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Phosphoenolpyruvate Carboxykinase 2 Could Be Used For distiguishment Of Hepatoblastoma And Affecting Ferroptosis

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KEYWORDS

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ABSTRACT

Hepatoblastoma (HB) is the most common malignant liver tumor in children, with low cure rates due to the lack of accurate predictive targets. Phosphoenolpyruvate carboxykinase 2 (PCK2) has been implicated in various tumors and may regulate ferroptosis, but its role in HB remains unexplored. This study analyzed patient tissues and integrated mRNA microarray and RNA-seq data to assess PCK2 expression and its diagnostic value. Using GSE104462 and enrichment analysis of PCK2 differentially co-expressed genes (DCEGs), we also examined its association with ferroptosis-related genes. PCK2 expression was significantly downregulated in HB (pooled SMD = -1.93), with sequencing data confirming the result. PCK2 showed strong diagnostic performance (AUC = 0.99, sensitivity = 0.97, specificity = 0.98) in distinguishing HB from normal tissues. Enrichment analyses suggested PCK2 may regulate HB progression via the HIF-1 and Ras signaling pathways. Furthermore, PCK2 expression increased in HB cells treated with erastin and correlated with ferroptosis-related genes, indicating its potential involvement in ferroptosis. In conclusion, PCK2 demonstrates excellent diagnostic potential and may play a key role in ferroptosis-related pathways in HB.

Introduction

In the past several years, the therapy of hepatoblastoma (HB) had obtained a considerable progress, multiple types of new adjuvant therapy combined with surgery could increase survival time and survival quality of patients significantly [1]. As the most common liver malignant tumors in children, HB is chiefly diagnosed before 2 years old and had a poor prognosis [2]. Although the existed therapies were able to significantly decrease the mortality of HB patients in early stages,

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the survival time of patients would be shorten once disease develops to advanced stages especially metastasis occurs [3]. Nowadays, the diagnosis of HB mainly depends on imaging methods, which caused parts of tumors in early stages could not be detected in time [4]. As a routine inspection and prediction index, alpha-fetoprotein (AFP) was applied to predict HB in early stages. However, the predicting of HB through inspecting AFP has great limitations as a result of the expression level of AFP usually upregulated physiologically in infants [5, 6]. AFP has a low specificity, so it is meaningless to predict HB by detecting expression level of AFP. Therefore, seeking for an target that could predict HB more accurately is vital for predicting HB and restricting its development in early stages.

Phosphoenolpyruvate carboxykinase 2 (PCK2, also known as PEPCK or PEPCK2), located on 14q11.2-q12, was perceived as having a function of encoding a mitochondrial enzyme [7]. To date, PCK2 had been reported that takes a place in metabolic of glucose in varies types of cancers. In a research managed by Grasmann et al. had pointed out that tumor cells were able to compose metabolites by regulating PCK2 expression when lacking of glucose, which inhibits cell apoptosis when energy deficiency [8]. Elisabeth et al. thought the expression of GLUT1 had a negatively correlated with PCK2, and high expressed PCK2 was benefit for gluconeogenesis pulmonary adenocarcinoma [9]. Moreover, Liu et al. also mentioned that PCK2 was probably related to liver tumors, and downregulated of PCK2 could induce apoptosis of liver cancer cells due to glucose deficiency [10]. Up to now, there has been more progress about regulating mechanisms of PCK2 in malignant tumors. Scott et al. induced ferroptosis through erastin in many tumors including HT-1080 and Calu-1, they reported there were different degrees of PCK2 expression, which means PCK2 might be related to ferroptosis in tumor cells [11]. Unfortunately, the relationship between PCK2 and ferroptosis was no further researched since it was first reported. In addition, though the expression levels and regulation mechanisms of PCK2 had been clarified in many tumors, we have not found any research about PCK2 and HB.

As mentioned earlier, PCK2 had a correlation with ferroptosis. Ferroptosis was first proposed by Dr. Scott and his colleagues in the study of RAS family enzume mutation [12]. As a newly reported way of apoptosis, researchers are committed to revealing the mechanisms of ferroptosis in the past decades. Jiang et al. reported GSH regulated by GPX would reduce the generation of ROS and reactive nitrogen, and inhibited the cellular antioxidant activity involved in Xc- system, which caused iron accumulates in cells and ferroptosis appeared [13]. In another researches carried by Jiang et al., the researchers reported that the activity of P53 might reduce the reactions of Xc- system, then regulated the process of ferroptosis [14]. At the same time, Yang et al. thought upregulated GPX4 was able to increase the antioxidant ability of tumor cells and impeded tumor cells ferroptosis [15]. Moreover, ferroptosis had been reported in many tumors such as head and neck cancer, gastric cancer and liver cancer [16-18]. However, the relationship between ferroptosis and HB had never been reported yet.

Therefore, we assesed the potential relationships between PCK2 and HB, and its potential regulating mechanisms involved. we herein analyzed the expression levels of PCK2 in common adults' and children's tumors, to systematically reveal the expression of PCK2 in a variety of tumors. Additionally, we discussed the expression level and prediction potentiality of PCK2 in HB through analyzing collectable RNA-seq and mRNA microarray. In order to explore the molecular mechanisms of differential expressed PCK2 in HB development, enrichment analysis were also applied to discuss the potential signaling pathways of PCK2 in HB. Ferroptosis was also been further discussed as an interesting point in the regulatory mechanisms.

Methods

Clinical Significnce of PCK2 in Common Adults' and Children's Tumors

First, we were trying to reveal the expression levels of PCK2 in adults' and children's pan-cancer, so we download and analyzed data from public databases. The Cancer Genome Atlas (TCGA) was managed by National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI), is a database collected thousands of patients with 39 types of cancers. Therapeutically Applicable Research To Generate Effective Treatments (TARGET) was an public database containing data of children's cancer, data of 7 cancer types common in children was downloaded available. We downloaded mRNA-seq series from the mentioned database and analyzed PCK2 expression levels. Moreover, to discuss the relationship between PCK2 and immune-related genes in varieties of adults' tumors, we also chose TISIDB database to analyze for the present study.j

Expression Level and Discrimination Potential of PCK2 in HB

We collected tumor and para tumor tissues from HB patients in the First Affiliated Hospital of Guangxi Medical University, and sequenced the obtained tissue samples. We performed the obtained sequencing data to analyze the expression of PCK2 in HB.

Previous study had never reported the expression level of PCK2 in HB, therefore, HB microarray and RNA-seq data series from Gene Expression Omnibus (GEO), Sequence Read Archive (SRA) and ArrayExpress database were searched and downloaded. 9 datasets were chosen for the followed analysis by using "hepatoblastoma" as the search keyword (Table.1).

Table 1 | The included HB related databases with PCK2 expression.

ID	Country	Platform	Year	Number of samples	
				HB	Non-HB
E-MEXP-1851	-	GPL571	2009	25	4
GSE75271	USA	GPL570	2016	50	5
GSE81928	USA	GPL16791	2018	23	9
GSE89775	USA	GPL16791	2016	10	3
GSE104766	France	GPL16791	2017	30	30
GSE131329	Japan	GPL6244	2019	53	14
GSE132037	Spain	GPL17586	2020	34	18
GSE133039	Spain	GPL16791	2020	34	32
GSE151347	Germany	GPL11154	2020	11	11
In-house	-	-	2021	3	3

Combining with samples from our hospital, 10 datasets were used for our study. We performed a $\log_2(x-1)$ conversion for above data in order to make the results more objective. Estimating standard mean difference (SMD) of 10 studies was performed by Stata 14.0. When $P < 0.05$ or $I2 > 50\%$, a random-effect model would be applied for the above studies, otherwise a fixed-effects model was adopted. Then, diagnostic test was applied to estimate the clinical significance of PCK2 in HB. Receiver operating characteristic (ROC) curves were plotted by IBM SPSS Statistics v23.0 and Graphpad Prism 8.0. Additionally, in order to objectively evaluate the potential of PCK2 in the diagnosis of HB, a summary receiver operating characteristic (sROC) was plotted. The area under the curve (AUC) of sROC represents the diagnostic value of PCK2.

The Exploration of Relationship Between PCK2 and Immune-Related Genes

Unlike adult's tumors, there is no straightforward analytical tool for analyzing the immune infiltration of PCK2 in HB. Therefore, we extracted immune-related genes from 9 public series and calculated Pearson's coefficient between immune-related genes and PCK2. In order to observe the results more intuitively, a heatmap was plotted, and some interesting points were highlighted.

The Identification of PCK2 Differential Expressed Genes (DCEGs)

Identification of PCK2 co-expressed genes (CEGs) in HB We aimed to find out those genes had close relationships with PCK2 and might be regulated or influenced by PCK2 in HB, thus, CEGs of PCK2 was screened. Genes expression data was extracted and Pearson's coefficients of genes were evaluated.We considered gene as one of the CEGs when $|r| > 0.3$ and $P < 0.05$. Moreover, PCK2 CEGs appeared more than three times in all 9 studies would be chosen for subsequent research.

Identification of PCK2 DEGs in HB According to the genes expression data from all of the studies, the SMD of all genes were extracted and calculated respectively. If lower 95%CI > 0 and SMD > 0, the gene would be considered to upregulate in HB. In the same light, If higher 95%CI < 0 and SMD < 0, the gene would be considered to downregulate in HB. The above genes was chosen as PCK2 DEGs.

CEGs and DEGs of PCK2 were intersected, the obtained genes would be identified as DCEGs of PCK2.

Functional Enrichment Analysis of PCK2 DCEGs

To further explore the molecular mechanisms and potential functions of differential expressed PCK2 regulated HB, DCEGs of PCK2 were entered into Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8. We chosen Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to plumb the potential signaling pathway. When $P < 0.05$, the GO terms and KEGG signaling pathways would be selected. Additionally, PPI networks was composed through Search Tool for the Retrieval of Interacting Genes (STRING). And Cytoscape v3.8.2 was applied to screened PCK2 hubgene.

The Exploring of Relationship Between PCK2 and Ferroptosis in HB

After enrichment analysis, we found some signaling pathways were correlated with ferroptosis in cancers. To reveal the relationship between PCK2 and ferroptosis in HB, we tried to collect public genes expression

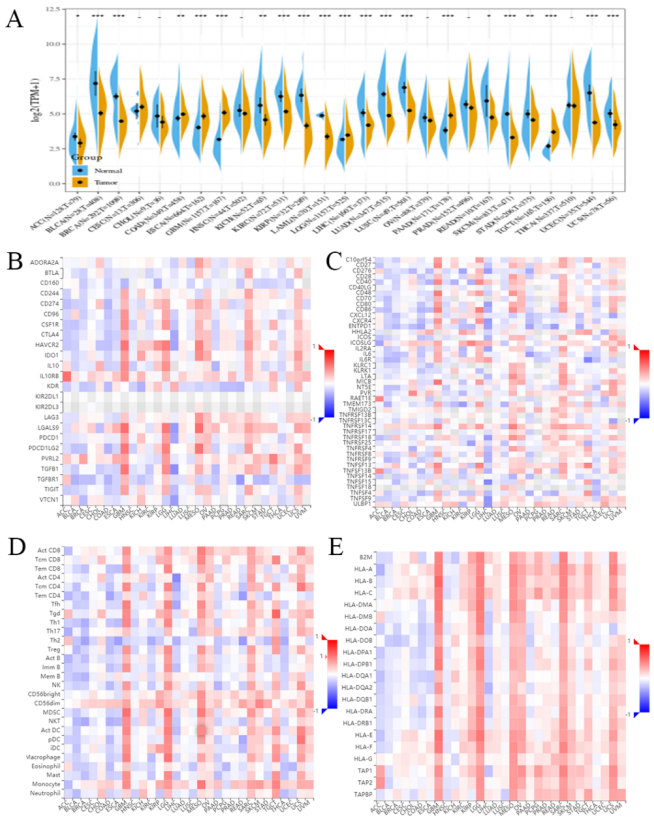

Figure 1 | (A) The mRNA expression levels of PCK2 in adults cancer. (B,C,D,E) Associations of the PCK2 expression levels with immunoinhibitors, Immunostimulators, lymphocytes and MHC molecules.

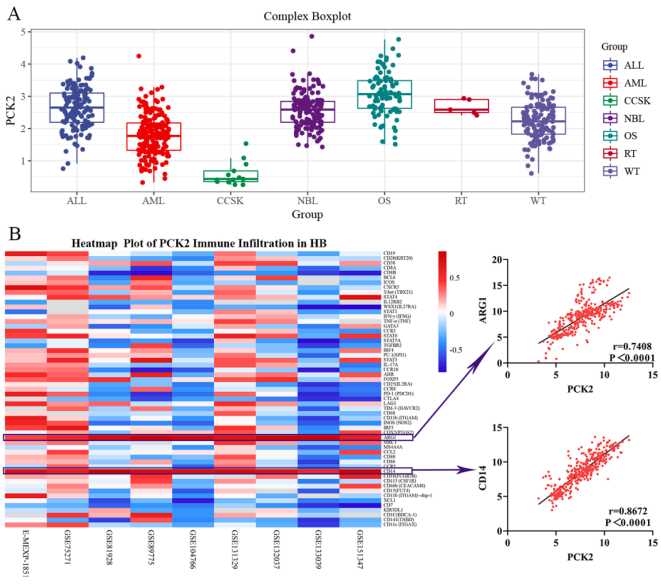

Figure 2 | (A) The expression levels of PCK2 in children cancer. (B) Heatmap plot of correlation between PCK2 and immune genes in HB based on 9 studies, ARG1 and CD14 was point out because of their high Pearson's r-value.

data about ferroptosis in HB. However, only 1 series was found, GSE104462 was chosen for the followed analysis. We first evaluated the expression level of PCK2 between ferroptosis inducer erastin and dimethyl sulfoxide (DMSO), and DEGs of PCK2 after treating with erastin were screened out. In addition, we plotted a heatmap, to visually observe the correlation between PCK2 and ferroptosis related genes in the all studies mentioned earlier.

Results

Clinical Significnce of PCK2 in Common Adults' and Children's Tumors

Based on TCGA database, the expression of PCK2 in common adults' tumors were extracted and assessed, the results show that PCK2 was differential expressed in multiple types of tumors. The violin plot displayed the results intuitively (Figure 1 A). Besides, correlation analysis between PCK2 and immune-related genes in various of cancers also be performed. Through TISIDB, the correlation analysis results indicated PCK2 was related to a various kind of immune-related genes in adults' cancers (Figure 1 B-E).

Showing only the expression levels of PCK2 in adults' tumors were still not convincing, so we collected and analyzed data from TARGET. The results were showed on Figure 2 A. Unfortunately, due to lacking of healthy control groups, we were not able to show whether the expressions of PCK2 in tumors tissues are different from that in normal tissues.

Immune-Related Analysis of PCK2 in HB

In order to explore the potential correlation between immune-related genes and PCK2 in HB, we extracted expression data of immune-related genes from collectable datasets and calculated Pearson's correlation coefficients between them. We plotted a heatmap plot to show our results, ARG1 and CD14 was highlighted because of thier high Pearson's r-values (Figure 2 B). The correlation analysis results indicated that PCK2 was

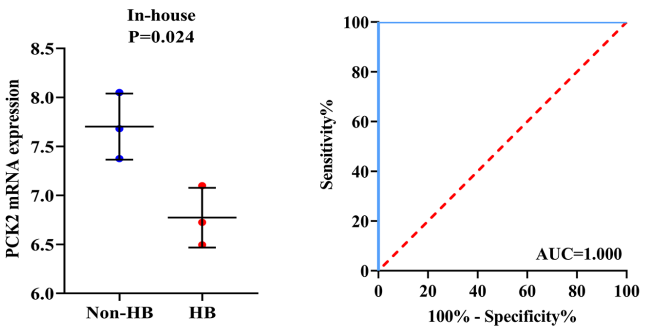

Figure 3 | The expression data and ROC curve of PCK2 from HB patients tumor and para tumor tissues, data from our hospital. The result indicated that PCK2 was significantly downregulated in HB.

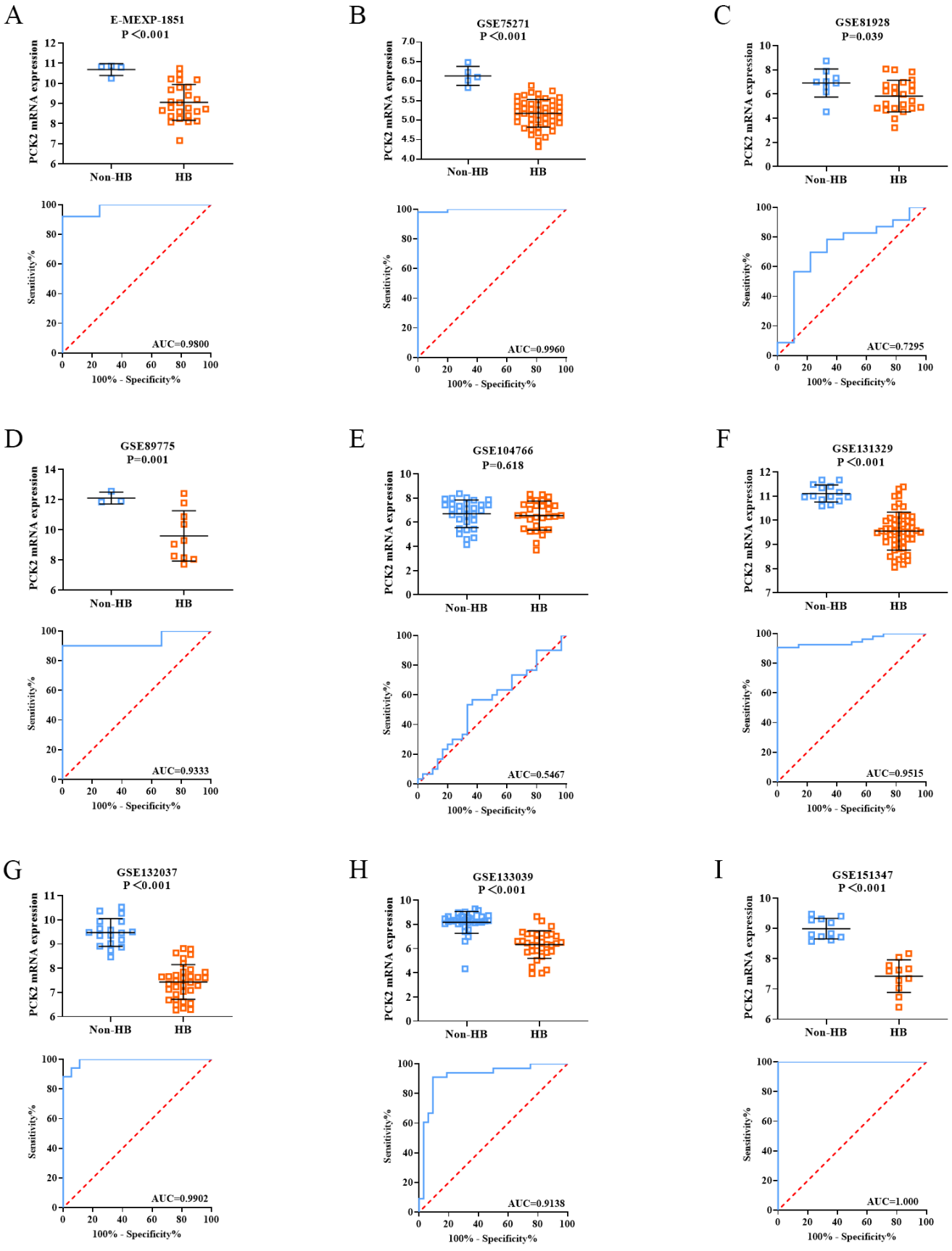

Figure 4 | The expression levels and diagnostic ability of PCK2 in 9 datasets. HB, hepatoblastoma.

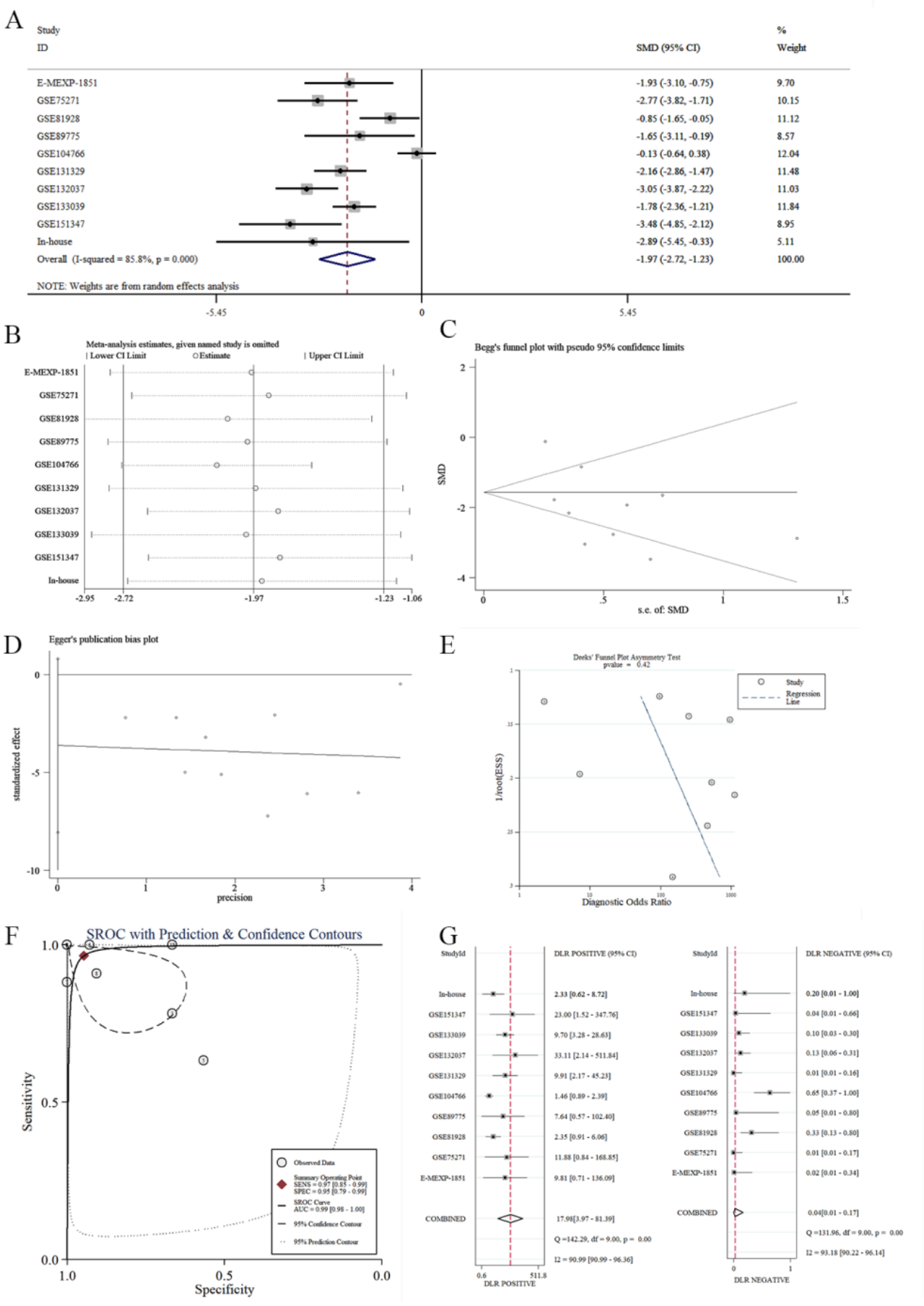

Figure 5 | The mRNA expression level and discrimination potential of PCK2 in HB based on 9 studies.

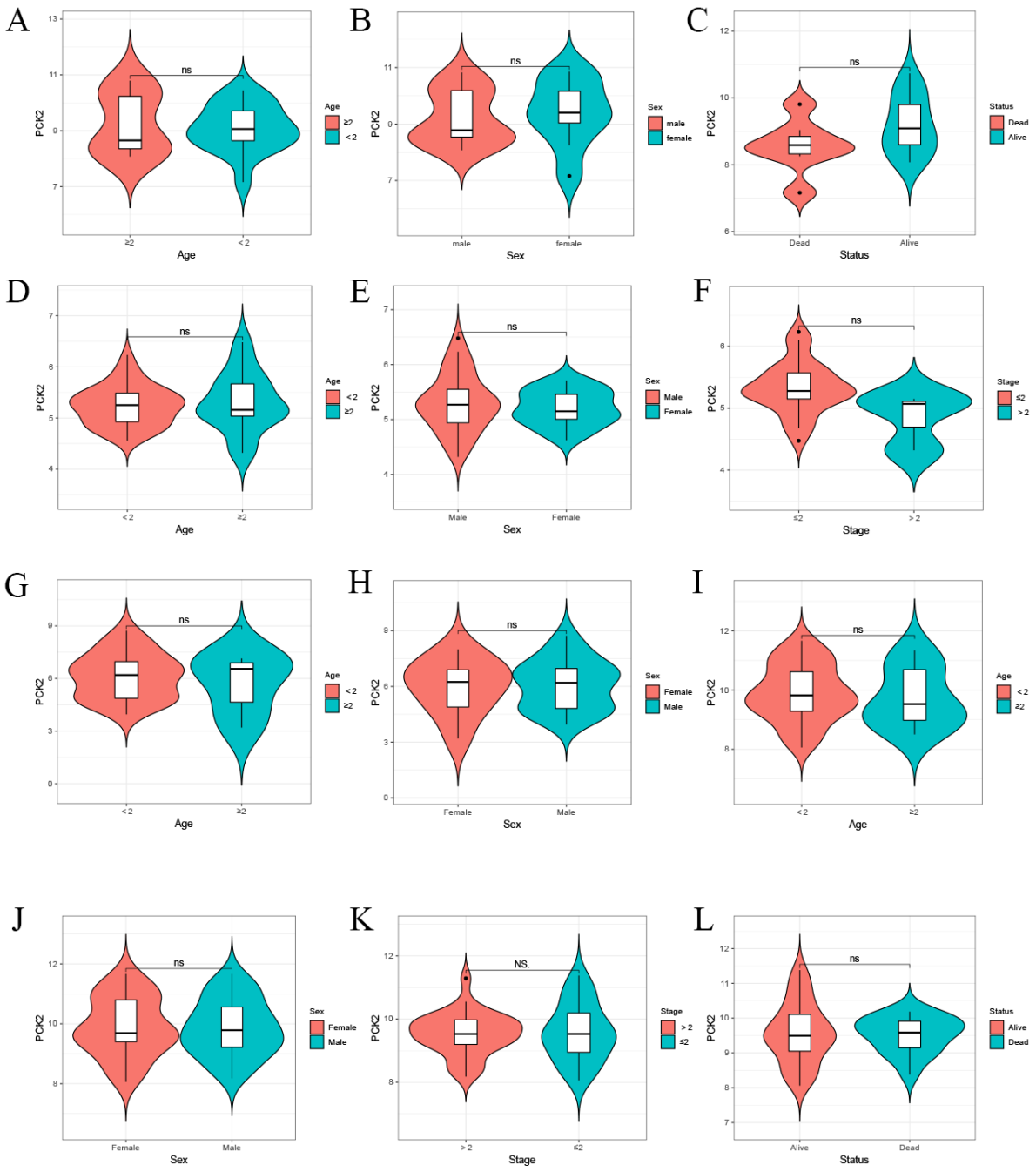

Figure 6 | Expression of PCK2 in tissues of patients with different clinical parameters. (A-C) Data from E-MEXP-1851. (D-F) Data from GSE75271. (G, H) Data from GSE81928. (I-L) Data from GSE131329.

correlated with many immune-related genes.

Expression Level and Discrimination Potential of PCK2 in HB

We analyzed the sequencing data from our hospital. The results showed that the expression of PCK2 was significantly downregulated in HB compared with normal tissues (Figure 3).

To further reveal the mRNA expression of PCK2 in HB, the mentioned collected series from public database and in-house sequencing data were performed to expression analysis. We plotted scatter plots and ROC curves to show the expression levels and discrimination potential of PCK2 in these HB datasets (Figure 4). After that, we estimated SMD to combine all of the datasets (Figure 5 A). A random-effect model was performed because of I^2 = 82.7% and $P < 0.001$. The forest plot results indicated that PCK2 was downregulated in HB (SMD = -1.93, and 95%CI = -2.70 to -1.15). Though we performed a heterogeneity analysis, the significant heterogeneity still could not been found out (Figure 5 B).

Table 2 | The means and standard deviations of PCK2 expression values for HB and non-HB based on 10 studies.

Study	Sample type	HB			Non-HB		
		N	M	SD	N	M	SD
E-MEXP-1851	Tissue	25	9.054	0.889	4	10.681	0.291
GSE75271	Tissue	50	5.176	0.354	5	6.134	0.242
GSE81928	Tissue	23	5.819	1.308	9	6.899	1.161
GSE89775	Tissue	10	9.597	1.667	3	12.101	0.394
GSE104766	Tissue	30	6.544	1.199	30	6.695	1.142
GSE131329	Tissue	53	9.554	0.783	14	11.106	0.354
GSE132037	Tissue	34	7.433	0.716	18	9.477	0.573
GSE133039	Tissue	34	6.328	1.138	32	8.165	0.904
GSE151347	Tissue	11	7.424	0.539	11	8.994	0.340
In-house	Tissue	3	6.773	0.305	3	7.702	0.338

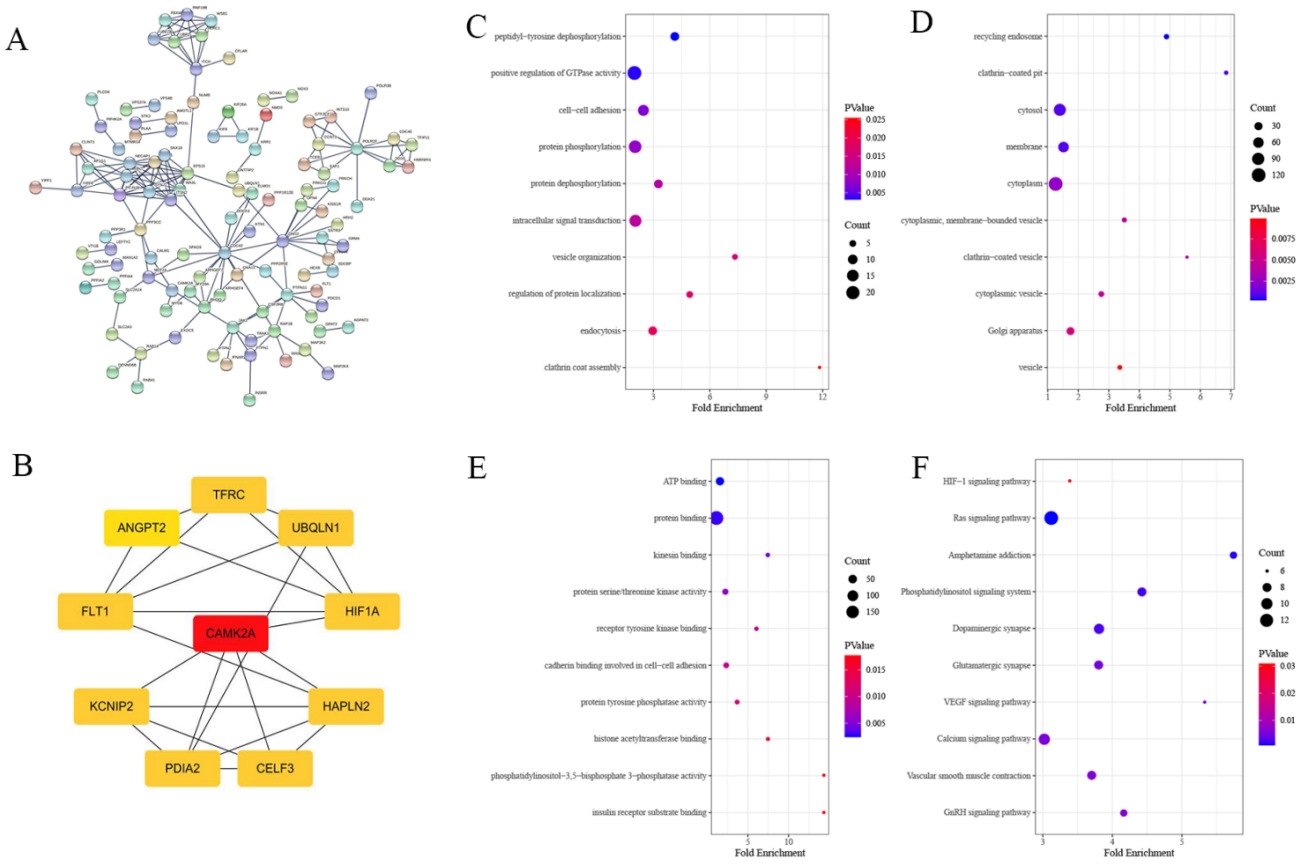

Figure 7 | Enrichment analysis. (A) PPI network analysis of PCK2 DCEGs. (B) CAMK2A was identified as PCK2 hub-gene through MCC calculation. (C) Enrichment terms of PCK2 DCEGs in biological process. (D) Enrichment terms of PCK2 DCEGs in cellular component. (E) Enrichment terms of PCK2 DCEGs in molecular function. (F) Enrichment terms of PCK2 DCEGs in Kyoto Encyclopedia of Genes and Genomes.

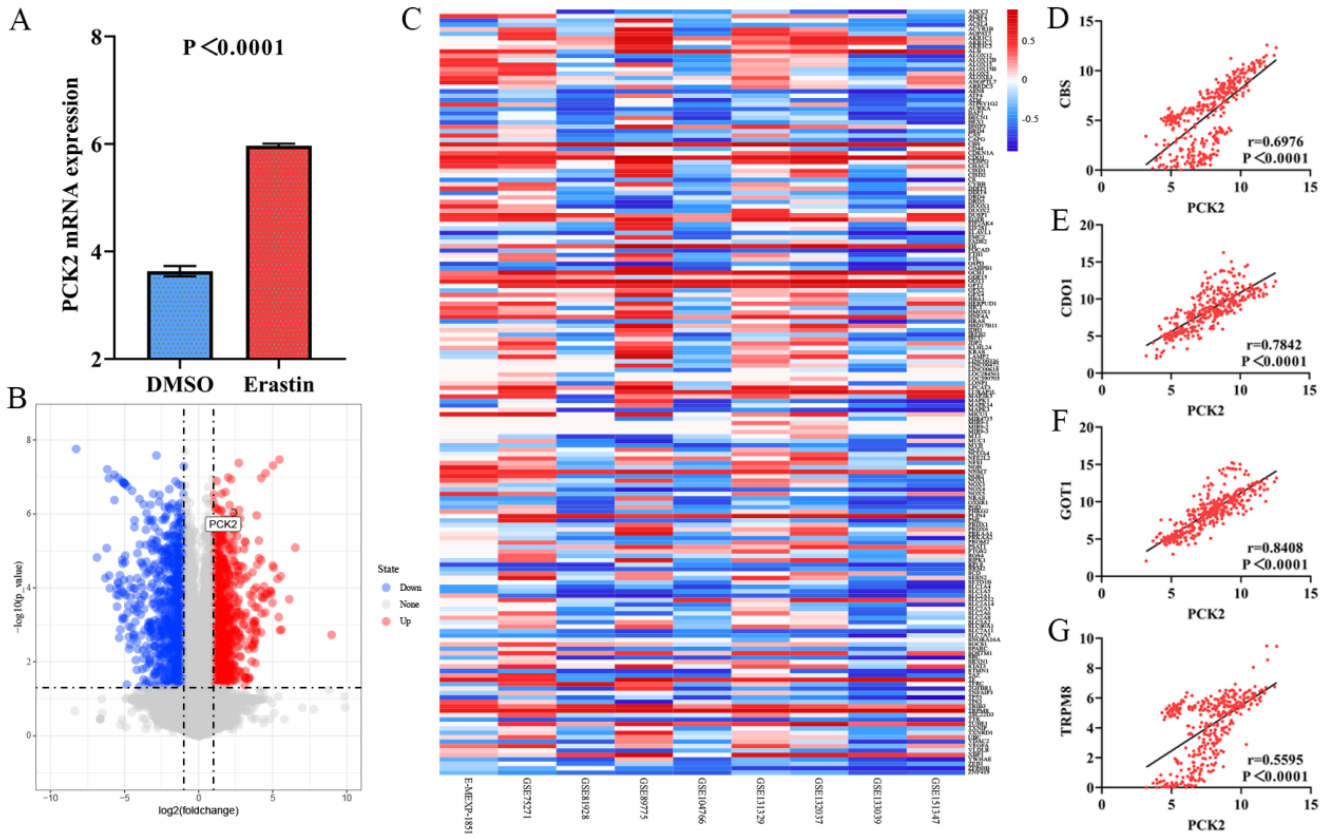

Figure 8 | Differential expressed PCK2 probably related to ferroptosis. (A) Compared with the HB tissue treated with DMSO, the HB tissue treated with erastin expressed higher levels of PCK2. (B) Volcano plot of DEGs in HepG2 cells treated with erastin. (C) Heatmap plot of correlation between PCK2 and other ferroptosis related genes in 9 studies. (D-G) PCK2 had high correlations with some ferroptosis-related genes.

Additionally, no publication bias was observed in funnel plots (Figure 5 C-E). A sROC curve was also plotted and the curve showed that the AUC of PCK2 value was up to 0.99 (95%CI = 0.98 to 1.00), which means PCK2 had an extremely high discrimination potential in HB (Figure 5 F). The raw mean and standard deviation values of PCK2 expression for both HB and non-HB samples across the 10 datasets are summarized in Table 2, which formed the basis for the SMD and diagnostic performance analyses. Besides, to reveal if different clinical parameters influence PCK2 expression, we collected and analyzed patients' clinical information. The violin plots were showed on Figure 6. Considering that PCK2 was significantly downregulated in HB and had no difference in patients with different clinical significance, PCK2 with high sensitivity and specificity is completely suitable for the prediction of HB.

Functional Enrichment Analysis of PCK2 DCEGs

DCEGs of PCK2 were entered into STRING and structured a PPI network to show the connectedness among screened DCEGs (Figure 7 A). Moreover, through calculating, CAMK2A was screened as hub-gene (Figure 7 B). Based on GO analysis, PCK2 DCEGs were enriched in peptidyl-tyrosine dephosphorylation, recycling endosome and ATP binding pathways (Figure 7 C-E). According to KEGG enrichment analysis, most of PCK2 DCEGs were mainly enriched in HIF-1 signaling pathway and Ras signaling pathway (Figure 7 F).

The Relationship Between PCK2 and Ferroptosis in HB

After searching for literature, we found PCK2 was related to ferroptosis. Interestingly, HIF-1 and Ras signaling pathway, which was identified as PCK2 potential signaling pathway in KEGG, was also reported correlated with ferroptosis. Therefore, we decided to further explore the relationship between PCK2 and ferroptosis in HB. First, the expression data of PCK2 was extracted from GSE104462, and the results demonstrated that PCK2 was upregulated in HepG2 cells after treating ferroptosis inducer erastin (Figure 8 A). By calculating correlation coefficient between PCK2 and reported ferroptosis-related genes, we surprised to find that PCK2 had significantly correlations with some ferroptosis-related genes such as CBS, CDO1, GOT1 and TRPM8

(Figure 8 C-G). All of our results implied that differential expressed PCK2 probably induces ferroptosis in HB.

Discussion

The present study had explored the expression levels of PCK2 in multiple adults' and children's cancer types, and revealed the expression and prediction potential of PCK2 in HB through large samples (n=401) . To our knowledge, this is the first time to propose PCK2 was downregulated and had an excellent potential to discriminate HB patients from healthy people. Moreover, through enrichment analysis, we predicted the potential signaling pathways of differential expressed PCK2 in regulating HB development, which providing a new thought for future researches. The relationship between PCK2 and ferroptosis in HB was also been further discuss for the first time.

From the previous researches, we found that the relationship between PCK2 and immune system in tumor cells were still a mystery. Through extracting the expression levels of immune-related genes from public datasets, we herein analyzed and discussed the relationship between PCK2 and immune-related genes. The results showed that PCK2 had high correlations with many types of immune-related genes, among these genes, PCK2 is particularly closely related to ARG1 and CD14. In the previous reports, we found CD14 was also related to HB, which showed up in regulating the development of HB through influencing immune-related pathways. Segerer et al. thought differential expressed CD14 was able to reverse the activity of thrombopoietin (TPO), then influenced the proliferation and migration of HB [19]. Wang et al. observed HepG2 cells could show immune responses to LPS in vitro, and liver would be damaged by upregulating immune-related genes such as iNOS, COS-2, CD14 and others [20]. However, the above articles were published long ago, we were regretted that no more research has been found to support our results. Interestingly, many studies had indicated that immune-related pathways might affects the development of HB. A report written by Aran et al. showed that CD5L was upregulated in HB cells, which would induce non tumor cell immune response, and promoted tumor colony formation and cell proliferation, played an inhibition role in the apoptosis of tumor cells [21]. In a study of Yang et al., the authors reported CXCL5 was overexpressed in HepG2 cells and enhanced the induced activation of granulocytes, and regulated expression of a variety of immune genes including IL-18 and IL-1β , then enhanced the proliferation and migration of HB cells [22]. Although exist evidences had showed correlations between immune-related pathways and development of HB, how PCK2 affects the proliferation and invasion of HB through immune-related pathways remains to be further studied.

To date, other researchers had revealed the clinical significance of PCK2 in various types of malignant tumors. Xiong and colleagues found Silenced PCK2 could inhibit the development of renal cell carcinoma through endoplasmic reticulum stress pathway, and improved sensitivity of targeted drugs [7]. Yun et al. reported that downregulated PCK2 would inhibit the invasion and metastasis of tumor cells in different laryngeal squamous cell carcinoma stages, this process probably related to hypoxia [23]. Furthermore, Luo's group also reported downregulated PCK2 was able to regulate tricarboxylic acid cycle pathway in melanoma cells, and involved the energy metabolism processes, proliferation pathways and development of melanoma cells [24]. Herein, we found PCK2 was lowexpressed in HB, sROC also showed that predicting HB with PCK2 had a high sensitivity and specificity. Moreover, through analyzing the expression levels of PCK2 in HB patients with different clinical parameters, we surprised to find the expression of PCK2 had no correlation with all collectable clinical parameters, which is undoubtedly conducive to the application of PCK2 in the clinical prediction of HB. However, the clinical parameters we could get were relatively limited and still need to be verified by follow-up studies.

Enrichment analysis showed that PCK2 DCEGs were significantly enriched in HIF-1 and Ras signaling pathways. When the authors wondered about the relationship between these two pathways and HB or PCK2, we had an unexpected found. HIF-1 signaling pathway was reported correlated with ferroptosis, PCK2 was also been reported related to ferroptosis in many types of tumor cells [11]. As mentioned earlier, ferroptosis is a kind of prospected cell apoptosis pathway. In Wu's study, through extracting expression of ferroptosis-related genes and predicted potential signaling pathways, the researchers found ferroptosis-related genes mainly enriched in HIF-1 signaling pathway, which is consistent with our results [25]. At the same time, Wu et al. also indicated that there was an interaction between ferroptosis-related genes and immune-related pathways [25]. Although the above results were based on different tumors, the high similarity among our results was still persuasive. By analyzing gene expression profile of leukemia cells induced by ferroptosis inducer, Luo et al. indicated that DEGs significantly enriched in HIF-1 signaling pathway and HIF-1 signaling pathway might be closely related to occurrence of ferroptosis [26]. Additionally, Hartman's research showed melanoma cells plasticity regulated by Ras signaling pathway,which mainly reflected in cells death and influencing ferroptosis process of melanoma cells [27]. Ye et al. also reported that HMGB1 regulated leukemia cells ferroptosis induced by erastin through Ras signaling pathway [28]. Unfortunately, we did not found report about the mentioned signaling pathways in HB, the present study is probably the first time to propose that HIF-1 and Ras signaling pathways are associated with iron death in HB.

Until now, the process and mechanisms of ferroptosis have not been clarified in HB, but abnormal iron metabolism in HB had been revealed. Hirayama et al. pointed out that the expression level of transferrin was different in HepG2 cells treated with different cell factors, this difference would lead to the accumulation of iron in Hb cells to varying degrees [29]. Kamiya et al. analyzed the expression of ferroptosis-related proteins in HepG2 cells and thought interacted effects of iron responsive elements (IRE) and iron responsive element-binding proteins (IRE-BP) probably participate in the metabolism and accumulation of iron in HB cells [30]. Both of the researches did not describe the outcome of iron accumulation in HB cells in detail, but according to research managed by Li, we have reason to believe that a large amount of iron accumulation will inevitably lead to cell ferroptosis [31].

Some limitations also existed in the present study. First, we could not confidently identified the function and molecular mechanisms of PCK2 in HB based on 401 samples, though our study included the largest sample size at present. Subsequently, in the analysis of PCK2 expression in children's common malignant tumors, we failed to compare the differences of PCK2 between tumors and healthy tissues due to lacking of control groups. Finally, the results on the potential mechanisms of PCK2 in HB including ferroptosis, HIF-1 and Ras signaling pathway still needed further experiments to verify.

In short, based on results of mRNA and sequencing data from public databases and our hospital, it was the first time to report PCK2 was downregulated in HB, and we also indicated PCK2 had an extremely high sensitivity and specificity. Moreover, PCK2 was also considered to be associated with ferroptosis in HB.

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