

Prognostic role of PHYH for Overall Survival (OS) in clear cell Renal Cell Carcinoma (ccRCC)

Zhengqi Qiu (✉ caspiansev@163.com)

Shenzhen University

Guo Zezhi

Shenzhen University College of Medicine

Jiang Lei

Shenzhen University College of Medicine

Qiu He

Shenzhen University College of Medicine

Pan Jinyao

Shenzhen University College of Medicine

Ao Ying

Shenzhen University College of Medicine

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Abstract

This study attempts to evaluate the prognostic role of PHYH for overall survival (OS) in clear cell renal cell carcinoma (ccRCC) by means of publicly available data from The Cancer Genome Atlas (TCGA). Clinical pathologic features and PHYH expression were downloaded from the TCGA database and relationships between them were analysed by Univariate and multivariate Cox regression analyses. Gene Set Enrichment Analysis (GSEA) and gene-gene interactions were also performed between tissues with different PHYH expression levels. PHYH expression levels were significantly lower in patient with ccRCC compared with normal tissues ($p = 1.156e-19$). Kaplan-Meier survival analysis showed that high expression of PHYH had a better prognosis than low expression ($p = 9e-05$). Moreover, PHYH expression was also significantly associated with high grade (G2-4, $p=0.025$), high stage (StageIII & IV, $p=5.604e-05$), High level of stage_T (T3-4, $p=4.373e-05$). Univariate and multivariate Cox regression analyses indicated that PHYH could be acted as an independent prognostic factor ($p<0.05$). Nomogram including Clinical pathologic features and PHYH expression were also provided. GSEA revealed that butanoate metabolism, histidine metabolism, propanoate metabolism, pyruvate metabolism, tryptophan metabolism, PPAR signalling pathway and Renin angiotensin system were differentially enriched in PHYH high expression phenotype. ICGC database was utilized to verify the expression level and survival benefit of PHYH (both $p<0.05$). We suspect that Elevated PHYH expression may be served as a potential prognostic molecular marker of better survival in ccRCC. Besides, alpha-oxidation was closely regulated by PHYH, and PPAR signalling, pyruvate metabolism, butanoate metabolism and RAS might be the key pathways regulated by PHYH in CCRC.

Background

Clear cell renal cell carcinoma (ccRCC) is a major type of kidney cancer accounting for 90–95% of cases [1]. It sporadically arises from proximal tubular epithelial cells of the renal cortex, characterized by malignant epithelial cells with typical clear cytoplasm. During the past decade, data have shown a 2–3% yearly increase in ccRCC incidence. Recent advances in scientific medical research have led to an increased perception of the underlying pathophysiological molecular mechanism of ccRCC [2, 3]. The most common and vital characteristic associated with ccRCC and cancer in general is hypoxia. A condition that initiates a cascade of molecular events including angiogenesis and involves cell-cycle control proteins, which are closely associated with tumour growth [4, 5]. With regards to renal cell carcinoma (RCC), past researchers have identified that the hypoxia inducing factors 1α (HIF-1α) and its linked pathways such as ubiquitin-proteasome and rapamycin pathways are major contributors in RCC tumorigensis [6–9]. More recent gene expression studies have identified some genes that predicts ccRCC aggressiveness and progression [10–13]. Yet despite our efforts, no molecular biomarkers have been verified and potentially applicable in a clinical setting to move toward precision medicine of RCC treatment.

Phytanoyl-CoA 2-Hydroxylase gene (PHYH) is gene of the PHYH family and critical in the formation of peroxisomal protein which in turn assists in the alpha oxidation of 3-methyl branched fatty acids. As

immune system evasion is the hallmark of cancer, peroxisomes have an emerging role in the regulation of cellular immune response with reports showing pro-tumorigenic functions of peroxisome. However, there exists a significant gap in knowledge in the role of peroxisome and its associated gene PHYH in the potential of tumour induction and development [14].

Thus, the objective of the current study was aimed to evaluate the prognostic value of PHYH expression in human ccRCC data obtained from TCGA. Indeed, gene set enrichment analysis (GSEA) was performed to gain a better understanding into the underlying pathophysiological pathway mechanisms associated with ccRCC pathogenesis and its relationship with PHYH regulatory network. Potentially, discovering links and mechanisms connected to tumorigenesis.

Methods

RNA-sequencing patient data and bioinformatics analysis

High throughput sequencing of gene expression data (HTSeq-counts) and clinical information of 538 cases of ccRCC and 72 paracancerous cases were downloaded from TCGA official website. Normal ccRCC samples were excluded, boxplots and whiskers plot were used to visualize expression differences for discrete variables [15].

Gene set enrichment analysis

GSEA is bioinformatics method aimed to identify whether prior sets of genes or proteins are significantly different between two phenotypes [16]. Our study applied GSEA to generate an order list of all genes according to their correlation with PHYH expression, significant survival differences observed between high and low PHYH groups were elucidated. Gene set permutations were performed 1000 times for each analysis. The expression level of PHYH was used as a phenotype label. The nominal p value and normalized enrichment score (NES) were used to sort the pathways enriched in each phenotype.

Gene-network analysis

To investigate associated genes in performing different molecular function and biological pathway, gene interaction analysis was performed for the PHYH gene. Gene cards database (<http://www.genecards.org>) was used for searching gene-gene interaction network to identify gene-gene association, then we selected those that have a confidence value of 0.7 (high confidence) or higher. Furthermore, these set of genes were displayed by using interactive gene view software (<http://software.broadinstitute.org/software/igv>).

Statistical analysis

R (v3.4.3) was used to perform all statistical related analysis. Relationship between clinical pathological features and PHYH expression were analysed via Wilcoxon signed-rank test and logistic regression. Nomogram construction was performed according to the guidelines proposed by Iasonos [17]. We randomly assigned four-fifths of patient information to the development cohort ($n = 430$) and one-fifth of patient information to the validation cohort ($n = 138$). To identify independent prognostic predictors, we used a Cox proportional hazards regression model for univariable and multivariable analyses by the

“Enter” method. The nomogram was developed to predict the 3- and 5-year prognosis mainly based on the results of the multivariable Cox regression model. The performance of the nomogram was estimated regarding discrimination and calibration. The C-index was applied to evaluate discrimination [18], which refers to the models’ ability to accurately distinguish the outcomes. A higher C-index indicates more precise model predictions [19]. Calibration curves were performed by comparing the means of the nomogram-predicted outcomes with the actual outcomes estimated with Kaplan-Meier. The bootstrapping (1000 repetitions) method was applied to reduce the estimate bias. In addition, model validations were performed using the data of the validation ccRCC cases as follows. First, we calculated the total points of the patients in the validation group using the established nomogram. Next, we used the total points as a factor to perform Cox regression analysis. Finally, the C-index and calibration curves were developed with the results of regression analysis. Receiver operating characteristics (ROCs) curve was used for the sensitivity and specificity of nomogram.

Results

Association with PHYH expression and clinicopathologic variables

As shown in (Fig. 1), expression of PHYH is significantly lower in patients with tumour ($p = 1.156e-19$ & $p = 2.634e-10$). Classic univariate ROC curve analysis was performed to assess true positive rate and false positive rate of the PHYH expression between normal patients and patients with tumours based on the area under the curve (AUC). The results revealed that PHYH expression had a reasonable AUC of 0.611. In addition, decreased expression of PHYH correlated significantly with grade of cancer cells (G1-2 vs. G2-4, $P = 0.025$), the Union for International Cancer Control (UICC) stage (Stage I&II vs. Stage III&IV, $p = 5.604e-05$) and size of primary tumour (T1-2 vs. T3-4, $p = 4.373e-05$) (Fig. 2A-C, Table 1).

Table 1

PHYH expression associated with clinical pathological characteristics (logistic regression).

Clinical characteristics	Total (N)	Odds ratio in PHYH expression	p-Value
Age (> 65 VS <= 65)	511	1.347(0.933–1.949)	0.112
Gender (Female VS Male)	511	0.594(0.410–0.856)	0.005
Race (African VS White)	511	0.368(0.069–1.670)	0.203
(Asian VS White)		0.586(0.317–1.060)	0.081
Grade (G3-4 VS G1-2)	511	0.591(0.416–0.840)	0.003
Stage (Stage I&II VS Stage III&IV)	511	0.506(0.352–0.725)	0.000
T (T1-2 VS T3-4)	511	0.529(0.365–0.762)	0.001
M (M0 VS M1&X)	511	0.976(0.635–1.501)	0.913
N (N1&X VS NO)	511	0.740(0.521–1.049)	0.091

Survival outcomes and multivariate analysis

The Kaplan-Meier survival analysis (Fig. 1C) showed that ccRCC with low expression of PHYH had a worse prognosis than that with high expression of PHYH ($p = 9e-5$). The univariate analysis revealed that positive distant metastasis is correlated significantly with a poor overall survivability (hazard ratio [HR]: 2.1; 95% confidence interval [CI]: 1.661–2.655; $p < 0.001$). Other clinicopathologic variables associated with poor survival include age, grade, UICC stage, size of primary tumour and PHYH expression (Fig. 3A, Table 2). At multivariate analysis, factors such as age, grade, stage and PHYH expression remained associated with overall survival (Fig. 3B, Table 2). Classical univariate ROC curve analyses revealed that grade, stage and size of primary tumour (T), showed a high AUC of 0.7, 0.779 and 0.723 respectively (Fig. 3C).

Table 2

Associations with overall survival and clinicopathologic characteristics in TCGA patients using univariate and multivariate cox analysis;

Clinical characteristics	univariate analysis.		Multivariate analysis	
	HR (95% CI)	p-Value	HR (95% CI)	p-Value
Age	1.033(1.020–1.047)	0.000	1.039(1.023–1.054)	0.000
Gender	0.933(0.680–1.282)	0.670	0.938(0.678–1.298)	0.700
Race	1.193(0.716–1.988)	0.498	1.043(0.611–1.779)	0.877
Grade	1.967(1.639–2.361)	0.000	1.460(1.162–1.834)	0.001
Stage	1.856(1.644–2.095)	0.000	1.778(1.245–2.538)	0.002
T	1.998(1.689–2.362)	0.000	1.039(0.785–1.375)	0.790
M	2.100(1.661–2.655)	0.000	0.797(0.417–1.525)	0.494
N	0.862(0.739–1.008)	0.063	0.848(0.722–0.995)	0.043
PHYH	0.963(0.941–0.986)	0.002	0.975(0.954–0.996)	0.021

A point ranking system was also developed to rank the association of each factor with survivability (Fig. 3D). The higher the point for a give factor, the lower the survivability. As the results show, grade (G2, G3, and G4) and stage (I, II and IV) are significantly associated with low survivability. In addition, higher age and lower PHYH expression are also significant related to low survivability. Interestingly, ethnic group African and white have lower survivability compared to Asian.

Gene network

We also investigate gene network in order to identify their gene-gene interaction. Our results showed that PHYH in connected to 10 different genes in gene-gene interaction (Fig. 4A). Among these genes, 5 are PEX genes that encode peroxin proteins (PEX2 PEX7 PEX10 PEX13 PEX14) which suggest the existence of protein interactions with PHYH in ccRCC. Figure 4B show the relationships between PHYH and microsatellite instability (MSI). The associations between PHYH and immune checkpoint inhibitors were also displayed in Fig. 4C. Figure 4D presented the relationships between PHYH and the methods of immunity.

GSEA identifies a PHYH-related signalling pathway

To identify signalling pathways that are differentially activated in ccRCC, we conducted Gene Set Enrichment Analysis (GSEA) between low and high TFAP2B expression data sets. GSEA reveal significant differences (FDR b 0.05, NOM p-val b 0.05) in enrichment of MSigDB Collection (c2.cp.biocarta and h.all.v6.1. symbols). We selected the most significantly enriched signalling pathways based on their normalized enrichment score (NES) (Fig. 5, Table 3). The Fig. 5 shows butanoate metabolism, histidine

metabolism, propanoate metabolism, pyruvate metabolism, tryptophan metabolism, PPAR signalling pathway and Renin angiotensin system are differentially enriched in high PHYH expression phenotype.

Table 3
Gene sets enriched in phenotype high;

MSigDB collection	Gene set name	NES	NOM p-val	FDR q-val
c2.cp.biocarta.v6.1.symbols.gmt	BUTANOATE_METABOLISM	2.506	0.000	0.000
h.all.v6.1.symbols.gmt	HISTIDINE_METABOLISM	2.503	0.000	0.000
	PPAR_SIGNALING_PATHWAY	2.124	0.000	0.006
	PROPANOATE_METABOLISM	2.500	0.000	0.000
	PYRUVATE_METABOLISM	2.551	0.000	0.000
	RENIN_ANGIOTENSIN_SYSTEM	2.071	0.002	0.008
	TRYPTOPHAN_METABOLISM	2.671	0.000	0.000

Verification of PHYH in ccRCC

To further verify the expression level and survival benefit of PHYH in ccRCC, the GTEx, ICGC and HPA databases were utilized respectively. As displayed in Fig. 6A, the expression level of PHYH in various cancers were shown including ccRCC with $P < 0.001$. In terms of ICGC database, the boxplot and survival analysis were consistent with in TCGA ($P = 5.214\text{e-}18$, $P = 1.51\text{e-}03$, respectively, Fig. 6B-6C). The HPA database indicated the difference of immunohistochemistry in normal and kidney cancers (Fig. 6D-6E).

Discussion

The expression of PHYH have been linked to multiple diseases such as Refsum Disease and Retinitis Pigmentosa [20]. Although there are no study associating human cancers to PHYH expression, The Human Protein Atlas have reported it to be a prognostic marker in renal cancer [21]. To our knowledge, expression of PHYH and its impact on ccRCC has not yet been explored. Therefore, the potential role of PHYH in ccRCC was the main focus point of our study.

We applied bioinformatics analysis using high-throughput RNA-sequencing data from TCGA to examine PHYH expression in ccRCC patients and its association with various advanced pathologic characteristics. We demonstrated that a decrease PHYH expression is associated with presence of tumour, grade of cancer, stage of cancer, primary size of tumour, age and presence of distant metastasis. To further investigate the functions of PHYH in ccRCC, we performed GSEA and gene-gent network using TCGA

data. GSEA showed that butanoate metabolism, histidine metabolism, propanoate metabolism, pyruvate metabolism, tryptophan metabolism, PPAR signalling pathway and Renin angiotensin system are differentially enriched in PHYH low expression phenotype. Gene-gen network analysis revealed association of PHYH with multiple PEX genes. These evidences highlighted the potential of PHYH serving as a prognostic marker of prognosis and therapeutic target in ccRCC.

Results from our study showed a decreased expression of PHYH gene in patients diagnosed with ccRCC. The PHYH gene encodes the enzyme phytanoyl-CoA hydroxylase, which is required for the alpha oxidation of branched chain [22, 23] and long chain [24] fatty acids such as phytanic acid in peroxisomes [25]. Researchers suspect that phytanoyl-CoA hydroxylase potentially participate in determining the number of peroxisomes within cells and is involved in regulating their activities [26]. Peroxisomes are membrane bound organelle within the cytoplasm that is conserved across eukaryotic cells [27], and plays a vital role in peroxisomal fatty acid beta-oxidation metabolism and ROS (reactive oxygen species) conversion [28]. Diseases such as the Zellweger syndrome and other genetic diseases occurs due to implications in the fatty acid beta-oxidation[29, 30]. Study have found that many chemicals designated as peroxisome proliferators can induce peroxisome proliferation, resulting in increase in fatty acid oxidation in liver cells which leads to tumours growth in rodents [31–33]. A study has observed an absence of peroxisome in epithelial cells of proximal tubule in cancer cells of renal cell carcinoma [34]. AS phytanoyl-CoA hydroxylase is coded by the PHYH gene and key component in peroxisome regulation, results of the present study agree with the provided evidence and suggests that decreased expression of PHYH gene is associated with absence of peroxisomes in ccRCC patients.

The gene-gene interactions form the results of our study have shown associations of multiple PEX genes (PEX2, PEX7, PEX10, PEX13, PEX 14) with PHYH. *PEX* genes encode peroxins, a class machinery protein required for proper peroxisome assembly [35]. Autosomal recessive loss of function mutations in the *PEX* genes can result in peroxisome biogenesis disorders in the brain bone kidney and liver [36–39]. Overexpression of PEX genes such as PEX2 can result in accumulation of ubiquitinated PEX5 which can promote pexophagy (autophagosomal degradation of peroxisomes) [14]. Decreased PEX5 levels are associated with both the onset of cancer in vivo [40], and sensitivity to exogenous H₂O₂ addition in hepatocarcinoma model systems in vitro [41]. Identification of PEX14-containg vesicles has connected peroxisomes biogenesis to mitochondrial mediation [42]. PEX7 facilitate matrix protein import, which significantly contributes to peroxisome membrane growth [43]. Notably, PEX7 has primarily been documented to directly shuttle PHYH to the peroxisomal matrix [25]. Given the importance of peroxisomal matrix protein import in normal cells it could be anticipated that the expression and/or function of peroxisome matrix proteins might become aberrant in tumour cells [14]. Combining the results from our analysis with the evidence presented, a clear association can be observed in which PHYH expression affects the expression of PEX genes. This in turn causes perturbations in peroxisomes biogenesis, function, and structure.

Results from the network analysis also revealed that alteration of PHYH expression in ccRCC phenotype implicates the alpha oxidation pathway. Genes HACL1, SLC27A2 (shown to be associated with PHYH) are

genes that code for protein 2-hydroxyacyl-CoA lyase 1 and very long-chain acyl-CoA synthetase, both enzymes along with PHYH are critical enzymes in converting phytanic acid to pristanic acid. Recent review have highlighted that peroxisomal disorders affect phytanic acid and alpha oxidation [44]. As most metabolism of phytanic acid occurs in the liver and kidney via alpha-oxidation, an alteration in PHYH expression will mostly likely implicate peroxisomal and subsequent alpha oxidation. The highlights the alpha oxidation as a target pathway for future studies in ccRCC.

GESA pathway analysis of TCGA data reveal multiple differentially expressed pathways in PHYH low expression phenotype. Among these altered pathways, the key peroxisome proliferator-activated receptor gamma (PPAR γ) pathway has been shown to be functionally expressed [45] in ccRCC and that increased PPAR γ abundance correlates with reduced patient survival [46]. Gluconeogenesis associated pathways pyruvate, and butanoate metabolism have also been shown to be downregulated in kidney cancer [47]. The renin angiotensin system (RAS) was also demonstrated to be under expressed in ccRCC. RAS is a hormone system known to maintain blood pressure and body fluids [48]. Recent literature has implicated a crucial role of the RAS in the development and maintenance of cancer, particularly its effects on cancer stem cells [49–52]. In addition, RAS deregulation was demonstrated as a renal cancer risk factor [53]. Collectively, evidences suggest that these altered pathways and metabolism are good association factors with ccRCC and starting points for understanding in depth underlying pathophysiological mechanism of ccRCC phenotype.

In conclusion, PHYH expression may be a potential prognostic molecular marker of poor survival in ccRCC. Low PHYH expression in ccRCC patients is closely related with dysfunction, degradation, and absence of peroxides. This occurs alters alpha -oxidation pathway which may potentially be a targeted pathway for future studies, Moreover, PPAR signalling, pyruvate metabolism, butanoate metabolism and RAS may be the key pathway regulated by PHYH in ccRCC. Further experimental validation should be performed to prove the biologic impact of PHYH.

Conclusion

Elevated PHYH expression could be served as a potential prognostic molecular marker of better survival in ccRCC. Besides, alpha-oxidation was closely regulated by PHYH, and PPAR signalling, pyruvate metabolism, butanoate metabolism and RAS might be the key pathways regulated by PHYH in CCRC.

Abbreviations

OS;Overall survival

ccRCC:Clear cell renal cell carcinoma

TCGA:The cancer genome atlas

GSEA:Gene set enrichment analysis

PHYH:Phytanoyl-CoA 2-hydroxylase

PPAR:Peroxisome proliferator activated receptor

ICGC:International cancer genome consortium

RCC:Renal cell carcinoma

HIF-1 α :Hypoxia inducing factors 1 α

NES:Normalized enrichment score

ROCs:Receiver operating characteristics

AUC:Are under the curve

UICC:Union for International Cancer Control

MSI:Microsatellite instability

GTEX:The genotype-tissue expression

HPA:Human protein atlas

RAS:Renin angiotensin system

PPAR γ :Peroxisome proliferator-activated receptor gamma

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Q.Z and A.Y conceived and designed the study; G.Z and Q.H acquired and analyzed the data; J.L and P.J. interpreted the data and drafted the manuscript; Q.Z., A.Y and G.Z modified the manuscript. All authors approved the final version to be published.

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Figures

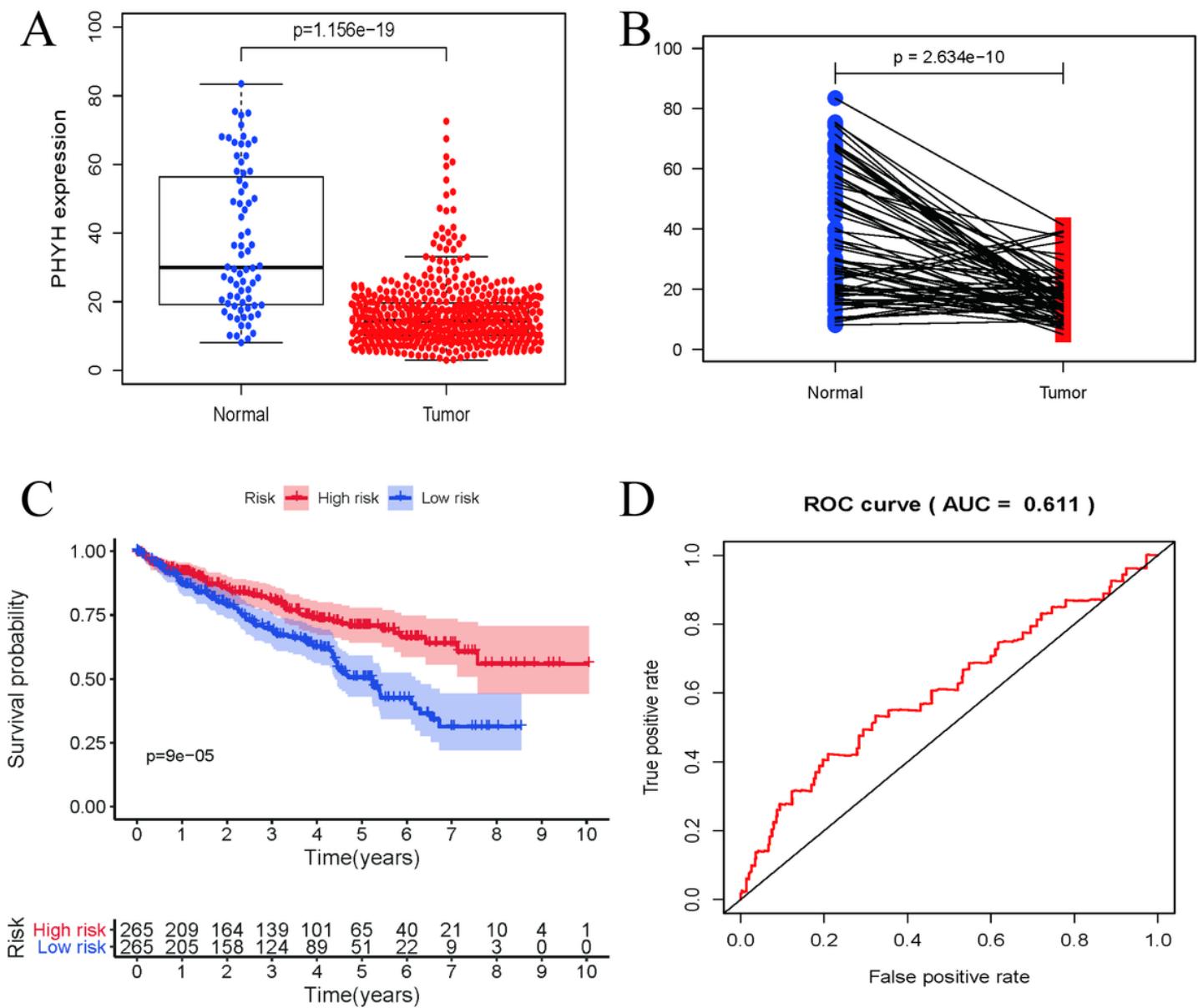


Figure 1

(A&B) Comparison of PHYH expression between normal patients and ccRCC patients with tumour. (C) Impact of PHYH expression on overall survival in ccRCC patients in TCGA cohort. (D) ROC curve analysis of significantly PHYH expression between normal patients and ccRCC patients with tumour.

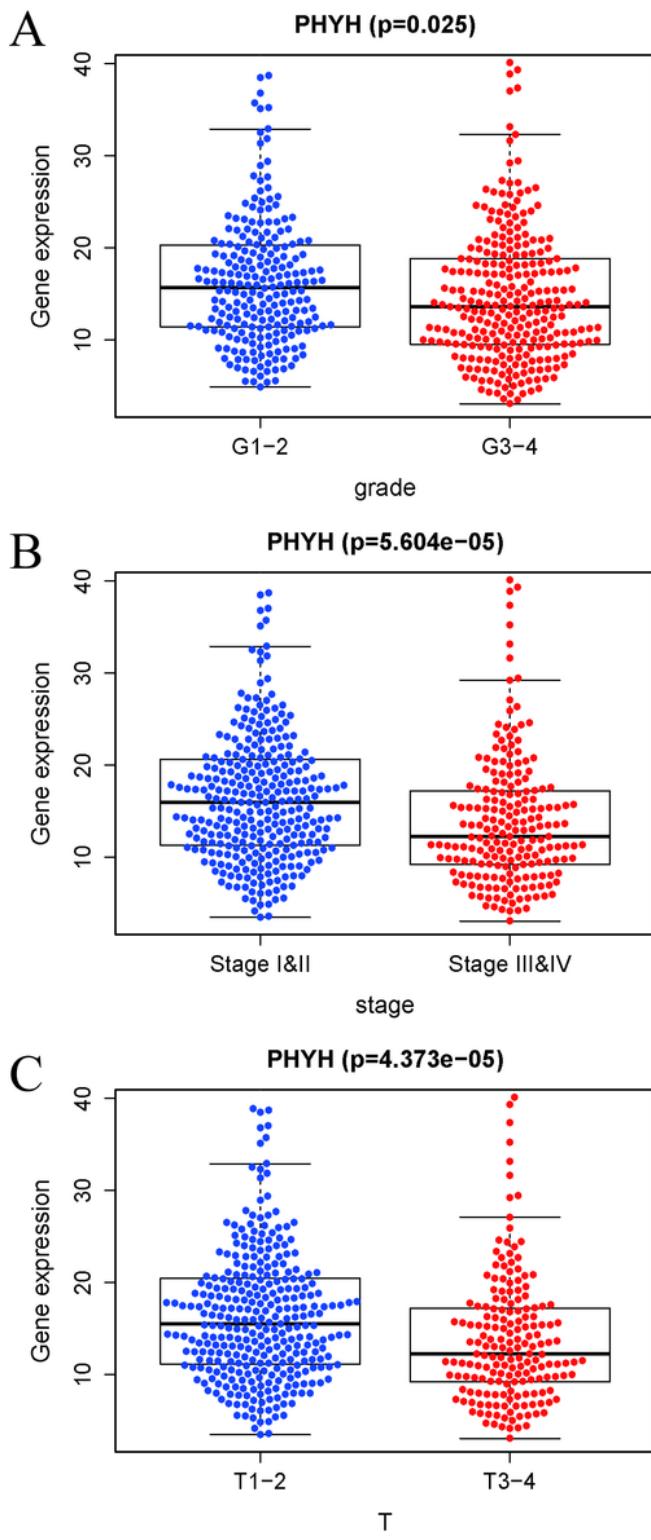


Figure 2

Association with PHYH expression and clinicopathologic characteristics, including (A) Grade, (B) Stage, (C) Primary tumour size (T).

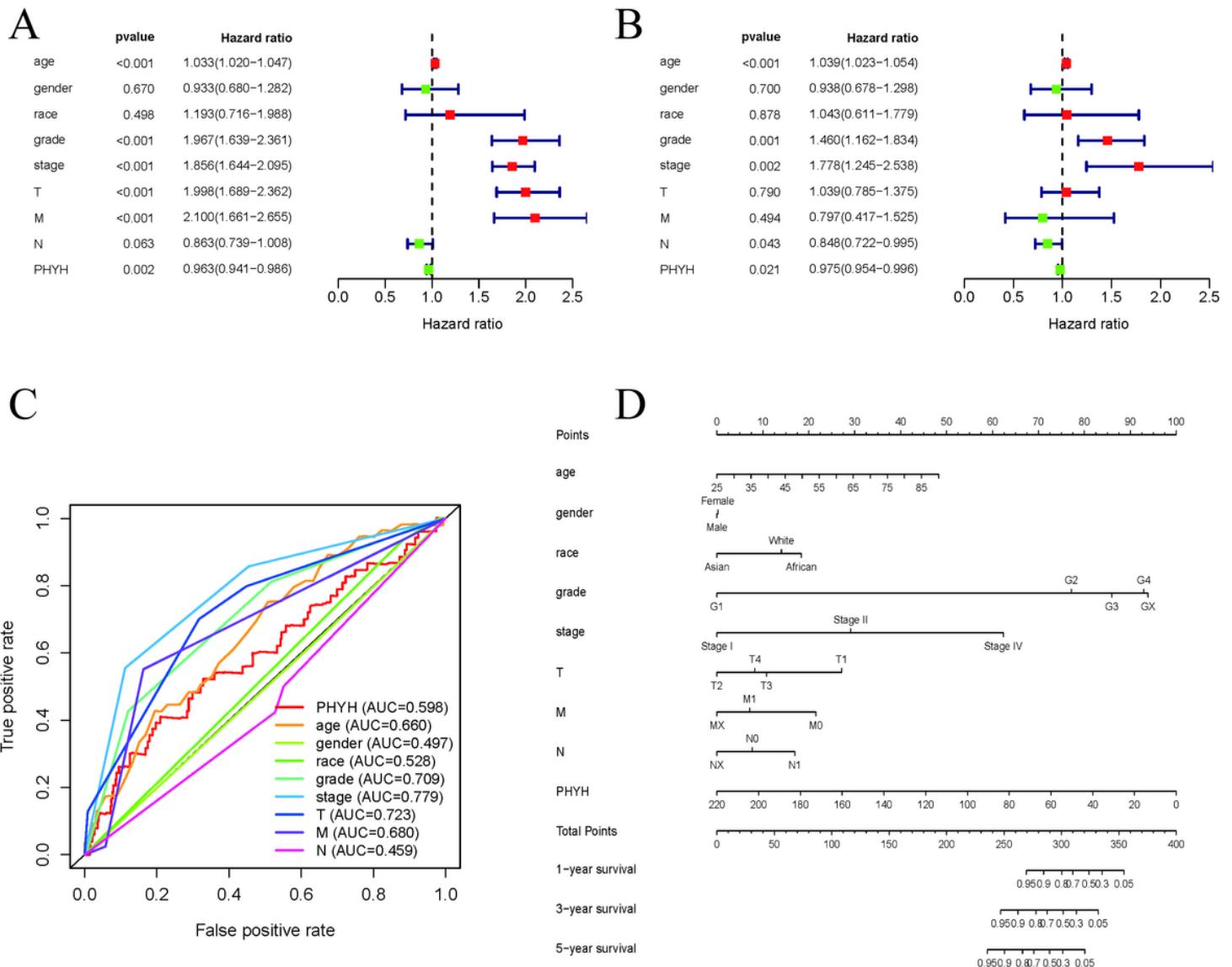


Figure 3

(A) Univariate Cox regression analysis of PHYH expression and clinicopathologic characteristics; (B) Multivariate Cox regression analysis of PHYH expression and clinicopathologic characteristics; (C) ROC curves analysis of PHYH expression and clinicopathologic characteristics. (D) Nomogram of PHYH expression and clinicopathologic characteristics.

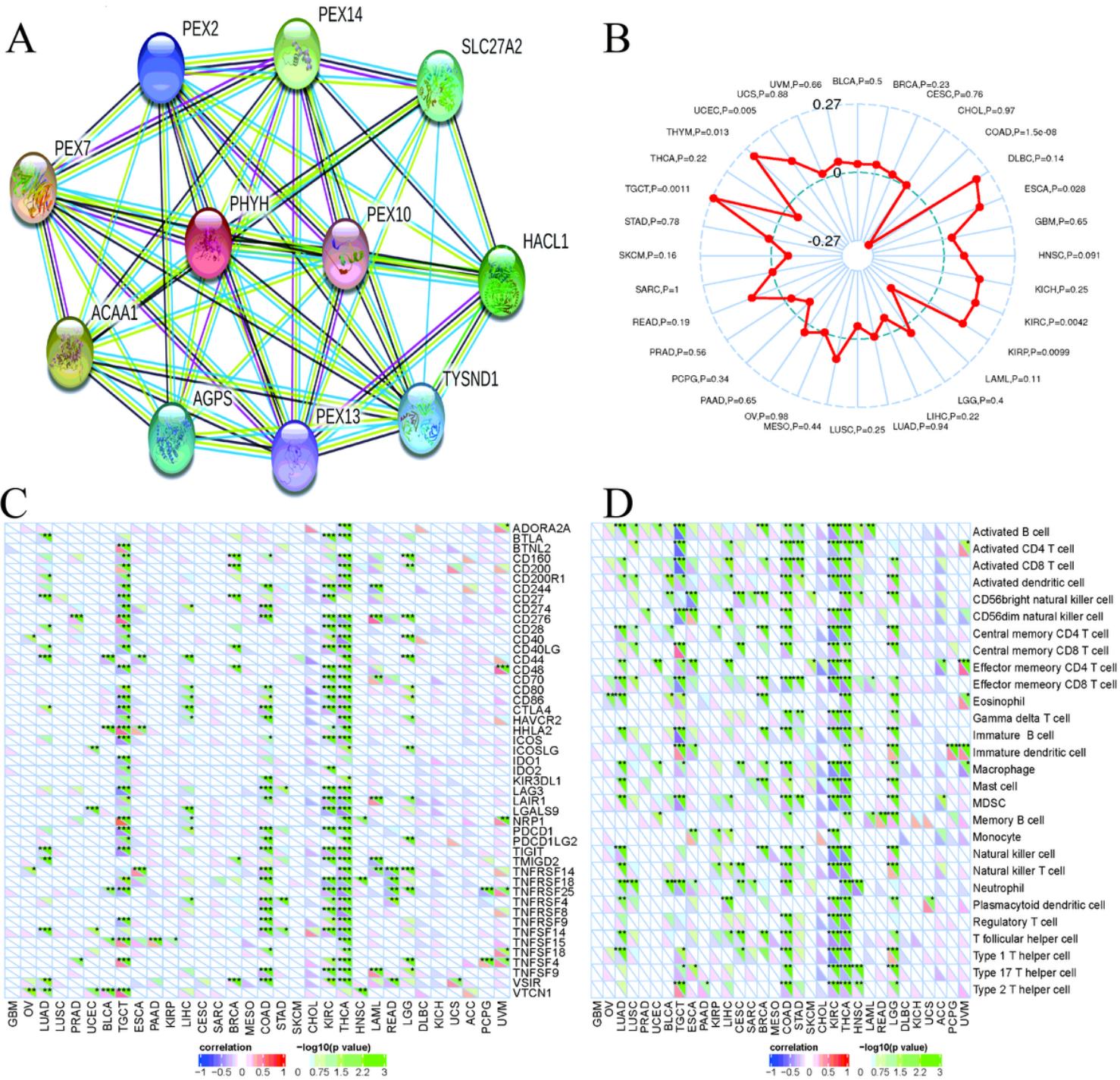


Figure 4

(A) PPI network o(A) PPI network of PHYH in ccRCC cases. (B) Relationships between PHYH and microsatellite instability (MSI). (C) Associations between PHYH and immune checkpoint inhibitors. (D) Relationships between PHYH and the methods of immunity.f PHYH in ccRCC cases. (B) Relationships between PHYH and microsatellite instability (MSI). (C) Associations between PHYH and immune checkpoint inhibitors. (D) Relationships between PHYH and the methods of immunity.

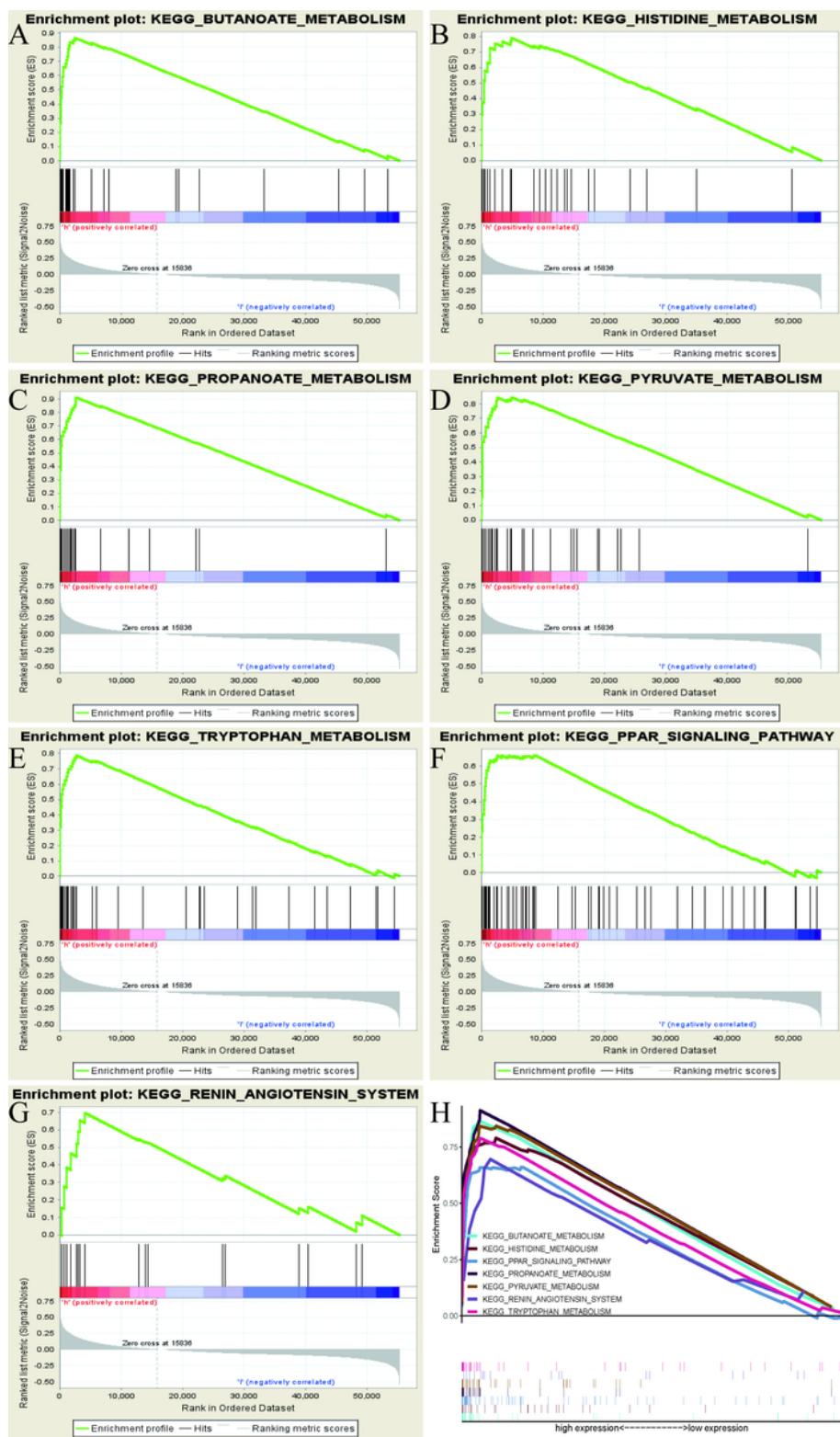


Figure 5

Enrichment plots from gene set enrichment analysis (GSEA). GSEA results showing (A) butanoate metabolism, (B) histidine metabolism, (C) propanoate metabolism, (D) pyruvate metabolism, (E) tryptophan metabolism, (F) PPAR signalling pathway and (G) Renin angiotensin system are differentially enriched in PHYH-related ccRCC. (H) Comparison of enrichment plots for all significant pathways.

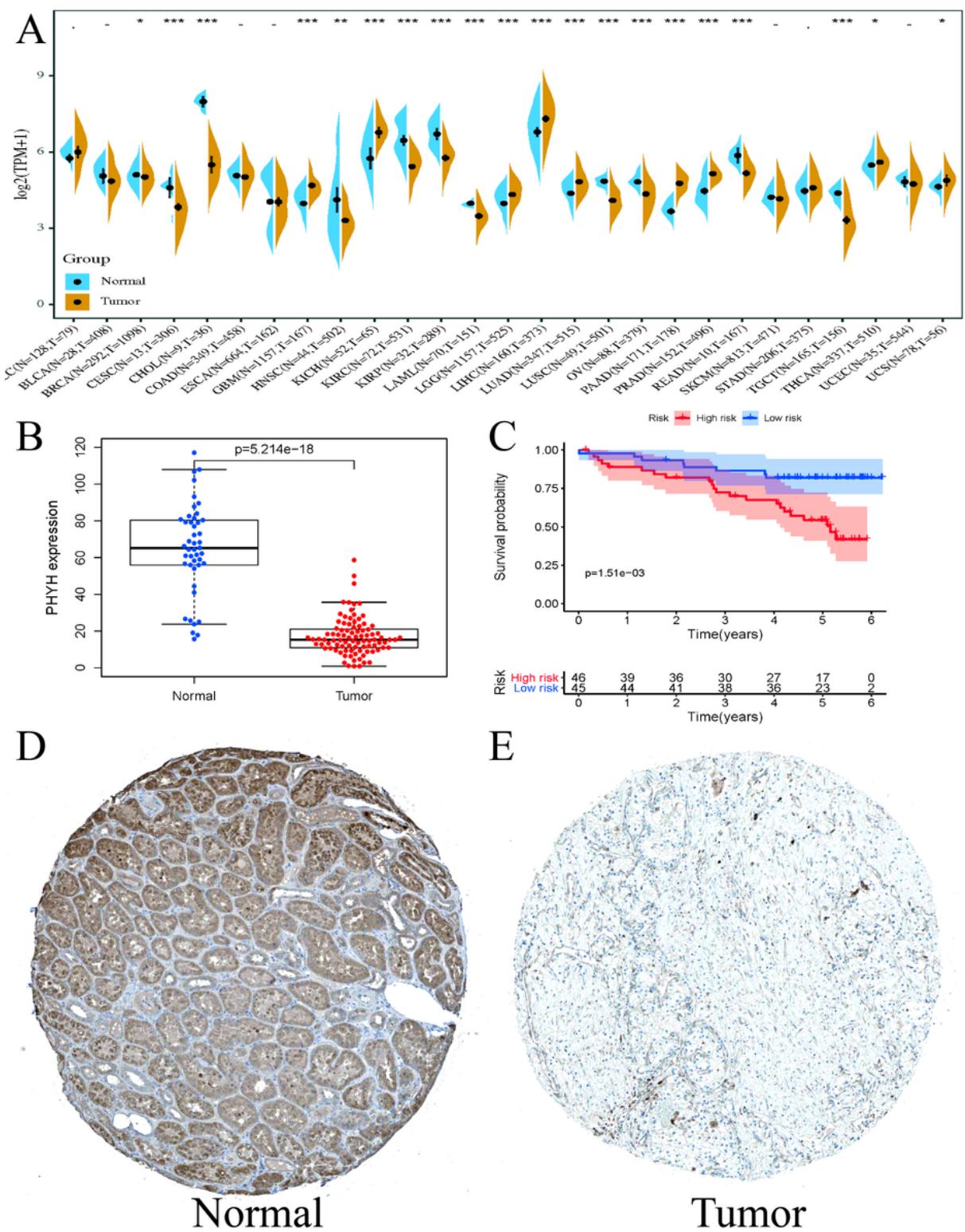


Figure 6

Verification of PHYH in ccRCC; (A) The expression level of PHYH in various cancers by GTEx and TCHA. (B) The boxplot of PHYH in ICGC database; (C) The survival analysis of PHYH in ICGC database; (D) The HPA database indicated the difference of immunohistochemistry in normal and kidney cancers.