Downregulation of GMPS inhibited the proliferation of hepatocellular cancer cells *via* the regulation of STAT3/c-Myc pathway

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ABSTRACT

Hepatocellular cancer (HCC) is the sixth most common type of cancer worldwide. Guanosine monophosphate synthase (GMPS) participates in the regulation of chromatin and genes in various organisms, and is highly expressed in a number of human malignant tumors. However, the role of GMPS in HCC has not yet been fully studied and clarified. In this study, the differential fold changes in gene expression levels between HCC cancer tissues and correspondent adjacent normal tissue in The Cancer Genome Atlas Program and GEO datasets were analyzed using R language. GMPS expression levels in HCC cells were knocked down using specific siRNAs. In addition, CCK-8, EdU, TUNEL and immunofluorescence staining were conducted to explore the effects of GMPS siRNAs on HCC cell viability, proliferation, apoptosis and the STAT pathway level, respectively. The results indicated GMPS expression was significantly increased in HCC tumor tissues compared with the corresponding adjacent normal tissues. In addition, high expression of GMPS is negatively associated with the survival rate of patients with HCC. *In vitro* studies illustrated the knockdown of GMPS notably prevented HCC cell proliferation and induced HCC cell (Hep3B2.1-7 and MHCC97H) apoptosis by regulating the STAT3/c-Myc pathway. The apoptosis-specific marker cleaved caspase was significantly upregulated by GMPS knockdown in HCC cells. The findings of the present study revealed the association between GMPS and the prognosis of HCC. The results suggested that GMPS may serve as a promising marker for the prognosis of HCC, and it may also be a potential therapeutic target for HCC. These findings may lay the theoretical foundation for the clinical application of GMPS.

Key words: Hepatocellular cancer; guanosine monophosphate synthase; STAT3; apoptosis.

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Introduction

Hepatocellular cancer (HCC) is the sixth most common type of cancer and the third leading cause of cancer-related mortality worldwide, with marked geographical variations in its incidence and mortality rates.[1] In China, HCC is becoming increasingly severe, with both the incidence and mortality rates continuously increasing over the past two decades, ranking fourth and second among all cancer types, respectively.[1] Currently, there are various treatment options for HCC. Patients with early-stage HCC usually undergo surgical treatments, such as hepatocellular resection, hepatocellular transplantation and ablation.[2,3] However, due to the lack of effective early monitoring methods, the majority of patients are diagnosed at an advanced stage when surgical intervention is no longer feasible.[4] Other treatment modalities, such as radiotherapy, chemotherapy, targeted therapy and immunotherapy, although capable of delaying disease progression to a certain extent, still have limitations such as low specificity, significant systemic toxicity, widespread drug resistance and limited improvement in patient survival rates.[5] Therefore, in the face of numerous challenges in the treatment of HCC, seeking safe and efficient anti-HCC treatment regimens has become a key area of research in the field of oncology.

Guanosine monophosphate synthetase (GMPS) catalyzes the final step of the purine biosynthesis guanosine monophosphate branch.[6] In this reaction, glutamine is hydrolyzed, and the resulting ammonia is incorporated into xanthosine monophosphate (XMP) to form GMP.[3] Therefore, GMPS plays a crucial role in normal cell proliferation and nucleotide biosynthesis in tumorigenesis. Early studies on GMPS were mostly conducted on bacteria, yeast and insects, where GMPS was found to play key roles in spore formation, pathogenicity and axon induction, respectively.[7] Research on mammalian GMPS has mainly been focused on the formation of a molecular cascade with TRIM21 and USP7, thereby controlling the stability of p53 in DNA damage or nucleotide deficiency.[8] However, due to the recent discovery that guanosine monophosphate metabolism plays a crucial role in the progression and prognosis of melanoma, HCC, breast cancer, and other tumors, GMPS has become an attractive target for anticancer therapy.[9,10]

STAT3 is a cytoplasmic signal transducer and transcription factor, belonging to the Janus kinase (JAK)-signal transduction and transcriptional activator (STAT) pathway. It plays a crucial role in the development of cancer cells. In brief, STAT3 is activated by various cytokines [including interleukin 6 (IL-6) and interleukin 10 (IL-10), epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF)]. Once these factors bind to their corresponding receptors, Janus kinases (JAKs) are activated. It is reported that GMPS upregulation mediates cervical cancer progression by regulating STAT3 pathway.[8] However, the functional interplay between GMPS and STAT3 in HCC remains to be elucidated.

In the present study, the HCC dataset was first analyzed and GMPS was found to exhibit a close association with the progression of HCC. However, only a few studies to date have reported the biological role of GMPS in HCC. Therefore, the present study aimed to explore the expression of GMPS in HCC and to elucidate its effects on the proliferation and apoptosis of HCC cells. We anticipate that our research results may offer novel therapeutic directions for clinical management of HCC.

Materials and Methods

Bioinformatics analysis

The HCC dataset from The Cancer Genome Atlas Program (TCGA) database includes transcriptome data and clinical information on 50 normal tissues and 368 tumor tissues. Data from the Gene Expression Omnibus (GEO) database comprised the following: i) the GSE14520 dataset included data on the RNA expression levels of 445 samples on the GPL3921 platform, with 220 normal samples and 225 tumor samples, as well as the survival information of the 225 tumor samples; ii) the GSE54236 dataset included the RNA expression levels of 161 samples, with 80 normal samples and 81 tumor samples. Metabolism-related genes (MRGs) were obtained from GeneCards (https://www.genecards.org/) using the keyword “Metabolism”, and genes with GIFtS >50 and Score >3 were selected to obtain a list of 4,907 genes (Table S1).

Differential gene analysis

Based on the limma function package of R language,[11] the differential fold changes of genes between groups were analyzed, and the *p*-value was corrected for multiple hypothesis tests using the FDR error control method. Genes with the absolute value of log2FC of the differential expression >1 and FDR <0.05 were selected as differentially expressed genes. Taking the final survival status of the patients as the binary response variable, a Lasso logistic regression analysis was conducted to identify the characteristic genes.

Cells and cell culture

The HCC cell lines, Hep3B2.1-7 (Cat. CBP60198), Huh7 (Cat. CBP30045L) and MHCC97H (Cat. CBP6027), were purchased from COBIER (Nanjing, China). DMEM medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific., Inc.) was used for MHCC97H and Huh7 cell culture, and MEM + 10% FBS + 1% non-essential amino acids + 1 mM Sodium Pyruvate was used for Hep3B2.1-7 cell culture. Liver epithelial cells (THLE-2) were provided with Meisen and cultured with BEGM + 10% FBS. All cells were incubated at 37°C with 5% CO_2.

CCK-8 assay

Hep3B2.1-7 or MHCC97H cells were seeded in 96-well plates (10,000 cells per well) and cultured overnight at 37°C. The cells were then incubated with 20 nmol GMPS siRNA or siRNA control (Ribobio, Guangzhou, China) using with Lipofectamine™2000 (Thermo Fisher Scientific, Inc.) for 24 h. The cells were then incubated with 10 μL CCK-8 solution at 37°C for a further 4 h. The absorbance values of each well were measured at 450 nm using a microplate reader.

RT-qPCR

Total RNA was collected from human colon epithelial cells using an RNA extraction kit (Beyotime Institute of Biotechnology, Shanghai, China). Subsequently, 1 μg RNA was reverse transcribed into cDNA using a cDNA Synthesis kit (ELK Biotechnology, Wuhan, China). The cDNA was then mixed with SYBR-Green PCR SuperMix (ELK Biotechnology) and qPCR was conducted (Life Technologies, Waltham, MA, USA). The information for the primers is listed in Table 1. GAPDH was used for the internal control and the $2^{-\Delta\Delta Cq}$ method was used for quantification.

Cell proliferation analysis

A 5-Ethynyl-2'-deoxyuridine (EdU) detection kit (Beyotime Institute of Biotechnology) was used to evaluate Hep3B2.1-7 and MHCC97H cell proliferation. Hep3B2.1-7 or MHCC97H cells were fixed using with 4% paraformaldehyde for 2 h at room temperature. The cells were then stained with EdU-488 solution (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) for 1 h at 37°C in the dark, and subsequently incubated with DAPI (Wuhan Servicebio Technology Co., Ltd.) for 15 min at room temperature. Finally, three random fields were selected for the EdU-positive cell quantification under a fluorescence microscope (Nikon Corporation, Tokyo, Japan).

TUNEL analysis

Hep3B2.1-7 and MHCC97H cell apoptosis was measured using with the TUNEL detection kit (Wuhan Servicebio Technology). Briefly, Hep3B2.1-7 or MHCC97H cells were fixed in 4% paraformaldehyde at room temperature for 2 h. The cells were then treated with 0.2% Triton X-100 for 2 min at room temperature and stained with the mixed solution at 37°C for a further 1 h. Subsequently, the cells were incubated with DAPI solution (0.1 μg/mL) for 30 min in the dark for nuclei staining. The mounting medium was polyvinyl alcohol mounting medium with DABCO® (Sigma-Aldrich, St. Louis, MO, USA). Finally, three random fields were selected for the TUNEL-positive cell quantification under a fluorescence microscope (Nikon Corporation).

Immunofluorescence staining

Hep3B2.1-7 and MHCC97H cell was washed wish PBS for three times (5 min for each time). The cells were then fixed with 4% paraformaldehyde for 20 min at room temperature. This was followed by blocking with 0.3% hydrogen peroxide for 15 min at room temperature. Subsequently, the cells were incubated with 5% bovine serum albumin for 60 min and incubated with primary antibodies cleaved caspase-3 (1:200; Abcam, Cambridge, UK), p-STAT3 (1:200; Abcam) and p-Myc (1:200; Abcam) at 4°C overnight. The samples were then incubated with a secondary antibody (1:200; Wuhan Servicebio Technology Co., Ltd.) for 30 min at 37°C. A fluorescence microscope (IX51, Olympus Corporation) was used to obtain the images. The intensity of staining and proportion of positive cells in three random images per section were quantified. The sample prepared by excluding the primary antibody and stained with PBS instead was used as the negative control.

Statistical analysis

The results are presented as the mean ± SD and GraphPad Prism 10.0 (Dotmatics) was used for data analysis. One-way analysis of variance (ANOVA) followed by Tukey's test was used for statistical analyses among multiple groups. A p-value <0.05 was considered to indicate statistically significant differences.

Results

Differential gene analysis between HCC tissues and adjacent normal tissues

For differential gene analysis between HCC tissues and adjacent normal tissues, TCGA and GSE14520 datasets were analyzed. In TCGA dataset, 2,651 genes were upregulated and 571 genes were downregulated in the tumor group compared with the normal tissues (Figure 1A). In the GSE14520 dataset, 419 genes were upregulated and 516 genes were downregulated in the tumor group comparing with the normal tissues (Figure 1B). The intersection of DEGs from these two datasets and metabolism-related genes (MRGs) yielded 133 key genes (Figure 1C). The final survival status of the patients was used as the binary response variable and Lasso logistic regression analysis was conducted, which resulted in 27 characteristic genes (Table S1). Through univariate Cox regression analysis of these 27 characteristic genes, the risk genes that significantly affected the prognosis of patients with HCC (p<0.05, HR >1) were identified. In TCGA dataset, there were 17 risk genes significantly affecting prognosis, while in the GSE14520 dataset, there were 7 (Figure 1D). The GMPS gene was found to be one of the intersection genes.

High expression of GMPS is negatively associated with the survival rate of patients with HCC

The present study then focused on exploring the role of GMPS during the progression of HCC. In TCGA dataset, the expression levels of GMPS were significantly increased in the HCC tumor tissues compared with the corresponding adjacent normal tissues (Figure 2A). In addition, the results of survival rate analysis suggested that a high expression of GMPS was negatively associated with the survival rate of patients with HCC (Figure 2B). Consistently, the level of GMPS was notably increased in HCC cell lines, including Hep3B2.1-7, Huh7 and MHCC97H, when compared with liver epithelial cells (THLE-2) (Figure 2C). GMPS siRNA was used to knockdown the expression of GMPS in Hep3B2.1-7 and MHCC97H cells (Figure 2 D,E). On the whole, the results demonstrated that GMPS expression was significantly increased in HCC tumor tissues, and its expression was negatively associated with the survival rates of patients with HCC.

GMPS knockdown inhibits the proliferation of HCC cells

Since GMPS expression was significantly increased in HCC tumor tissues and its expression was negatively associated with the survival rate of patients with HCC, the effects of GMPS siRNA on the viability of HCC cells were evaluated using CCK-8 assay. The results demonstrated that GMPS siRNA significantly suppressed the viability of Hep3B2.1-7 and MHCC97H cells (Figure 3 A,B). The results from EdU staining also suggested that the knockdown of GMPS inhibited the proliferation of HCC cells (Figure 3 C,D). In summary, these data illustrated that GMPS siRNA was able to inhibit the proliferation of HCC cells.

Table 1. List of primers used in this study.

Name	Primer	Sequence (5'-3')
Homo GAPDH	Forward	CATCATCCCTGCCTCTACTGG
	Reverse	GTGGGTGTCGCTGTTGAAGTC
Homo IFN-β	Forward	CTGGCAATCAGAGTAATATGTGCT
	Reverse	CAGTGAATGCAGACTGGTAATTTG

Downregulation of GMPS induces HCC cell apoptosis

The present study then examined the effect of GMPS siRNA on HCC cell death using TUNEL assay. The results of TUNEL staining demonstrated that GMPS siRNA significantly increased the TUNEL-positive cell rate in Hep3B2.1-7 and MHCC97H cells (Figure 4 A,B). In addition, the knockdown of GMPS notably upregulated the expression of the key executor of apoptosis, cleaved caspase-3, in HCC cells (Figure 4 C,D). In summary, the knockdown of GMPS induced the apoptosis of Hep3B2.1-7 and MHCC97H cells.

GMPS knockdown inhibits the STAT3/c-Myc pathway in HCC cells

A previous study reported that GMPS knockdown was able to induce gastric cancer cell apoptosis *via* the STAT3/P53 molecular pathway,[8] indicating the potential interaction between GMSP and the STAT3 pathway. The present study found that the knockdown of GMPS inhibited the phosphorylation of STAT3 in Hep3B2.1-7 and MHCC97H cells (Figure 5 A,B). In addition, its downstream oncoprotein, c-Myc, was detected. The results illustrated that GMPS knockdown significantly inhibited the phosphorylation of c-Myc as well. Collectively, GMPS knockdown was able to inhibit the STAT3/c-Myc pathway in HCC cells.

Discussion

GMPS is a typical biosynthetic enzyme that can promote cell proliferation and DNA replication. The majority of current research on GMPS has been conducted on bacteria and insects. It has been reported that GMPS is involved in the regulation of chro-

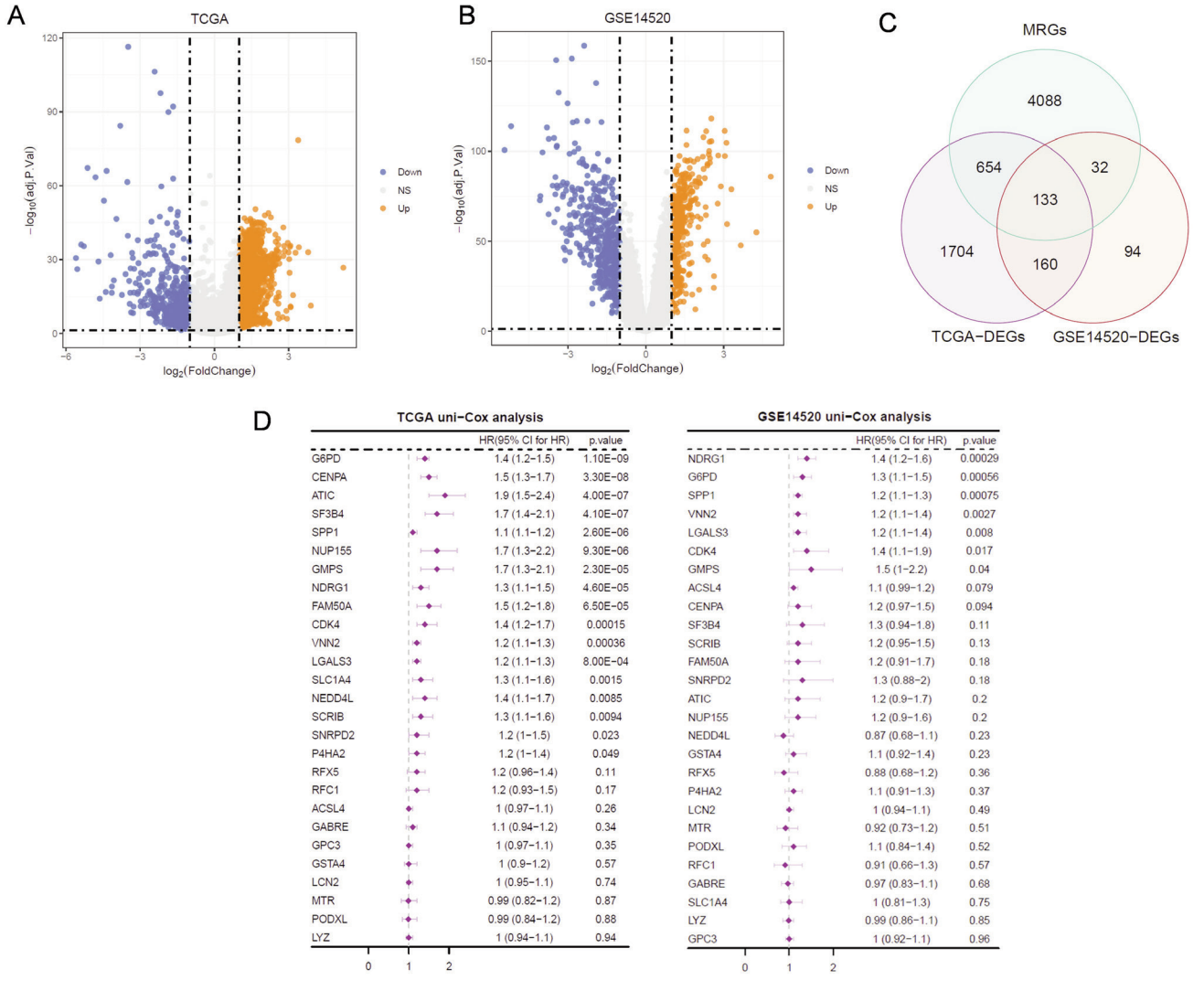

Figure 1. Differential genes analysis between hepatocellular cancer tissues and adjacent normal tissues. **A,B**) Limma function package of R language was used to analyze the differential fold changes of genes between groups in TCGA and GSE14520 datasets. **C**) The intersection of DEGs from these two datasets and MRGs were showed in Venn diagram. **D**) The 27 risk genes that significantly affected the prognosis of HCC were analyzed with univariate Cox in TCGA and GSE14520 datasets.

matin and genes.[12,13] Guo *et al* reported GMPS was highly expressed in non-small cell lung cancer, and that GMPS promoted lung cancer cell proliferation and migration via the SERPINB2/DNMT1 axis.[14] Consistently, GMPS has been reported to promote the progression of cervical cancer by regulating the STAT3/P53 pathway.[8] In the present study, it was demonstrated that GMPS expression was significantly increased in HCC tumor tissues compared with corresponding adjacent normal tissues and that a high expression of GMPS was negatively associated with the survival rate of patients with HCC. All these outcomes suggest GMPS may play a promoting role in the occurrence and development in a number of cancer types.

GMPS may be involved in key processes, such as DNA replication, cell proliferation and abnormal division.[12] Therefore, this enzyme with multiple functions may become a potential target for novel therapeutic strategies in cancer and other diseases. In the present study, it was found GMPS knockdown inhibited the proliferation of HCC cells and induced the apoptosis of HCC cells. As is known, apoptosis refers to the process of programmed cell death. It is one of the core mechanisms for maintaining tissue homeostasis and eliminating damaged cells in organisms.[15] In the event that cell apoptosis is dysregulated, it is often a signal of cancer occurrence. The dysregulation of apoptosis is not only related to the occurrence and development of cancer, but also directly leads to the issue of drug resistance in cancer treatment.[16] The present study found that GMPS knockdown was able to induce HCC cell apoptosis. This finding is supported by the findings of a previous study demonstrating that small nucleolar RNA SNORD50A and SNORD50B promoted the growth of p53 wild-type breast cancers *via* mediating the TRIM21-GMPS interaction.[17] These results suggest that GMPS may be associated with the biological behavior of cancer. The STAT3/c-Myc pathway serves as a crucial switch for cells to transition from the quiescent phase (G0/G1) to the division phase (S/G2/M). c-Myc activates cell cycle-related proteins, such as Cyclin D1/E, forcing the cells to bypass the growth checkpoints and thereby enabling rapid proliferation.[18] In addition, in this pathway, anti-apoptotic proteins such as Bcl-2 and Survivin are upregulated, while the expression of FAS is inhibited, blocking the cell death process. Additionally, it regulates vascular growth factor (VEGF) and matrix metalloproteinases (MMPs), promoting tumor angiogenesis and matrix degradation, thereby facilitating tumor metastasis.[19] Recent research has demonstrated that GMPS is not only a metabolic enzyme, but its overexpression or activated state in tumor cells may regulate the STAT3 signaling pathway, thereby participating in the regulation of cell proliferation, survival and apoptosis in cervical cancer cells.[8] Similar to the present study, it

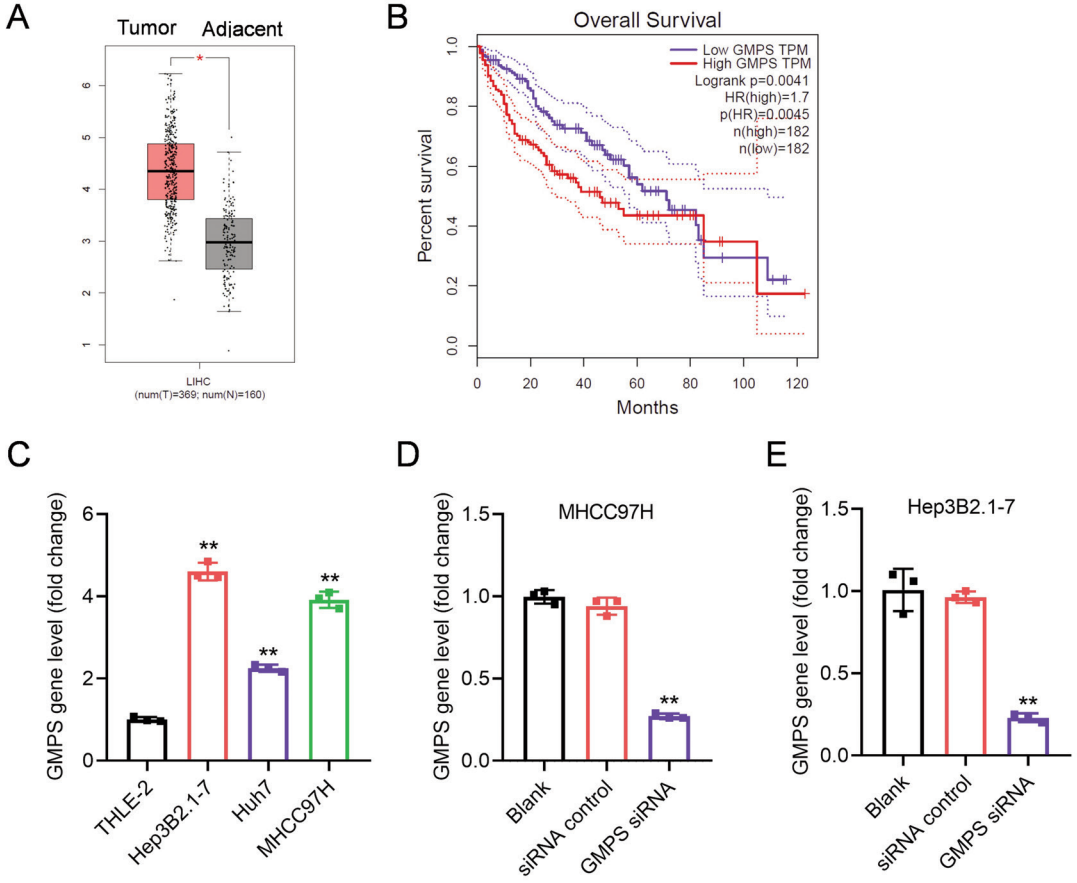

Figure 2. High expression of GMPS was negatively correlated with the survival rate of patients with HCC. **A**) The expression of GMPS in HCC tissues in TCGA dataset was analyzed using online tool GEPIA (http://gepia.cancer-pku.cn/detail.php). **B**) The correlation between GMPS expression and the survival rate of patients with HCC was analyzed using GEPIA. **C-E**) The expressions of GMPS in Hep3B2.1-7, Huh7, MHCC97H and THLE-2 were detected with RT-qPCR.

was previously found that GMPS knockdown inhibited the STAT3/c-Myc pathway in HCC cells. c-Myc promotes proliferation, while p53 monitors the risk of mutations. They form a negative feedback loop.[20] In the present study, it was demonstrated that GMPS knockdown inhibited the STAT3/c-Myc pathway in HCC cells. Thus, it can be deduced that GMPS knockdown significantly induces the apoptosis of HCC cells. This process of inducing apoptosis is dependent on the regulation of the STAT3/c-Myc signaling pathway by GMPS. In conclusion, the present study demonstrated the association between GMPS and the prognosis of patients with HCC. GMPS knockdown inhibited the proliferation of HCC cells and induced apoptosis. These results suggest that GMPS may serve as a promising marker for the prognosis of patients with HCC, and it may also be a potential therapeutic target for HCC. The findings presented herein may lay a theoretical foundation for the clinical application of GMPS.

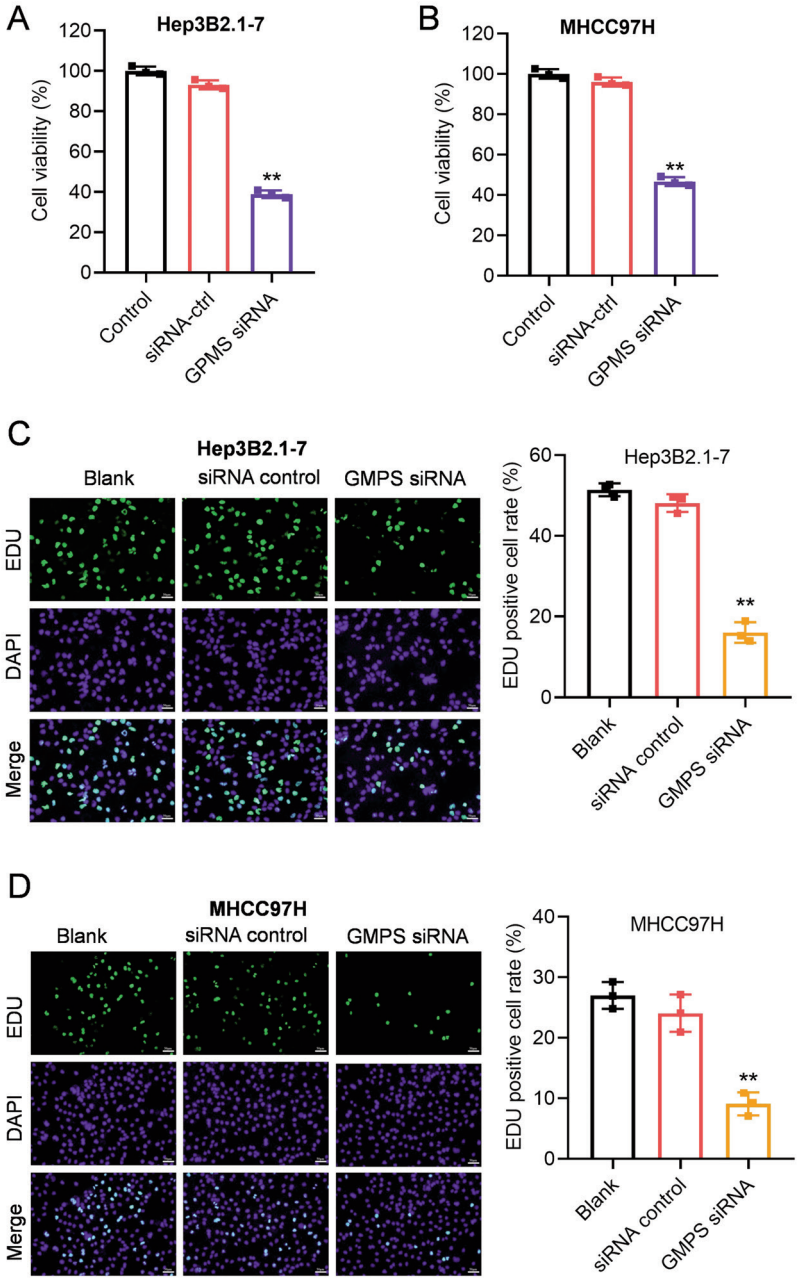

Figure 3. Downregulation of GMPS inhibited the proliferation of HCC cells. Hep3B2.1-7 or MHCC97H cells were incubated with 20 nmol GMPS siRNA or siRNA control using with Lipofectamine™2000 for 24 h. **A,B**) The cell viability was measured with CCK8 assay. **C,D**) The cell proliferation was evaluated using with EdU immunofluorescence staining method; the EdU-positive cells were quantified. n=3; **p<0.01 comparing with siRNA control group.

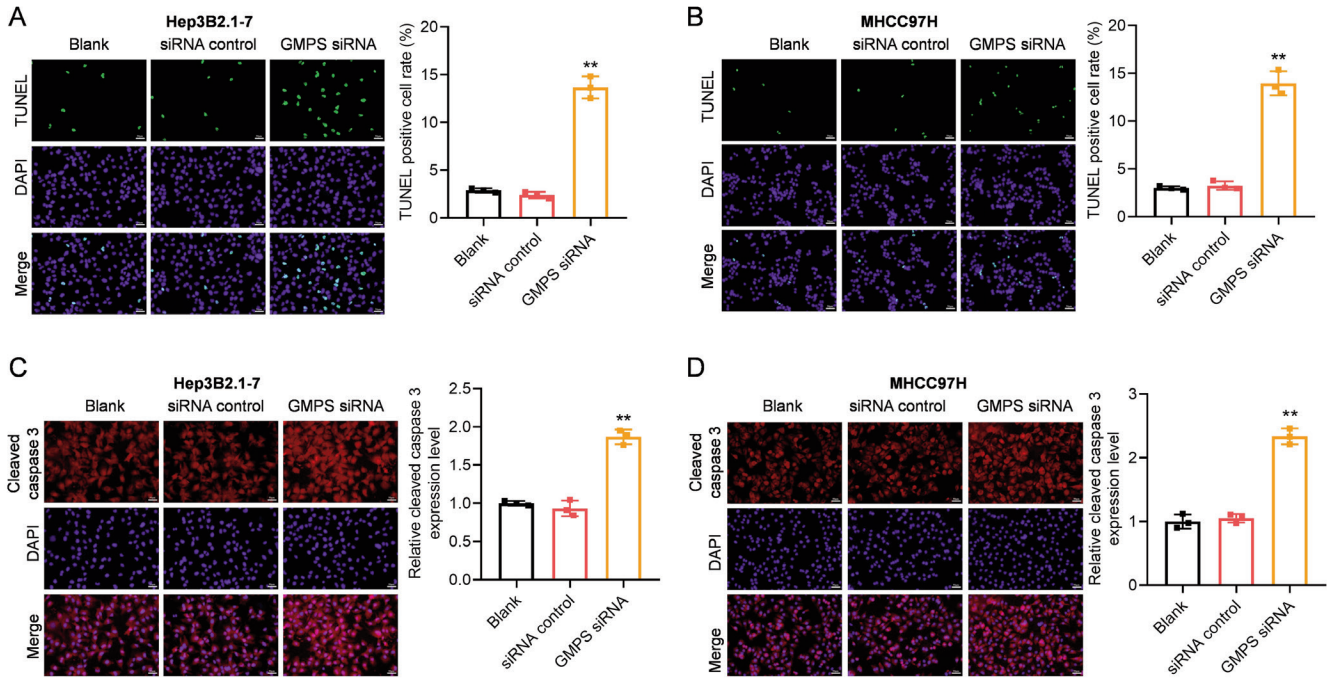

Figure 4. Downregulation of GMPS induced HCC cell apoptosis. Hep3B2.1-7 or MHCC97H cells were incubated with 20 nmol GMPS siRNA or siRNA control using Lipofectamine™2000 for 24 h. **A,B**) The cell proliferation was evaluated using with TUNEL immunofluorescence staining method. The TUNEL-positive cells were quantified. **C,D**) The protein expression of cleaved caspase 3 in HCC cells was evaluated with cleaved caspase 3 immunofluorescence staining and quantification was conducted. n=3; $^{**}p<0.01$ comparing with siRNA control group.

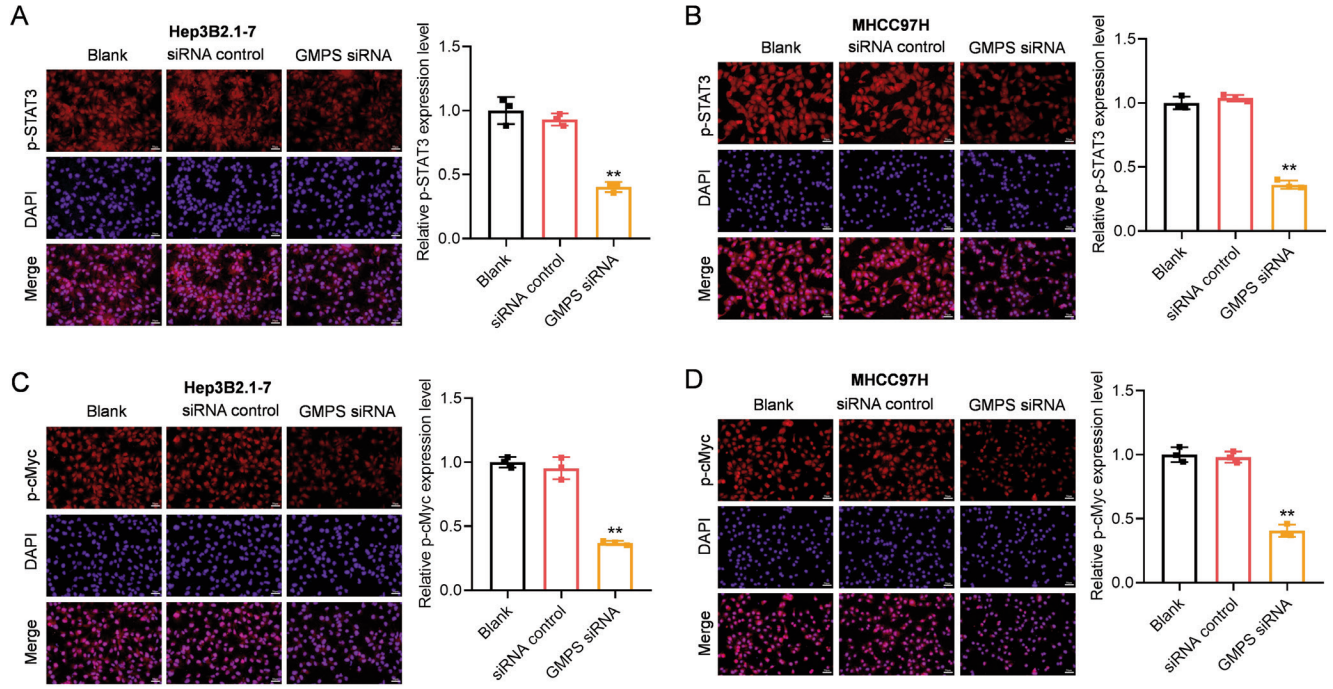

Figure 5. GMPS knockdown inhibited STAT3/c-Myc pathway in HCC cells. Hep3B2.1-7 or MHCC97H cells were incubated with 20 nmol GMPS siRNA or siRNA control using Lipofectamine™2000 for 24 h. **A,B**) The expression of p-STAT3 was evaluated using with immunofluorescence staining method; the p-STAT3-positive cells were quantified. **C,D**) The expression of p-cMyc was evaluated using with immunofluorescence staining method; the p-cMyc-positive cells were quantified. n=3; $^{**}p<0.01$ comparing with siRNA control group.

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Online Supplementary Material
Table S1. Lasso logistic regression analysis.

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