GSE15366 and GSE41666 were obtained from NCBI GEO database (https://www.ncbi.nlm.nih.gov/gds), with the key words "hypoxia" and "hepatocellular carcinoma" as the search terms. GSE15366 contains the gene expression profile data of HepG2 cells which were cultured in normoxic (20% O_2) or hypoxic (2% O_2) conditions for 72 h. GSE41666 contains the gene expression data of HepG2 cells which were cultured in normoxic (21% O_2) or anoxic (0% O_2) condition. The DEGs in the two datasets were analyzed with the GEO2R tool, with $|\text{Log}_2$ fold change (FC)$| > 1$, $p < 0.05$ as the screening criteria, respectively. The list of prognostic genes in HCC was obtained from Human Protein Atlas (HPA) database (https://www.proteinatlas.org).

Tissue Samples Collection

All samples (primary HCC tissues and the adjacent liver tissues) were from Renmin Hospital of Wuhan University. All samples were histopathologically diagnosed as primary HCC. All tissue samples were instantly stored in an ultra-low temperature refrigerator after removal from the tumor during surgery. Informed consent was obtained from the patients before surgery. The collection and use of human tissue samples were approved by the Ethics Committee of Renmin Hospital of Wuhan University (No. 202100045), and the workflow was in accordance with *Declaration of Helsinki*.

Cell Culture

HCC cell lines (HepG2, Hep3B) were available from ATCC (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Sigma) with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (All were from Gibco) at 37 °C in 5% CO_2. Hypoxic cell models were constructed when HepG2 and Hep3B cells were cultured under normoxic conditions to 60–70% confluences, followed by hypoxic conditions at 1% O_2 for 6, 12, and 24 h, respectively. Before the experiments, mycoplasma testing was performed to confirm that the cells were not contaminated.

Cell Transfection

PROZ overexpression plasmid (PROZ), blank plasmid (NC), small interfering RNA (siRNA) targeting PROZ (si-PROZ) and negative control siRNA (si-NC) were from Gene Pharma Co., Ltd. When cell confluences reached 70-80%, the above vectors/oligonucleotides were transfected into HCC cell lines by Lipofectamine^{TM} 2000 (Invitrogen). 24 h later, the transfection efficiency was verified by quantitative real-time polymerase chain reaction (qRT-PCR).

qRT-PCR

RNA was extracted from HCC cells by TRIzol reagent (Invitrogen), and reversely transcribed into cDNA according to the manufacturer's instructions of the reverse transcription kit (Shiga, Japan), and then qRT-PCR was conducted on an ABI 750 0FAST Real-Time PCR machine (Applied Biosystems) with the SYBR Green PCR Kit (TaKaRa). The results were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences: PROZ (forward, 5′-GCCCTCCATCGTGTGGAGCC-3′; reverse, 5′-TAAGCTTTTCCTGGACGCCTGTGC-3′); GAPDH (forward, 5′-TCAAGATCATCAGCAATGCC-3′, reverse, 5′-CGATACCAAAGTTGTCATGGA-3′).

Cell Counting Kit-8 (CCK-8) Assay

The proliferation of HepG2 and Hep3B cells was detected by a CCK-8 kit (Beyotime). The transfected cells were inoculated into 96-well plates at a density of 1×10^3 cells/well. According to the manufacturer's instruction, 10 μL of CCK-8 reagent was loaded per well after transfected cells were cultured for 12, 24, 48, and 72 h, respectively, and then the cells were incubated for 2 h. The wells only containing the CCK-8 agents and medium were set as the blank control wells. The optical density (OD) values at 450 nm wavelength per well were probed by a microplate reader (Molecular Devices). The viability of the cells was indicated by $OD_{450nm\ test\ well} - OD_{450nm\ blank\ control\ well}$.

Terminal Deoxyribonucleotide Transferase Mediated dUTP Nick End Labeling (TUNEL) Assay

The apoptosis of HepG2 and Hep3B cells was determined by TUNEL assay. The cells were inoculated on 6-well plates at a density of 3×10^5 cells/well. The apoptosis was probed using a TUNEL assay kit (Roche Diagnostics, Basel, Switzerland), followed by nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). The positive cells were observed and counted under a fluorescence microscope (Nikon), with the apoptosis rate calculated.

Glucose Consumption and Lactate Production

Transfected or non-transfected HepG2 and Hep3B cells were seeded into 6-well plates (at a density of 1×10^5 cells/well) and cultured overnight and then incubated in hypoxia or normoxia condition for 24 h. The detection of

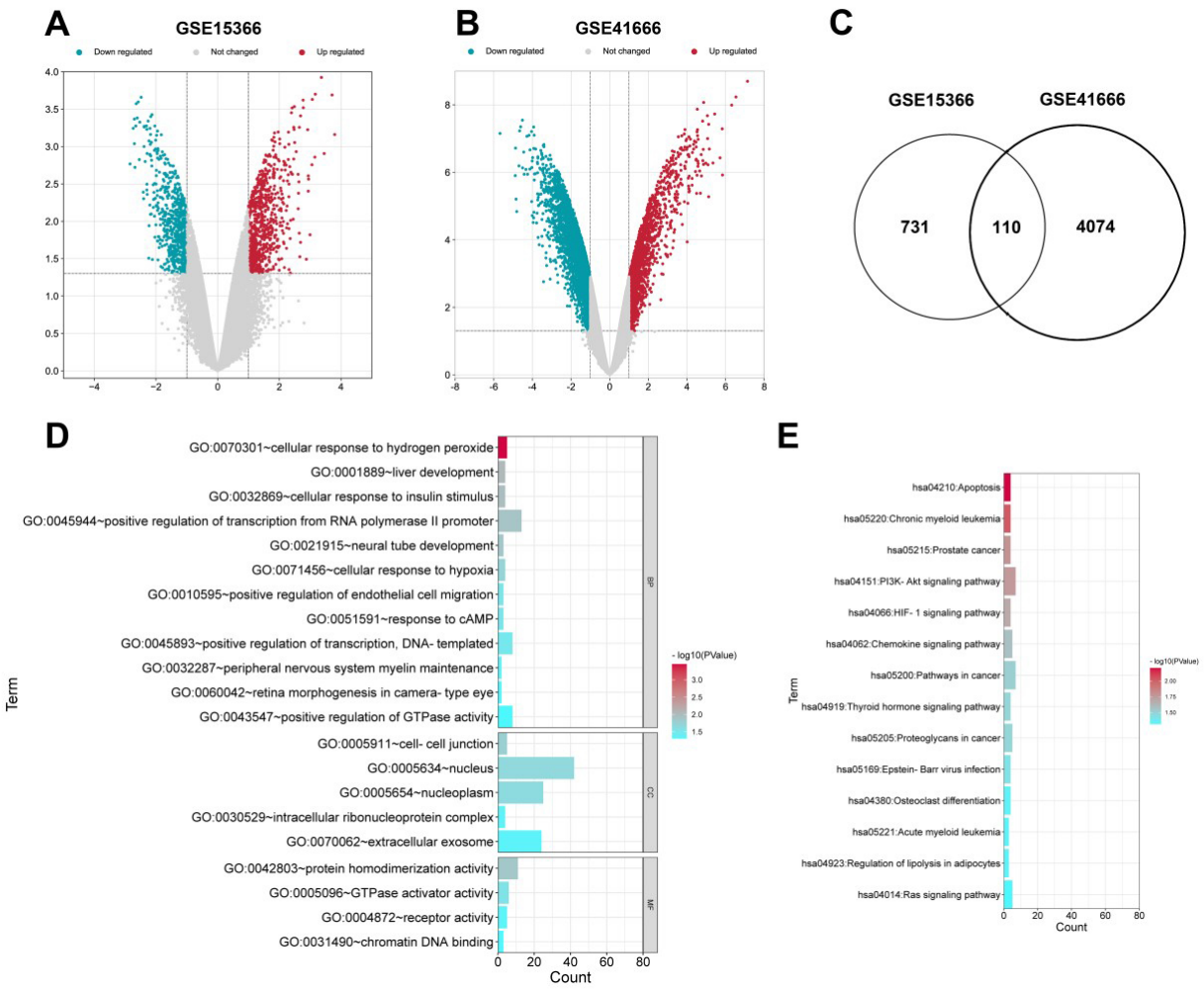

Fig. 1. Functional enrichment analysis of DEGs in HCC. (A,B) The GSE15366 and GSE41666 datasets were downloaded to show the DEGs in HCC cells under hypoxic and normoxic conditions. The red dots in the volcanic diagram indicated up-regulated genes, and the green dots reflected down-regulated genes. (C) The up-regulated genes in hypoxia-induced HCC cells from the above two datasets were screened out, with a Venn diagram drawn. (D) GO analysis was performed on the 110 common DEGs with DAVID database. (E) KEGG analysis was performed on the 110 common DEGs with DAVID database.

glucose consumption and lactate production was conducted with Glucose Assay Kit and Lactate Assay Kit (Sigma) respectively according to the protocols provided by the manufacturer. The relative levels of glucose consumption and lactate production in hypoxic groups were normalized to normoxia group.

Immunoblotting

The protein was extracted by RIPA lysis buffer (Roche) and quantified using a bicinchonic acid (BCA) kit (Pierce). The extracted protein was subjected to polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), which were then blocked with tris buffered saline tween (TBST) containing 5% skimmed milk for 1h at room temperature. The membranes were subsequently incubated with the primary antibodies against HIF1A (ab179483, 1:1000, Abcam), PROZ (ab85317, 1:1000, Abcam), cyclin D1 (ab226977, 1:1000, Abcam), cyclin B1 (ab215436, 1:1000, Abcam), Hexokinase 2 (HK2) (ab227198, 1:1000, Abcam), Notch receptor 1 (Notch1) (ab52627, 1:1000, Abcam), Hes Family BHLH Transcription Factor 1 (HES1) (ab71559, 1:1000, Abcam), and β-actin (ab8227, 1:1000, Abcam) overnight at 4 °C. Subsequently, horseradish peroxidase-labeled secondary antibody (Ab6721, 1:1000, Abcam Inc.) was added and the membranes were incubated for 2 h at ambient temperature, and protein bands were visualized by an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific), with β-actin as the internal reference. Finally, the quantification of the protein bands was performed with Image J software (NIH, Bethesda, MD, USA).

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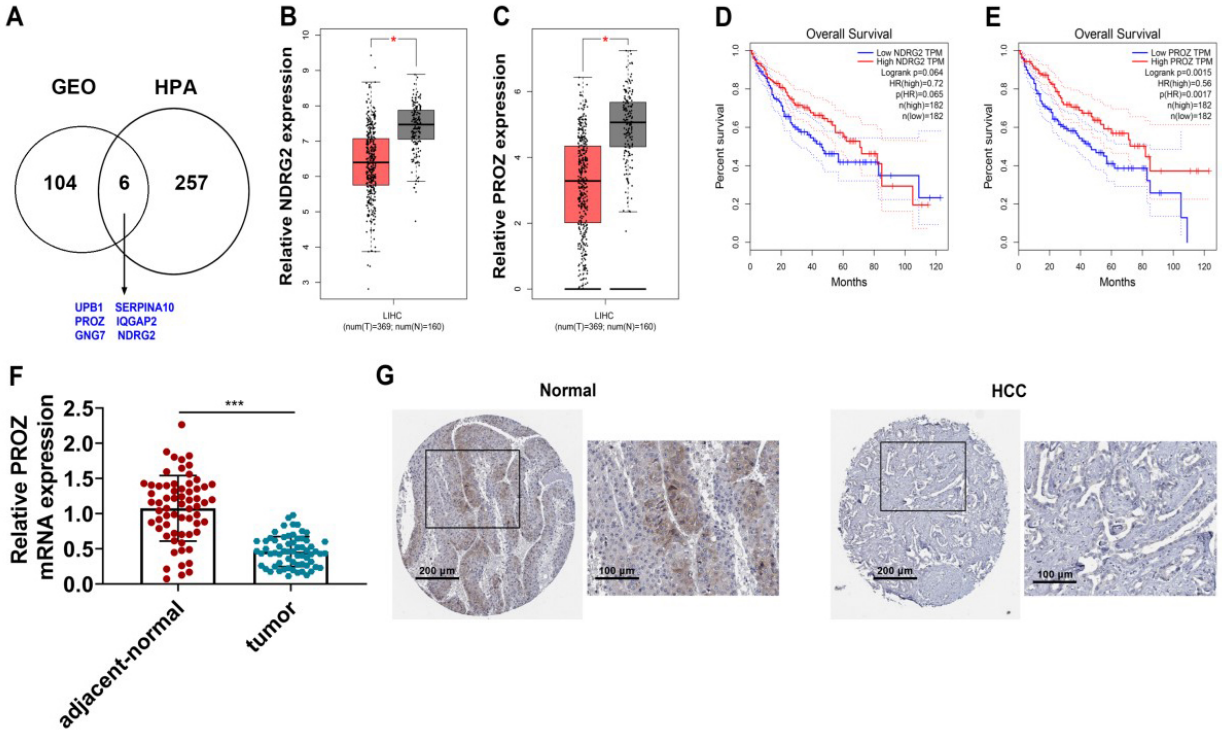

Fig. 2. Key genes associated with prognosis of HCC patients were obatained. (A) 263 prognostic genes downloaded from the HPA database were intersected with 110 genes with up-regulated expression in hypoxia-induced HCC cells and a Venn map was drawn. (B,C) NDRG2 and PROZ expression in HCC was analyzed in the GEPIA database. (D,E) GEPIA database was adopted to analyze the relationship between NDRG2 and PROZ expressions and the survival of patients. (F) qRT-PCR assay showed the mRNA levels of PROZ in HCC. (G) Immunohistochemical staining results of PROZ in HCC tissues were obtained from HPA database and the staining differences were compared. *$p < 0.05$ and *** $p < 0.001$. HCC, Hepatocellular carcinoma; HPA, Human Protein Atlas; PROZ, protein Z; GEPIA, Gene Expression Profiling Interactive Analysis.

Statistical Analysis

SPSS 22.0 (IBM, NY, USA) was used for statistical analysis. Measurement data were expressed as "mean ± standard deviation". The unpaired *t* test was utilized for the comparison between two groups, and one-way ANOVA for the data comparison among multiple groups. Chi-square test was performed to analyze the clinicopathological correlation of PROZ expression. $p < 0.05$ is reflective of a statistically significant difference.

Results

DEGs of HCC Cells in Hypoxic Condition

To identify hypoxia-related DEGs in HCC, GSE15366 and GSE41666 datasets were downloaded from the GEO database and DEGs in hypoxia-induced HCC cells were analyzed using the GEO2R online analysis tool, with $|\text{Log}_2\text{FC}| > 1$, $p < 0.05$ as the screening criterion (Fig. 1A,B). In the intersection of the above two datasets, 110 common DEGs were obtained (Fig. 1C). Moreover, 110 DEGs were subjected to GO enrichment analysis and KEGG analysis in the DAVID database. GO enrichment analysis showed that these DEGs were significantly enriched in Biological Process (BP) including Cellular cellular response to hypoxia, liver development; in cellular component (CC) including extracellular exosome, nucleoplasm; in molecular function (MF) including protein homodimerization activity, GTPase activator activity (Fig. 1D). The KEGG enrichment analysis suggested that these DEGs were significantly associated with the apoptosis, PI3K-Akt signaling pathway, and HIF-1 signaling pathway (Fig. 1E).

PROZ is a Hypoxia-Related Gene with Prognostic Value in HCC

Then the genes with prognostic value in HCC were obtained via HPA database (https://www.proteinatlas.org), and 263 genes were obtained. These 263 genes were intersected with the above 110 genes down-regulated in hypoxia-induced HCC cells, and the results showed that SERPINA10, PROZ, IQGAP2, and NDRG2 associated with HCC development were common targets in the above two sets (Fig. 2A). Next, the expression levels of these four genes in HCC and their relationship with patients' prognosis were validated with GEPIA database. The re-

Table 1. The clinicopathologic relationship of PROZ in HCC patients (n = 64).

Clinical characteristics		PROZ expression		χ^2	*p* value
		High	Low		
		(n = 32)	(n = 32)		
Gender	Male	14	17	0.563	0.453
	Female	18	15		
Age (years)	≥50	25	23	0.333	0.564
	< 50	7	9		
Tumor size	≥5 cm	16	18	0.251	0.616
	<5 cm	16	14		
Cirrhosis	Absent	12	19	3.065	0.080
	Present	20	13		
Lymph node metastasis	Yes	13	22	5.107	0.024*
	No	19	10		
Tumor grade	Low	19	8	7.751	0.005**
	High	13	24		

*Represent statistically significant. PROZ, protein Z; HCC, hepatocellular carcinoma.

sults showed that NDRG2 and PROZ were lowly expressed in HCC (Fig. 2B,C). NDRG2 expression was not significantly associated with the prognosis of patients, and the low expression of PROZ suggested the unfavorable prognosis of the patients (Fig. 2D,E). qRT-PCR showed that PROZ mRNA expression in HCC tissues was significantly down-regulated (Fig. 2F). Next, the patients were grouped into high- and low-expression groups according to the average expression level of PROZ mRNA, and the results of chi-square test suggested that low expression of PROZ was associated with tumor lymph node metastasis and higher tumor grade (Table 1). In addition, immunohistochemical staining results for PROZ were obtained in the HPA database, showing that PROZ staining was weaker in HCC tissues as against the liver tissues in normal control group (Fig. 2G).

PROZ Expressions were Down-Regulated in Hypoxia-Induced HCC Cells

HCC cells (HepG2, Hep3B) were cultured under hypoxic conditions for 6 h, 12 h, and 24 h. Western blot assay showed that, HIF1A protein expression level in HCC cells after hypoxic induction was markedly increased time-dependently (Fig. 3A), suggesting that the hypoxia model was successfully established. Accordingly, the protein expression of PROZ was significantly reduced time-dependently after hypoxia induction (Fig. 3B).

PROZ Repressed the Growth and Glycolysis of HCC Cells and Promoted Apoptosis

Next, PROZ overexpression cell model was constructed by transfecting PROZ overexpression plasmid and blank plasmid into HepG2 cells, while the knock-down model was constructed by transfecting si-PROZ and si-NC into Hep3B cells (Fig. 4A). CCK-8 assay showed that, overexpression of PROZ markedly repressed the viability of HepG2 cells, while knockdown of PROZ significantly facilitated the proliferation of Hep3B cells (Fig. 4B). TUNEL assay showed that compared with the control group, exogenous expression of PROZ promoted the apoptosis of HepG2 cells, while depletion of PROZ significantly restrained the apoptosis of Hep3B cells (Fig. 4C). In addition, as against the control group, PROZ overexpression markedly suppressed glucose consumption and lactic acid production in HepG2 cells, while knockdown of PROZ remarkably promoted glucose consumption and lactic acid production in Hep3B cells (Fig. 4D,E). HK2 is the key enzyme involved in glycolytic pathway. Western blot assay highlighted that overexpression of PROZ remarkably repressed HK2 protein expression, while knockdown of PROZ worked oppositely (Fig. 4F).

PROZ Regulated Cell Cycle and Notch Signaling Pathways

Next, gene set enrichment analysis (GSEA), with LinkedOmics database, was performed to analyze the signaling pathway associated with PROZ dysregulation in HCC. The results showed that high expression of PROZ was negatively correlated with the activation of DNA republican, and negatively correlated with cell cycle, Notch, Wnt, and Hippo signaling pathways, but positively correlated with the activation of PPAR signaling pathway (Fig. 5A–F). Subsequently, Western blot experiment was conducted to detect the effect of PROZ on cell cycle-related proteins (cyclin D1, cyclin B1) and Notch signaling pathway-related proteins (Notch1, Hes1). As shown, PROZ overexpression significantly inhibited cyclin D1, cyclin B1 and Notch1 expression levels, while PROZ knockdown functioned oppositely (Fig. 5G).

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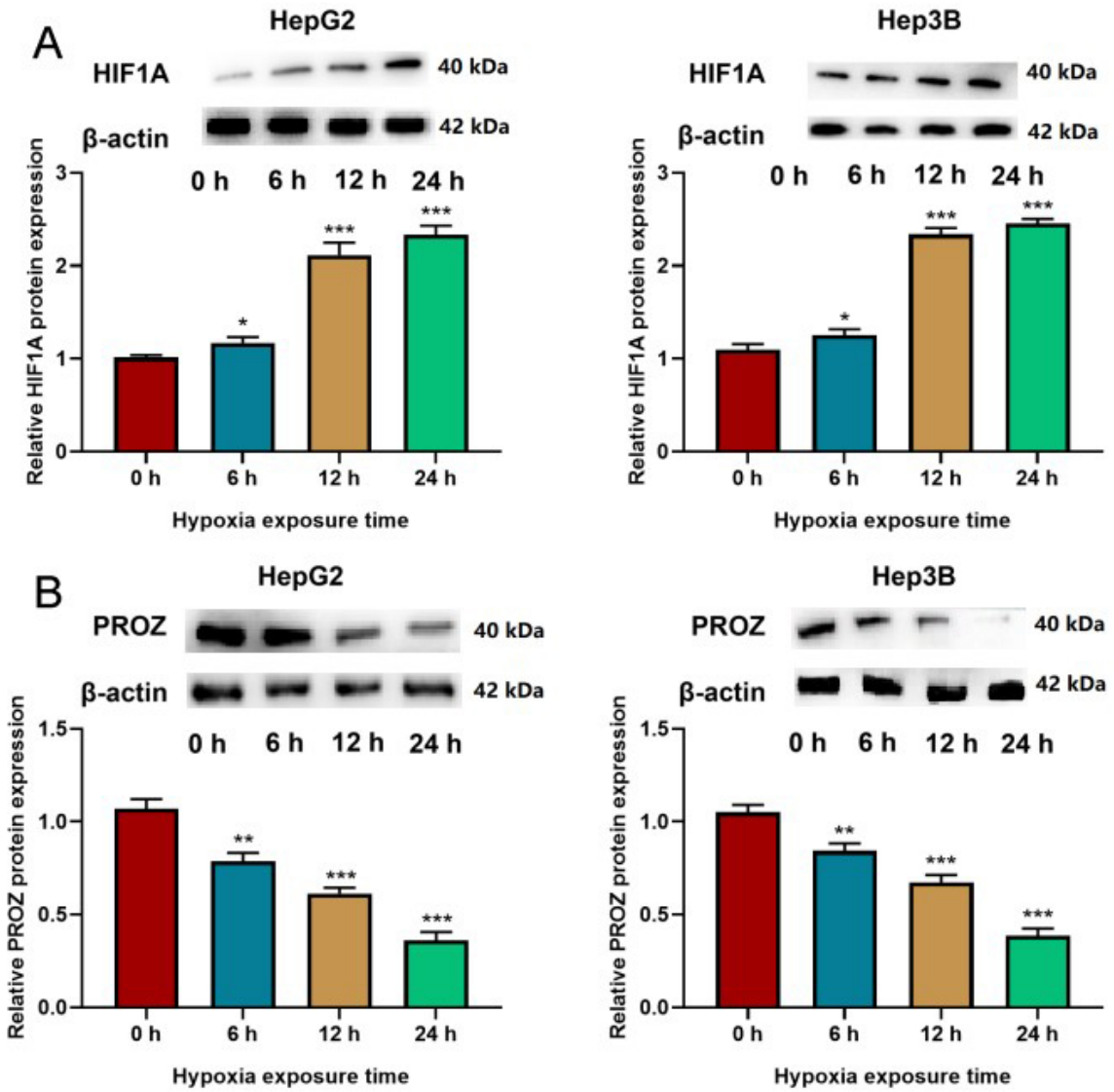

Fig. 3. PROZ expressions were down-regulated in HCC cells under hypoxia. (A) HepG2 and Hep3B cells were cultured under hypoxic conditions for different times. Western blot experiment was operated to detect the protein level of HIF1A. (B) HepG2 and Hep3B cells were cultured under hypoxic conditions for different times. Western blot experiment was conducted to detect PROZ expression. *$p < 0.05$ and ***$p < 0.001$.

Discussion

Hypoxia is one of the characteristics of solid tumors' microenvironment [3]. More and more studies have shown that a variety of molecules are implicated in the regulation of hypoxia process [15]. In this study, we identified 110 DEGs associated with hypoxia in HCC through bioinformatics analysis, and confirmed that these genes were closely associated with multiple pathways associated with tumor development. We then further analyzed the prognostic value of the 110 DEGs in HCC and finally identified the target gene: PROZ.

The role of PROZ in a variety of tumors has been gradually revealed. For example, as reported, in lung adenocarcinoma, compared with that in normal lung tissue, the expression levels of PROZ mRNA and protein are greatly elevated in lung adenocarcinoma tissue, and PROZ features prominently in angiogenesis [10]. In colon cancer, PROZ mRNA expression in colon cancer cells is associated with endothelial cells, and thus blood coagulation activation outside tumor sites are affected [12]. PROZ, reportedly, is implicated in the pathogenesis of bacterial meningitis and increases the risk of cerebral infarction [16]. The role of PROZ in HCC has also been reported. PROZ expression in tissues of the patients with early HCC is reduced, and its low expression is correlated with low overall survival rate of early HCC patients, and PROZ may be a prognostic biomarker of early HCC [17]. Similarly, another study reports that PROZ is identified as the central gene of HCC

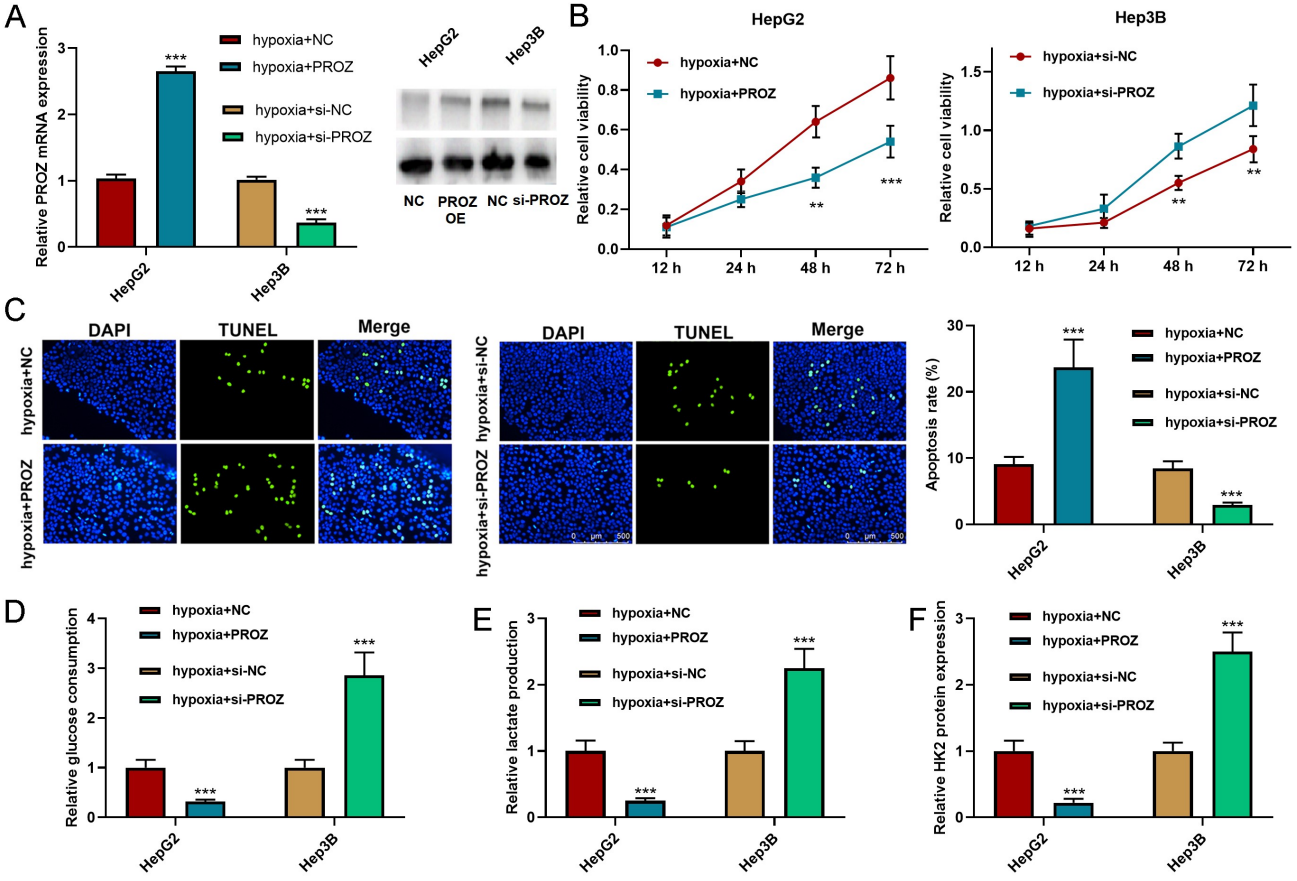

Fig. 4. PROZ restrained the viability and glycolysis of HCC cells and promoted apoptosis. PROZ overexpression plasmid and empty plasmid were transfected into HepG2 cells, and si-NC and si-PROZ were transfected into Hep3B cells: (A) PROZ mRNA expression was detected by qRT-PCR and Western blot. (B) The cell viability was evaluated by CCK-8 asssay. (C) TUNEL assay was used to detect the apoptosis of HCC cells. (D) Glucose consumption levels in HCC cells were measured. (E) The lactic acid production levels in HCC cells were measured. (F) The HK2 protein levels in HepG2 and Hep3B cells were examined by Western blot assay. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

tumorigenesis and its low expression is interrelated with poor prognosis of patient [18]. In addition, hypoxia leads to reprogramming of glycolysis metabolism, which is mainly characterized by increased levels of glucose consumption and lactic acid production, as well as increased levels of key glycolysis-related enzymes such as HK-2 [19,20]. Here we found that PROZ expression was down-regulated in HCC and its underexpression suggests higher tumor grade and lymph node metastasis. Further analysis showed that PROZ expression was significantly inhibited in HCC cells under hypoxic conditions, and that exogenous expression of PROZ inhibited hypoxia-induced proliferation and glycolysis of HCC cells and promoted apoptosis.

We performed GSEA after the samples were grouped according to expression level of PROZ, and the results showed that PROZ expression was related with cell cycle progression and activation of Notch signaling pathway. Cell cycle disorder of cancer cells is accompanied by the up-regulation of cyclin D1, cyclin B1 and other cyclins: during the cell cycle, cyclins activate cyclin-dependent kinases to drive the phase transition, and this process is also involved in repairing DNA damage and cell death [21,22]. Interestingly, our study found that PROZ greatly restrained the expression of cyclin D1 and cyclin B1. As reported, Notch signaling pathway can accelerate the progression of HCC via multiple different mechanisms [23]. For example, Notch signaling pathway can interact with HIF-1 to control mitochondrial biogenesis of cancer cells, thereby regulating mitochondrial function of HCC cells [24]. It has also been found that the abnormal activation of Notch signaling pathway in HCC leads to the increased levels of Notch pathway-activated biomarkers such as Notch1 and Hes1 [25]. In this study, we found that PROZ inhibited the expression of Notch1 and Hes1. Therefore, we concluded that PROZ plays an inhibitory role in the progression of HCC by inhibiting expressions of cell cycle-related proteins as well as the activation of Notch signaling pathway.

In conclusion, we identified the key gene, PROZ, in the progression of HCC and confirmed its down-regulation in HCC tissues is associated with hypoxia condition; it

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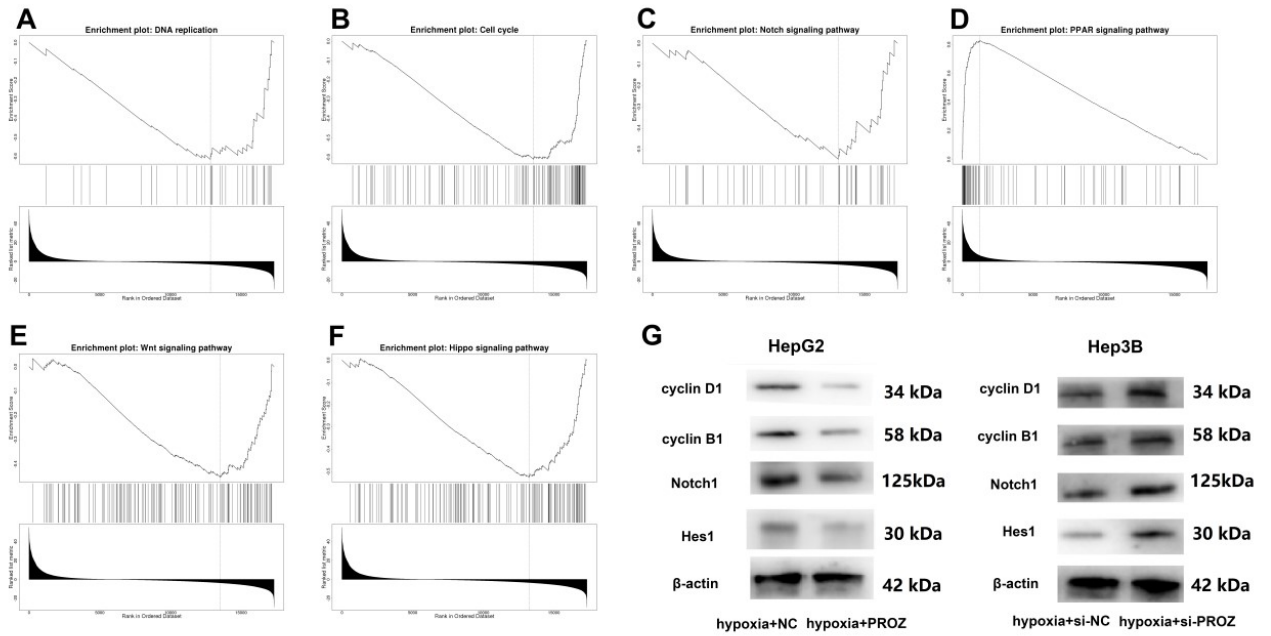

Fig. 5. PROZ regulated cell cycle and Notch signaling pathways. (A–F) Relevant signaling pathways regulated by PROZ in HCC were analyzed using the LinkedOmics database. (G) The PROZ overexpression plasmid and the empty plasmid were transfected into HepG2 cells, and si-NC and si-PROZ were transfected into Hep3B cells. Western blot assay was adopted to detect cell cycle-related proteins (cyclin D1, cyclin B1) and Notch signaling pathway-related proteins (Notch1, Hes1) expressions.

was further found that PROZ significantly suppressed the growth and glycolysis of HCC cells under hypoxia conditions, and promoted apoptosis. In term of mechanism, PROZ can inhibit the development of HCC by inhibiting expressions of cell cycle-related proteins and interfering with the activation of Notch signaling pathway. This study paves a new way for understanding the progression of HCC under hypoxic conditions and implies that PROZ can be a potential therapeutic target for HCC treatment.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

XY and TD conceived and designed the study; XY and ML performed the experiments and analysis; XY and XW wrote the paper. All authors approved the final version of the manuscript and agreed to submit the manuscript to this journal.

Ethics Approval and Consent to Participate

The collection and use of human tissue samples were approved by the the Ethics Committee of Renmin Hospital of Wuhan University (No. 202100045).

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Conflict of Interest

The authors declare no conflict of interest.

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