

PHTF2 regulates lipids metabolism in gastric cancer

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ABSTRACT

Identification of hub genes and key pathways of gastric cancer was recognized to be essential to elucidate the tumorigenesis of GC. This study was aimed to identify the differentially expressed genes (DEGs) in GC via bioinformatics methods and their related pathways involved in the pathological process of GC. Gene expression profile datasets acquired by microarray chips or RNA-seq were downloaded from GEO dataset and TCGA, and 298 differentially expressed genes was identified. The Gene Ontology (GO) and Kyoto Gene and Genomic Encyclopedia (KEGG) pathways of DEGs were then analyzed by the DAVID database to elucidate the potential molecular functions of DEGs. The protein-protein interaction (PPI) network of DEGs was further analyzed with the STRING database and PHTF2 was identified as a hub gene in the PPI network. Subsequently, PHTF2 was found to be highly expressed in different subtypes of gastric cancer tissues obtained from TCGA database or clinical patients, resulting with a poor prognosis. By GSEA, PHTF2 was found to significantly enrich the fatty acid metabolism pathway in gastric cancer. Moreover, PHTF2-regulated lipids metabolism significantly affected the tumorigenesis of GC cells. In summary, this work identified a new mechanism by which PHTF2 precipitated in the pathological process of GC by regulating cellular lipid metabolism.

INTRODUCTION

Epidemiological data have indicated that gastric cancer (GC), a malignant tumor in the digestive system, was the third cause for tumor-related death owing to its high recurrence rate, and was also accompanied with an increasing incidence and mortality worldwide [1, 2]. Although multiple treatment strategies have been comprehensively utilized, GC remained one of the more risk-prone tumors due to the poor prognosis of patients over the past few decades [3]. The significant role of changes at intracellular molecular levels, such as genetic mutations, epigenetic changes and abnormal molecular signaling pathways caused by helicobacter pylori infection, obesity, nitrite and other carcinogenic factors, was gradually being recognized in the development and metastasis of GC cells [4, 5]. Therefore, identification of biomarkers and their molecular functions in GC was essential for the development of the specific treatment for GC.

Interestingly, studies have revealed that more lipid metabolism-related proteins, identified as oncogenes in eukaryotic cells, were highly expressed in many human cancers [6, 7]. A high prevalence of gastric cancer was found in overweight individuals by epidemiological studies, and the significance of fatty acid metabolism in the development and progression of GC was increasingly recognized by scholars [8, 9]. Moreover, lipids accumulation was also observed in the pathological tissues in GC [10]. In the mouse model of gastric cancer, it was found that tumor tissues were often accompanied by up-regulated fatty acid metabolism-related proteins expression [11, 12]. Interestingly, down-regulation of lipid-proteins in these mice can significantly delay the occurrence of GC [11]. However, the association between fatty acid metabolism and the pathological development of gastric cancer remained confusing, and the relationship between lipid metabolism and clinical prognosis in GC was not explored.

Putative homeodomain transcription factor 2 (PHTF2), an evolutionarily conserved gene, was speculated as a transcription factor, involved in mediating stress transcription -. Gene Ontology (GO) annotations related to PHTF2 included DNA-binding transcription factor activity [13–15]. However, studies on the involvement of PHTF2 in regulating cancer pathology have not been reported.

In this study, we first identified PHTF2 as a hub gene and revealed its expression pattern in GC tissues by bioinformatics methods, and further confirmed its function in the tumorigenesis of gastric cancer cells via mediating intracellular lipids metabolism. Our research provided a new perspective for intracellular fatty acid metabolism in the development and progression of gastric cancer.

RESULTS

PHTF2 is identified as a hub gene in gastric cancer

In order to identify the potential hub genes involved in the pathological process of gastric cancer, two gene expression profile datasets of gastric cancer were selected from GEO. The results revealed that GSE79973 dataset contained 2406 differentially expressed genes (DEGs) and GSE19826 was identified to include 1924 DEGs according to the established criteria for screening DEGs. Furthermore, 7919 genes were found to be differentially expressed in gastric cancer tissues compared with para-cancerous samples collected from TCGA datasets. Calculated by FunRich software, 298 genes were identified to overlap in the three datasets, such as PHTF2, SPARC, TIMP1, LOX, VCAN, MMP14, and SPP1 (Figure 1A).

By enriching the Gene Ontology terms and KEGG pathways of DEGs, we noted that 298 DEGs were significantly enriched in processes associated with cancer development and progression, such as regulation of cellular protein metabolism processes (GO: 0032268), signal transduction regulation (GO: 1902531), cell growth (GO: 0007049), cancer center carbon metabolism (Hsa05230), insulin signaling pathway (Hsa02520) and mTOR signaling pathway (Hsa04150) (Figure 1B–1D).

By generating PPI network of 298 DEGs by the STRING online tool, the inter-molecular correlation index was calculated to identify hub genes in the PPI network. Interestingly, PHTF2 presented a complex interaction network and was identified as a hub gene in the PPI network (Figure 1E, 1F).

PHTF2 is significantly up-regulated in gastric cancer

To explore the potential role of PHTF2 in the development and progression of GC, we next analyzed the expression pattern of PHTF2 in tumors. The “Oncomine” database showed that PHTF2 was up-regulated in most tumors, especially in gastric cancer, where PHTF2 mRNA was detected significantly higher than para-cancerous tissues (Figure 2A). Considering the differences in subtypes of gastric cancer tissues, gastric cancer samples acquired from TCGA were classified according to histological typing, and it was found that PHTF2 in different gastric cancer subtypes mostly showed an up-regulated expression pattern compared with adjacent tissues (Figure 2B). Furthermore, the expression pattern of PHTF2 was found to gradually increase with the tumor pathological grade increased (Figure 2C). Surprisingly, gastric cancer patients with high expression of PHTF2 showed poor prognosis survival compared to patients with low expression of PHTF2 (Figure 2D). To further explore a comprehensive overview of the expression pattern of PHTF2 in gastric cancer, we searched the CCLE online database to further examine the expression variation of PHTF2 between different cell lines. The results showed that PHTF2 was generally over-expressed in more than half of the gastric cancer cells, especially in GCIY and NUGC3 cells.

In addition, 45 clinical samples were collected to further confirm the expression pattern of PHTF2 in gastric cancer. Paired student-*t* test showed that the expression of PHTF2 was statistically up-regulated in gastric cancer tissues (Figure 2F). Consistently, IHC results indicated PHTF2 expressed in gastric cancer tissues was significantly different from that in paracancer tissues ($\chi^2=11.429$, $P<0.001$). Obviously, the proportion of cases with high PHTF2 expression in gastric cancer tissues was significantly higher than that in adjacent tissues (Figure 2H, 2I). Whatever, these results strongly suggest that the expression of PHTF2 is up-regulated in gastric cancer.

PHTF2 affects the intracellular contents of lipids in GC cells

To further verify the role of PHTF2 in the pathological process of gastric cancer, GSEA assay was used to analyze the TCGA dataset, and PHTF2 was found to be significantly associated with the fatty acid metabolic pathway (NES value=1.22, $P<0.01$) (Figure 3A). Numerous studies have revealed significant risk of tumorigenesis in obese populations, and fatty acid metabolism played a key role in the tumorigenesis of various types of tumor cells [16–18]. These findings derived us to speculate that PHTF2 may be involved in

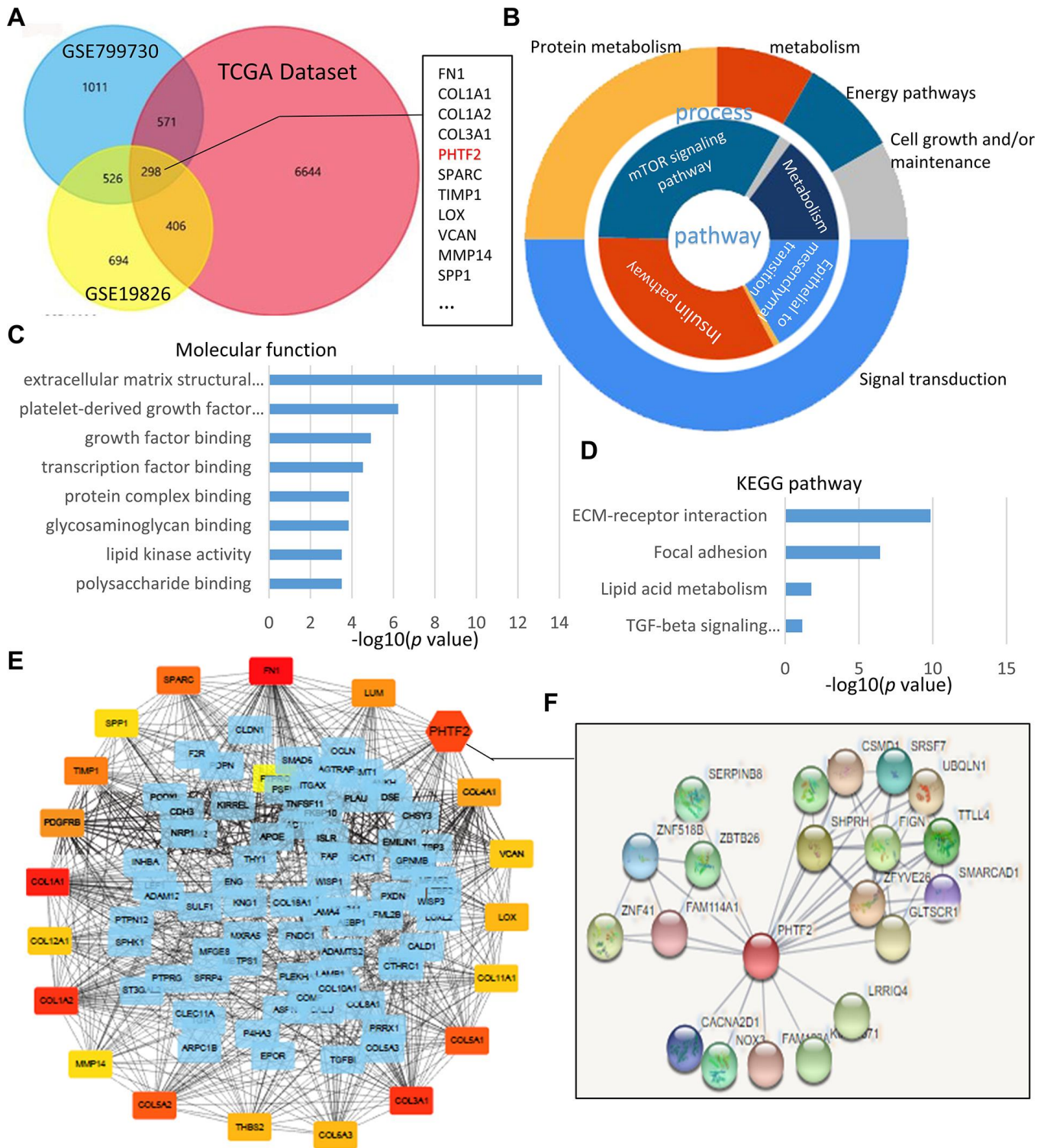


Figure 1. PHTF2 was identified as a hub gene in GEO datasets and TCGA database. (A) Venn chart of the different-expressing genes in GSE79973, GSE19826 and TCGA gastric cancer datasets, which overlapped according to the analysis of FunRich software. (B) Pie chart for illustration of different-expressing genes molecular pathways and process analyzed by DAVID online tools. (C, D) Molecular function and KEGG pathway enrichment of differentially expressed genes analyzed by DAVID online tools. (E) On line tool of STRING database analysis of the PPI network for the different-expressing genes. (F) Amplification of the network for PPI associated with PHTF2.

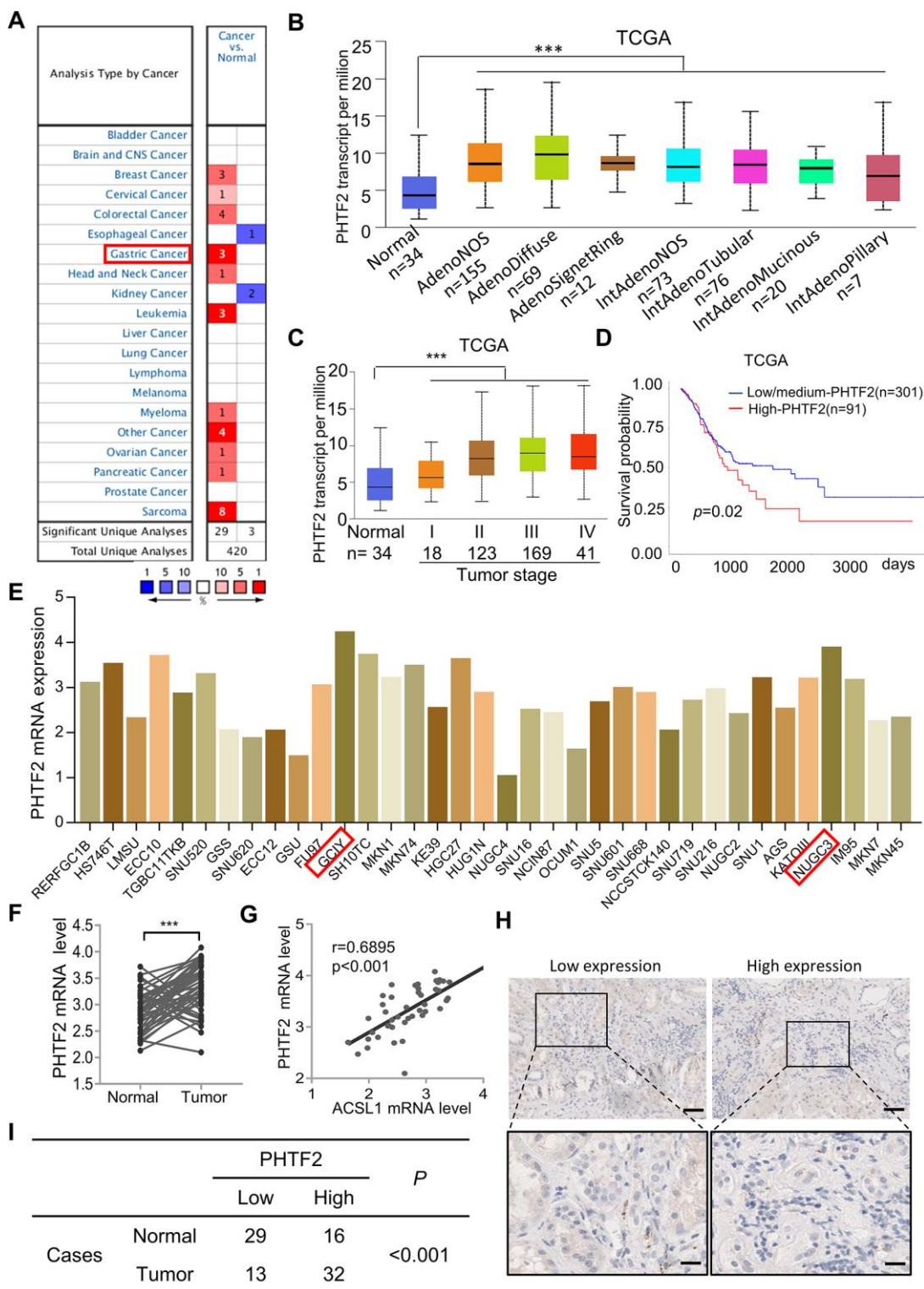


Figure 2. PHTF2 was significantly up-regulated in gastric cancer. (A) Expression pattern of PHTF2 in differential human malignancies from “Oncomine” database. (B) PHTF2 was significantly up-regulated in different histological subtypes of gastric cancer. (C) PHTF2 mRNA expressed in four different stages of cancerous tissues compared to para-cancerous tissues based on TCGA gastric cancer dataset. (D) Overall survival of patients with gastric cancer was calculated using Kaplan–Meier analysis according to the PHTF2 mRNA expression lever. (E) PHTF2 is commonly up-regulated in various gastric cancer cell lines from CLE database. (F) PHTF2 mRNA expression pattern in 50 pairs of human gastric cancer tissues (Cancer) and adjacent tissues (Normal). (G) The expression pattern of ACSL1 and PHTF2 was associated in gastric cancer tissues. (H) Representative IHC image show PHTF2 protein expression pattern in gastric cancer tissues. (I) Different distribution of PHTF2 was statistically analyzed with Chi-square test.

the development and progression of gastric cancer via mediating lipids metabolism.

According to the genes enriched in fatty acid metabolism pathways by GSEA assay (Figure 3B), several key fatty acid metabolic enzymes were detected in PHTF2 knockdown gastric cancer cells in order to assess the effects of PHTF2 on fatty acid metabolism. Interestingly, it was found that fatty acid metabolic enzymes such as ACSL1, ACSL3, HADH, ADH4 and ALSH1B were significantly down-regulated at the mRNA level resulted by silencing PHTF2 in GCIY and NUGC3 cells. However, exogenously overexpressing PHTF2 significantly restored the expression of these fatty acid metabolizing enzymes (Figure 3C).

In addition, fatty acids and their metabolic intermediates in gastric cancer cells were detected in order to confirm whether PHTF2 played a role in lipid metabolism. The results showed that PHTF2 knockdown significantly down-regulated the levels of TAG and PLs in GCIY and NUGC3 cells, while restoration of PHTF2 increased TAG and PLs contents in GCIY and NUGC3 cells (Figure 3D, 3E). Consistently, it also showed that knockdown PHTF2 significantly reduced the content of neutral lipids in GCIY and NUGC3 cells, while overexpression of PHTF2 could restore neutral lipid levels (Figure 3F). These results indicated that PHTF2 can affect the expression of fatty acid metabolism-related enzymes, and then regulate the fatty acid metabolism process in GC cells.

In addition, to further determine whether PHTF2 regulates lipid metabolism in gastric cancer cells, ACSL1 was selected as a target and depleted in gastric cancer cells. Not only because PHTF2 was involved in regulating ACSL1 expression in GCIY and NUGC3 cells (Figure 3C), but because ACSL1 was a key factor in lipid biosynthesis and fatty acid degradation in tumors, whose expression was also confirmed to be significantly correlated with the expression pattern of PHTF2 in gastric cancer (Figure 2G). The results showed that ACSL1 knockdown significantly reduced the contents of triglycerides, phospholipids, and neutral lipids in GCIY and NUGC3 cells. Surprisingly, exogenous PHTF2 overexpression significantly restored intracellular levels of triglycerides, phospholipids, and neutral lipids, as restorative expression of ACSL1 did (Figure 3G–3I). Furthermore, exogenous PHTF2 overexpression restored ACSL1 expression to some extent in ACSL1-depleted cell lines (Figure 3J). These results indicate that PHTF2 can affect the expression of fatty acid metabolism related enzymes, which further regulate the fatty acid metabolism in gastric cancer cells.

PHTF2 promotes tumorigenesis of gastric cancer cells via mediating lipids metabolism

To evaluate the role of PHTF2 on the tumorigenic ability of gastric cancer cells, PHTF2 was stably silenced in GCIY and NUGC3 cell lines, and cell viability was observed to be significantly downregulated caused by PHTF2 knockdown, but restoration of PHTF2 expression was found to rescue cell viability to some extent (Figure 4A). Furthermore, knockdown PHTF2 caused a significantly declining in colony formation, while more clones were observed in the PHTF2 re-expression group (Figure 4B, 4C). Therefore, the above results can be inferred that the up-regulated PHTF2 expression in gastric cancer cells was a risk of tumor-promoting factor.

The effect of PHTF2 on tumorigenic ability in GC was further examined in a xenograft mouse model. Interestingly, PHTF2 knockdown in the NUGC3 cell line caused a much slower tumor growth rate, a smaller size, and a lighter weight of formed tumors, while recovering PHTF2 expression significantly reversed the stagnant tumorigenesis of PHTF2 silenced NUGC3 cell line (Figure 4D–4F). Interestingly, PHTF2 knockdown similarly resulted with a declining expression pattern of above-mentioned fatty acid metabolic enzymes enrich by GSEA assay (Figures 3B, 4G). However, recovering PHTF2 expression significantly reversed the expression pattern of those genes (Figure 4G). Therefore, the above results can be speculated that the up-regulated PHTF2 in GC was identified as tumor promoter which participated in mediating intracellular lipids metabolism.

DISCUSSION

Gastric cancer was one of the malignant tumors with a high prevalence and heterogeneous characteristic in human digestive system, and was accompany with a various histological subtypes with multiple genotype changes [19, 20]. In conclusion, mutations in protein-coding genes or dysfunctions in signaling pathways caused by abnormal gene expression patterns were always thought to be significant factors associated with the development and pathological progression of gastric cancer [5]. Identification of abnormally expressed genes in the pathological process of gastric cancer, and further revealing its molecular functions or associated biological processes, may bring light on discerning valuable biomarkers, and providing guidance for the diagnosis or treatment for gastric cancer [21, 22]. With the development of high-throughput biotechnology, including microarray chips, transcriptome sequencing, and genome sequencing, which have been widely used to acquire gene expression information in different

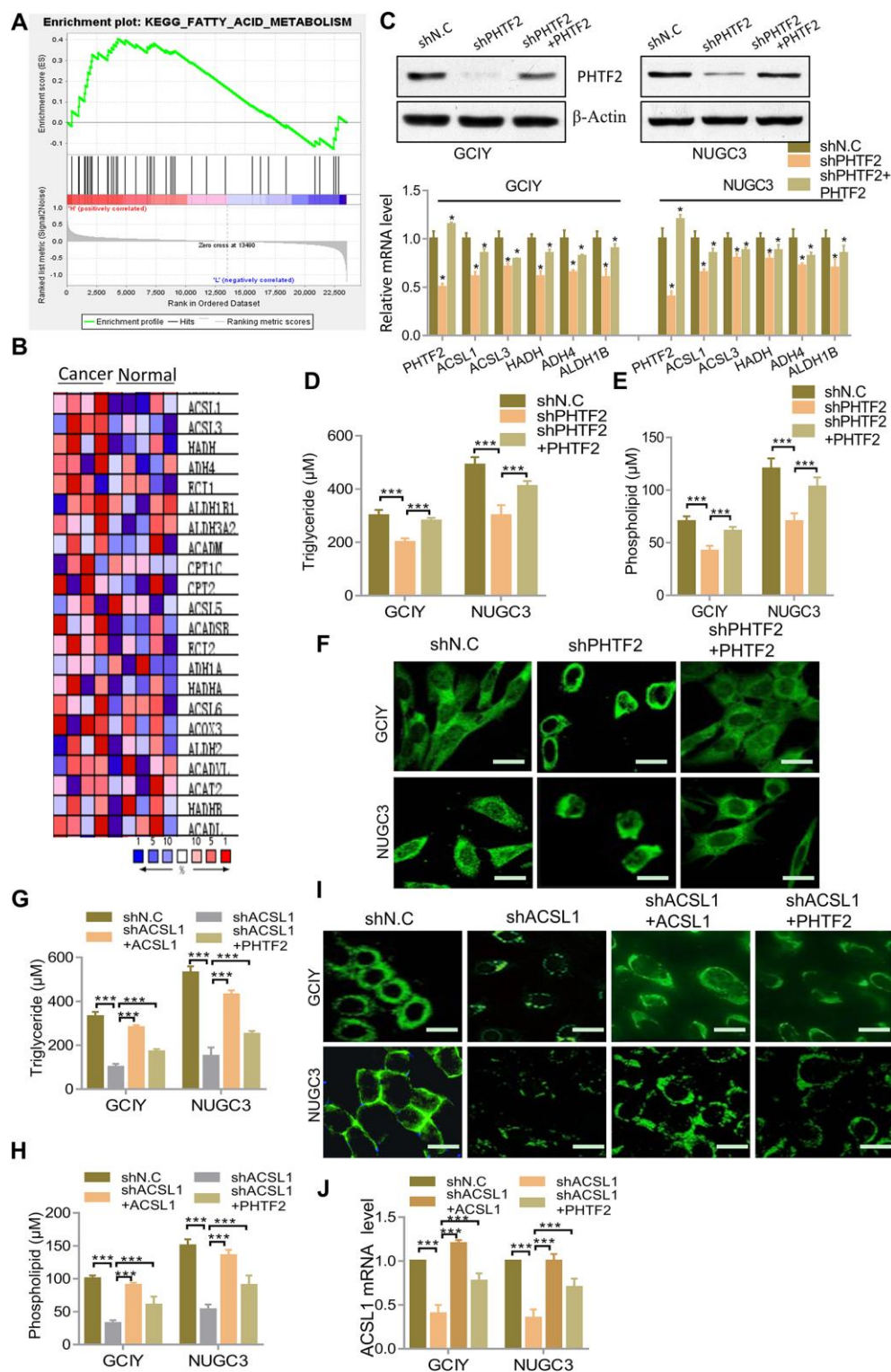


Figure 3. Effects of PHTF2 on the intracellular contents of lipids and key lipid metabolic enzymes in gastric cancer cells. (A) Identification of gene sets enriched in phenotypes correlated with PHTF2 by GSEA using TCGA data. (B) Heat map of core enrichment genes in the gene set KEGG FATTY ACID METABOLISM. (C) Up: Western blot showed PHTF2 protein expression in indicated cells. Down: RT-qPCR analysis for mRNA levels of the key lipid metabolic enzymes ACSL2, ACSL3, HADH, ADH4 and ALDH1B in the indicated cells. (D, E) Cellular content of triglycerides (D) and phospholipids (E) was detected in the indicated cells. (F) The neutral lipids content was detected by double staining with BODIPY 493/503 dye in the indicated cells. Scale bars (10 μ m). (G, H) Cellular content of triglycerides (G) and phospholipids (H) was detected in the indicated cells. (I) The neutral lipids content was detected by double staining with BODIPY 493/503 dye in the indicated cells. Scale bars (10 μ m). (J) RT-qPCR analysis for ACSL1 mRNA levels in the indicated cells.

stages of human gastric cancer tumors, many databases have collected those genes expression datasets for further analysis [23, 24].

In this study, we explored gene expression patterns in two microarray datasets involved 22 pairs of gastric cancer tumor samples paired with para-cancerous tissues and 78 tumor samples and 45 non-cancer samples acquired from TCGA gastric cancer database. Finally, 298 differentially expressed genes were obtained, and the expression patterns of all DEGs in

tumor samples and non-cancer samples were significantly up-regulated or down-regulated, indicating that the tumor samples have specific gene expression patterns, and these genes were likely to participate in various biological processes.

In order to better understand the functions of DEGs in gastric cancer cells, GO and KEGG pathway enrichment analysis revealed that DEGs were mainly involved in intracellular signal transduction, energy metabolism, and fatty acid metabolism. This was

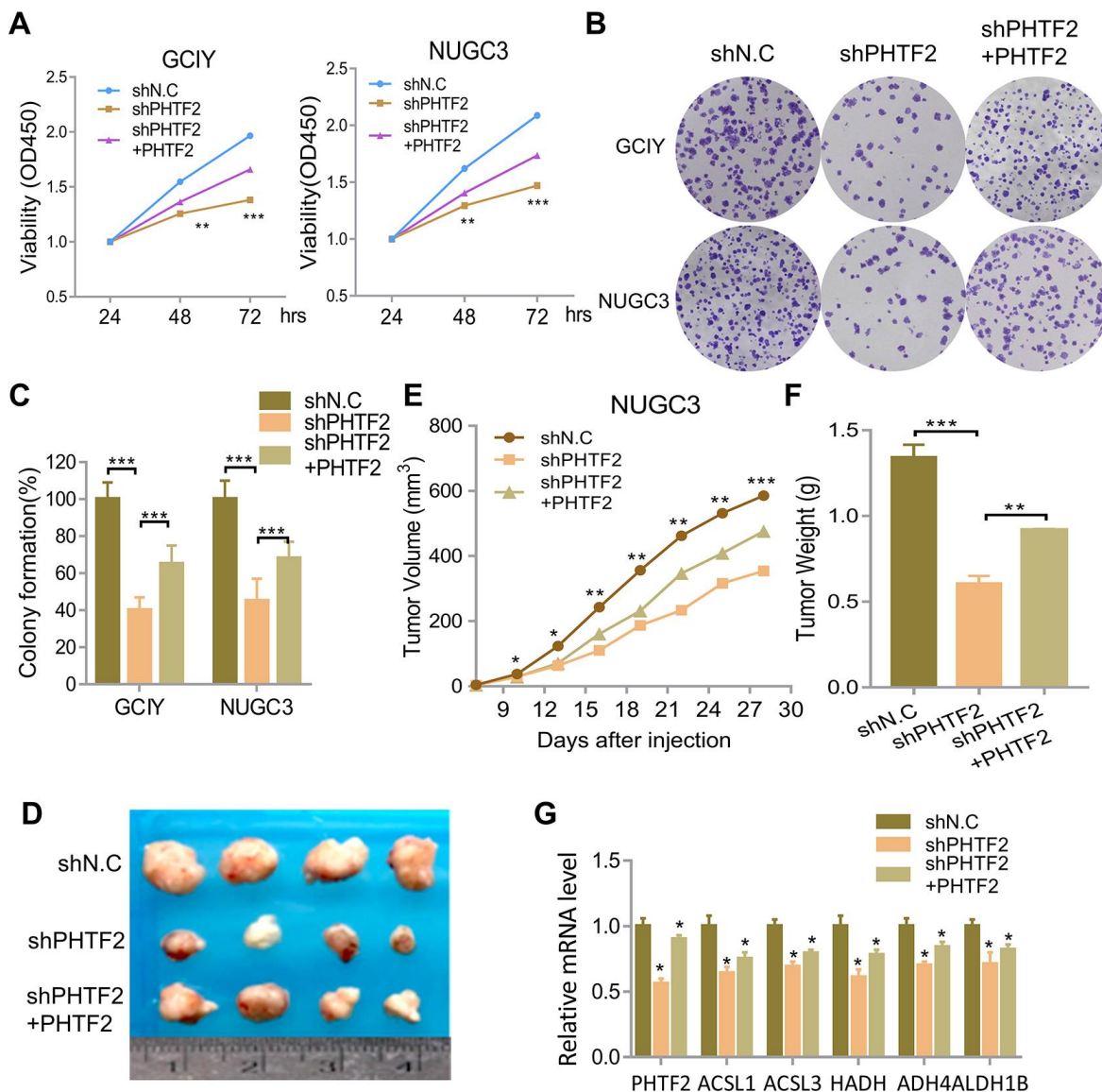


Figure 4. PHTF2-regulated fatty acid metabolism promotes tumorigenic ability of gastric cancer cells in vitro and in vivo. (A) CCK8 assay showed cells viability in the indicated cells. **(B)** Colony formation assay showed cell growth of the indicated cells. **(C)** Qualification of the colony formation shown in B (n=3). **(D)** The representative pictures of dissected tumors from nude mice transplanted with indicated cells. **(E)** Subcutaneous tumor growth curves of mice in different treatment groups was presented. **(F)** The average weight of tumors at the time the animals were sacrificed in the indicated groups. **(G)** Relative mRNA expression pattern of associated gene in tumor tissue acquired from nude mice.

consistent with the idea that the pathological process of gastric cancer was usually accompany with abnormalities in fatty acid metabolism [9, 25]. Through computing the relevance of the constructed PPI network, 20 highly central genes were found to be the key nodes. Further analysis by GSEA revealed that PHTF2, one of the hub genes in the PPI network of DEGs, was -significantly associated with the fatty acid metabolic pathway. Furthermore, PHTF2 silencing in GC cells can significantly down-regulate genes expression involved in fatty acid metabolism pathways, and affect the content of fatty acids and their metabolic intermediates in GC cells. Whatever, PHTF2 can also change the tumorigenicity of GC cells by affecting the expression of key regulators in fatty acids metabolism. These all indicated that the lipid metabolism regulated by PHTF2 was of great significance in the diagnosis and treatment for gastric cancer.

In conclusion, we identified PHTF2 as a hub gene in gastric cancer by bioinformatics methods, which was involved in cellular lipid metabolism in gastric cancer. Furthermore, it was also verified that fatty acid metabolism regulated by PHTF2 can significantly affect the tumorigenic ability of gastric cancer cells. In summary, our research provides more information for exploring the mechanisms for the development of gastric cancer.

MATERIALS AND METHODS

Gene expression profile data

GSE79973 and GSE19826 datasets were downloaded from the GEO database [26]. In the GSE79973 dataset, tumor tissues of 10 gastric cancer patients and paired para-cancerous tissues were included. GSE19826 dataset incorporated 12 tumors and matched adjacent gastric tissues. Another gastric cancer gene expression profile dataset and clinical information was acquired from the cancer genome atlas program (TCGA) through the Genomic Data Commons (GDC) Data Transfer Tool 1.3.0, which contains 78 gastric cancer tumor samples and 35 adjacent non-cancer samples (<https://tcga-data.nci.nih.gov/tcga>).

Data preprocess and DEGs identification

The downloaded gene expression profile was performed with R (version 3.3) to correct background, batch, convert probe to gene symbol, and normalization. The *t*-test package was used to analysis the linear model of the microarray between the GC samples and the non-cancerous tissues. The "limma" package was used to identify differentially expressed genes (DEGs) according to the following criteria: (I) $|\log_2FC| > 2$; (II) $P \text{ value} < 0.05$; (III) false discovery rate (FDR) < 0.05 .

The overlapping features of the DEGs in the three datasets were analyzed by Funrich 3.0 Software. Genes commonly expressed in three gastric cancer samples were set as a group.

An online database of Cancer Cell Line Encyclopedia (CCLE: <https://portals.broadinstitute.org>) was used to determine the expression pattern of PHTF2 in different gastric cancer cell lines.

Functional network establishment of DEGs

To explore the potential molecular functions, biological processer and key pathways of DEGs in the pathology of GC, Gene Ontology (GO) and the Kyoto Gene and Genomic Encyclopedia (KEGG) pathway enrichment analysis were performed in data annotation visualization and integration discovery (DAVID 6.7, <https://david.ncifcrf.gov/>). The threshold for significant function and pathway screening was set at $p < 0.05$.

Exploration of potential interactions between candidate proteins was performed in the STRING V10.0 database (<http://string-db.org>) and Protein-Protein Interaction (PPI) networks were drawn. The interaction score of 0.04 was set as a cut-off criterion.

Cytoscape V3.5.0 software (<http://www.cytoscape.org/>) was used to reconstruct the PPI network and screen the Hub genes in the PPI network.

Cell lines, virus packaging and infection

GCIY and NUGC3 cell lines used in this study were purchased from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). All experiments were repeated at least 3 times with two cell lines.

The packaging plasmid pMD, pCMV and the pRSV plasmid containing the indicated shRNA were co-transfected into 293T cells using lipo3000 reagents (Invitrogen), and the supernatant containing the virus was collected. GCIY and NUGC3 cells were screened for 3 days with puromycin (800 ng / mL) after infection with virus for 24 hours to obtain stably PHTF2 knockdown cell lines.

Clinical specimens and immune-histochemical staining

In order to confirm the expression pattern of PHTF2 in gastric cancer, tumor and adjacent normal tissues were collected from 45 patients, who were not treated with radiotherapy or chemotherapy. All patients included in

Table 1. Primers used for qPCR.

Gene	Primers (5' to 3', forward/reverse)
PHTF2 mRNA	TCTGACCAGAAAGGGAATTGTTTC / AGCCATATCGGCCCAATCAC
ACSL1 mRNA	CCATGAGCTGTTCCGGTATTT / CCGAAGCCCATAAGCGTGTT
ACSL3 mRNA	GCCGAGTGGATGATAGCTGC / ATGGCTGGACCTCCTAGAGTG
HADH mRNA	ACCAGGCAGTTCATGCGTT / ACGTGCTTGACGATTATCTTCTT
ADH4 mRNA	AGTTCGCATTTCAGATCATTGCT / CTGGCCCAATACTTTCCACAA
ALDH1B11 mRNA	GGAGTCTTATGTCTTGGATCTGG / TGTCGGGTGAAGCAGAAATG
β-Actin mRNA	CATGTACGTTGCTATCCAGGC / CTCCTTAATGTCACGCACGAT

the study or their guardians signed the informed consent and the study was approved by our Hospital Ethics Committee.

The sections of the paraffin-embedded tissue were performed for immune-histochemical staining according to standard experimental procedures. Images of the staining results were captured by Leica microscope and analyzed by Image Plus 5.0 software.

Quantitative real-time PCR analysis

Total RNA in gastric cells was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and then 2 µg of total RNA was reverse-transcribed by reverse transcriptase kit (TOYOBO) with oligo d(T) primer. The indicated mRNA was detected by SYBR green method, and β-Actin mRNA level was used as an internal reference. Three replicates were performed in each set of experiments. The primer primers used in this study are shown in Table 1.

Quantification of neutral lipids, triglycerides and phospholipids

Intracellular neutral lipids were detected by the lipophilic dye BODIPY 493/503 (Invitrogen) according to the manufacturer's instructions. Intracellular content of triglyceride (TAG) or phospholipid (PL) were determined by EnzyChrom™ Triglyceride Assay Kit (BioAssay Systems) and EnzyChrom™ Phospholipid Assay Kit (BioAssay Systems) according to the manufacturer's instructions.

Mouse xenograft

2×10⁷ cells were suspended in PBS buffer containing 50% Matrigel Matrigel (BD Biosciences) and then

injected into the back of BALB/c nude mice (5 weeks old, male). When tumor-like products were present (about 4 mm in diameter), the tumor size was measured according to the project and growth curve was drawn. Four weeks later, the mice were sacrificed to acquire tumors for further analysis of tumor size, weight and gene expression patterns. All operations are approved by our Institution's Animal Care and Use Committee.

Statistical analysis

The data was statistically analyzed with Graphpad prism 7.0, and were presented as mean ± SD from at least three independent experiments. Quantitative data was analyzed by one-way ANOVA or two-sided *t*-test under conditions of normal distribution and homogeneity of variance, or otherwise by wilcoxon rank-sum test. *P* < 0.05 was considered to be statistically significant.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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