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ORIGINAL RESEARCH

Analysis of Key Genes Regulating the Warburg Effect in Patients with Gastrointestinal Cancers and Selective Inhibition of This Metabolic Pathway in Liver Cancer Cells

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Xinyue Zhang,[1–3,*] Jinan Guo,[4,5,*]
Parham Jabbarzadeh Kaboli,[2,6]
Qijie Zhao,[2] Shixin Xiang,[2] Jing Shen,[2,6]
Yueshui Zhao,[2,6] Fukuan Du,[2,6] Xu Wu,[2,6]
Mingxing Li,[2,6] Huijiao Ji,[2,6] Xiao Yang,[2]
Zhangang Xiao,[2,6] Qinglian Wen[1,3]

[1]Department of Oncology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, People's Republic of China; [2]Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou, Sichuan, People's Republic of China; [3]Academician (Expert) Workstation of Sichuan Province, Luzhou, Sichuan, People's Republic of China; [4]The Department of Urology, The Second Clinical Medical College of Jinan University (Shenzhen People's Hospital), The First Affiliated Hospital of South University of Science and Technology of China, Shenzhen Urology Minimally Invasive Engineering Center, Shenzhen, Guangdong, People's Republic of China; [5]Shenzhen Public Service Platform on Tumor Precision Medicine and Molecular Diagnosis, Shenzhen, Guangdong, People's Republic of China; [6]South Sichuan Institute of Translational Medicine, Luzhou, Sichuan, People's Republic of China

*These authors contributed equally to this work

Correspondence: Zhangang Xiao
Laboratory of Molecular Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou 646000, Sichuan, People's Republic of China
Email zhangangxiao@swmu.edu.cn

Qinglian Wen
Department of Oncology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, People's Republic of China
Email wql73115@163.com

Objective: The Warburg effect, also known as aerobic glycolysis, plays a dominant role in the development of gastrointestinal (GI) cancers. In this study, we analyzed the expression of key genes involved in the Warburg effect in GI cancers and investigated the effect of suppressing the Warburg effect in vitro in liver cancer cell lines.

Methods: The Cancer Genome Atlas (TCGA) RNA-Seq data were used to determine gene expression levels, which were analyzed with GraphPad Prism 7.00. Genetic alterations were queried with cBioPortal. The influence of the Warburg effect on liver cancer cell viability, migration and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was determined by means of MTT, transwell and GAPDH activity assays.

Results: The levels of expression of genes associated with the Warburg effect were increased in tumors. To our knowledge, this is the first report of upregulated expression of *CUEDC2*, *HMGB2*, *PFKFB4*, *PFKP* and *SIX1* in liver cancer. Clinically, overexpression of these genes was associated with significantly worse overall survival of liver cancer patients. In vitro, selective inhibition of GADPH suppressed the growth and metastasis of Huh-7, Bel7404 and Hep3B hepatocellular carcinoma cell lines.

Conclusion: The Warburg effect may play an important role in GI cancers, especially in liver cancer.

Keywords: Warburg effect, gastrointestinal cancers, liver cancer, bioinformatics, GAPDH

Introduction

According to GLOBOCAN, liver cancer is the sixth most common form of cancer in the world. Among the estimated 9.6 million cancer deaths that occurred in 2018, liver cancer was the second cause of death in men and was among the top five cancer types resulting in death, with an estimated 8.2% mortality rate for both genders.[1,2] Liver cancer is a highly heterogeneous disease with a wide range of causes, including HBV, HCV, fungal toxins, alcohol consumption, obesity, and diabetes.[2]

The Warburg effect is a metabolic phenotype commonly seen in tumors. Even in the presence of sufficient oxygen, cancer cells produce about 60% of their ATP through glycolysis instead of oxidative phosphorylation.[3,4] The Warburg effect allows dividing cells to use intermediate glucose metabolites to double their biomass and suppress apoptosis.[5] This phenomenon was first discovered by Otto Warburg in the 1920s and was called aerobic glycolysis. A number of studies have shown that hypoxia-inducible

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factor 1a (*HIF1A*) and PI3K/AKT signaling regulate key enzymes of aerobic glycolysis and therefore modulate the Warburg effect in different cancers.[6] It has been shown that *AKT1*, in response to cellular stress and drug treatments such as lapatinib, activates *Nrf2* and *HIF1A* signaling in breast cancer.[7] Altered *HIF1A* increases the chance of recurrence in patients suffering from HCC.[8] In addition, aerobic glycolysis is significantly increased in liver cancer stem cells (CSCs). Nicotinamide adenine dinucleotide (NAD^+) is required for increased activity of mitochondria. Higher levels of ribosomal protein S5 (*MRPS5*) and NAD^+ dependent deacetylase sirtuin-1 (*SIRT1*) effectively increase the expression of glycolytic proteins and of the Warburg effect in liver CSCs.[9] Similarly, glycolysis inhibitors such as hexokinase (HK) inhibitors, suppress tumor xenograft progression.[10] In addition to HK isoenzymes, pyruvate dehydrogenase E1α (PDHA1) is another key enzyme involved in triggering aerobic glycolysis in HCC.[11]

On the other hand, there are other enzymes which reportedly act as positive regulators of the Warburg effect in liver or other cancer cells, so their inhibition might be an effective means of treating liver cancer (Figure 1). For example, metastasis-associated in colon cancer protein 1 (*MACC1*) and EGFR-phosphorylated platelet isoform of phosphofructokinase 1 (*PFKP*) enhance glycolysis via PI3K/AKT-dependent positive feedback regulation.[12,13] Liver cancer cell metastasis and motility are reduced by inhibition of *MACC1* expression.[14] Transcription factor sine homeobox 1 (*SIX1*) is also a key enzyme involved in the regulation of glucose uptake, lactate production, ATP generation, and increased oxygen consumption rate (OCR).[15]

Cancer stem cells are responsible for drug resistance, so targeting cancer cell stemness is important to overcome drug-resistant phenotypes. Inhibition of *SIX1* reduces stemness of HCC cells and therefore, sensitizes HCC cells to chemotherapy. *SIX1* can bind to Sox2, which regulates stemness.[16] In addition to Sox2, high-mobility-group protein 2 (*HMGB2*) is upregulated in liver cancer and is a key regulator of stem cell pluripotency.[17] Proliferation of HCC cells depends on 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 (*PFKFB4*) which is a key enzyme of glycolysis.[18] Upregulation of Pim1 proto-oncogene (*PIM1*), a serine/threonine kinase, promotes glycolysis in HCC cells by enhancing Akt activation.[19] CUE domain-containing 2 (*CUEDC2*) also plays a crucial role in promoting the Warburg effect by interacting with the

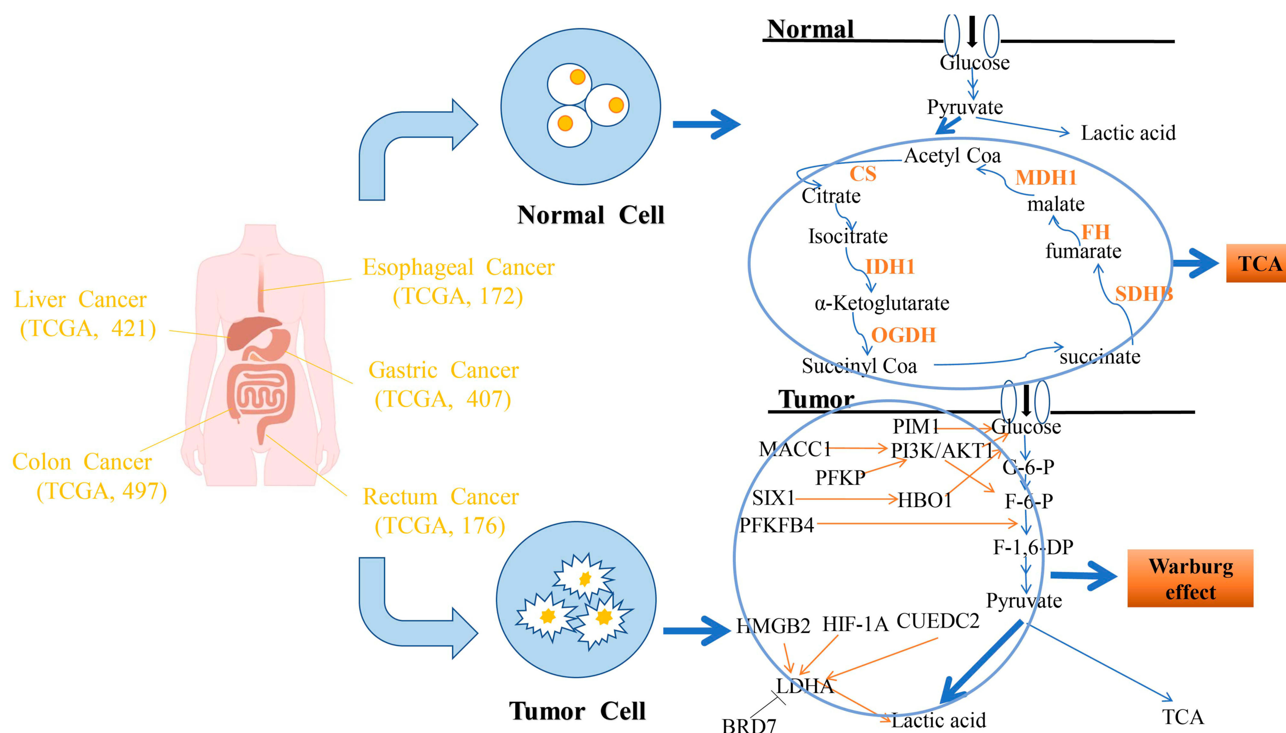

Figure 1 Schematic illustration of the roles played by the TCA cycle and the Warburg effect in gastrointestinal cancers and normal tissues. Normal tissues: The TCA cycle incorporates glucose metabolic products and transform them. Tumors: Under the influence of the Warburg effect, glucose metabolism is predominantly shunted towards pyruvate and lactic acid. *PIM1*, PI3K/AKT and HBO1 directly promote the conversion of glucose into G-6-P. *PFKFB4* enhances the conversion of F-6-P into F-1, 6-DP. With the exception of *BRD7*, multiple factors can promote the production of lactic acid by *LDHA*.

glucocorticoid receptor and upregulating glucose transporter 3 (*GLUT3*) and lactate dehydrogenase A (*LDHA*), two key glycolytic proteins[20]; however, the effect of *CUEDC2* on glycolysis in HCC cells has not been studied. Although aerobic glycolysis has been extensively studied, its precise details and mechanisms are not completely understood. The role of the Warburg effect in gastrointestinal cancers, especially in liver cancer, has not been systematically investigated. In addition to studying Warburg effect-related genes, we also compared the expression of genes involved in the tricarboxylic acid (TCA) cycle between normal and cancerous GI tissues.

Materials and Methods

Data and Samples

Clinical and gene expression data of patients with GI cancers were obtained from The Cancer Genome Atlas (TCGA), specifically from the TCGA-LIHC, TCGA-ESCA, TCGA-STAD, TCGA-READ and TCGA-COAD datasets, including information from both tumoral and normal tissues (number of samples: liver cancer, 421; esophageal cancer, 172; gastric cancer, 407; rectal cancer, 176; colon cancer, 497). Differential analysis of genes involved in the TCA (deep deletions, amplifications, and missense mutations) in GI cancer was performed by means of the cBioPortal for Cancer Genomics database (1700 samples). Protocols for data analysis were based on Zhao et al.[21]

Chemicals and Reagents

RPMI-1640, DMEM, FBS, antibiotics (penicillin and streptomycin, 0.25% w/v), trypsin, EDTA, and phosphate-buffered saline (PBS) were all obtained from Life Technologies (Grand Island, USA). Koningic acid (KA) was purchased from Cayman (Michigan, cat # 14,079). The GAPDH Activity Assay Kit was purchased from BioVision (Milpitas, cat # K680). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Tocris Bioscience (Bristol, cat # 5224/500).

Cell Lines and Cell Culture

HepG2, Hep3B, hepatoblastoma and HCC cell lines were purchased from the American Type Culture Collection (Manassas, USA). The Huh-7 HCC cell line was provided by Dr H. Nakabayashi (Hokkaido University School of Medicine, Japan).[22] Bel7404[23] was obtained from the Cell Bank of the Chinese Academy of Sciences. MIHA-immortalized hepatocytes were obtained from Dr J.R. Chowdhury (Albert Einstein College of Medicine; New York, USA). The normal human liver cell line, LO2, was obtained from the cell bank of the Shanghai Institute of Cell Biology (Chinese Academy of Sciences; Shanghai, China). Use of these cell lines was approved by the ethics committee of Southwest Medical University. Cells were cultured in RPMI-1640 or DMEM with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL streptomycin and 100 μg/mL penicillin) and incubated at 37°C in a humidified atmosphere with 5% CO2.

MTT Assay

Cell viability was analyzed by means of the MTT method. Briefly, after 12 hours of incubation to permit cell adherence, the experimental group was treated with 5 μM or 10 μM KA, whereas 0.01% dimethyl sulfoxide (DMSO) was added to the control group. After 24 hours, 10 μL MTT was added to each well. Following 4 hours of incubation, the solution was removed and DMSO was added. The absorbance at 490 nm was measured to calculate the average inhibition. The experiment was repeated three times with each cell line.

Transwell Migration Assay

Cell migration was measured by the transwell method, using 8-μm pore chambers (Corning, NY, cat # 3244). Briefly, different numbers of cells were seeded onto the upper chamber in serum-free medium. The lower chamber was filled with 600 μL of medium containing 1 μM KA (or not, in the control group). After 24 hours of incubation, the cells of the upper chamber were gently removed with cotton swabs, and the lower side of the membrane was dipped in 95% methanol and stained with crystal violet for 30 minutes. Five random fields were counted under a microscope.

GAPDH Activity Assay

Briefly, cells (10^6) were seeded onto 60mm dishes containing 1 μM KA or 0.01% DMSO (as control) and incubated for 24 hours. Trypsinized cells were subjected to the procedures described in the instructions of the GAPDH Activity Assay Kit and the protein content of the samples was determined. The absorbance was measured at 450 nm in kinetic mode for 30 minutes.

Statistical Analysis

TCGA data were first normalized and log2 transformed. Next, one-way ANOVA and the unpaired *t*-test were used to compare multiple groups and two groups, respectively,

using SPSS 21.0 and GraphPad Prism 7.00 software programs. Overall survival was analyzed based on Kaplan–Meier curves using the Log rank test. *P* values <0.05 were considered statistically significant.

Results and Discussion

Expression of Genes Associated with the Warburg Effect

Cancer cells maintain high rates of glucose uptake, metabolism and fermentation to lactate.[24] In this study, we conducted a systematic analysis of the expression of proteins, factors and enzymes associated with enhancement or inhibition of the Warburg effect in GI cancers. Our results indicate that the expression of genes associated with the Warburg effect are increased in tumors. To our knowledge, this is the first report of upregulated expression of *CUEDC2*, *HMGB2*, *PFKFB4*, *PFKP*, and *SIX1* in liver cancer.

We analyzed the mRNA expression levels of genes associated with the Warburg effect in GI cancers using the TCGA database. The results confirmed a significant trend towards upregulation of all genes in tumors, especially in liver and esophageal cancer (Figure 2). In colon and gastric cancer, most genes showed similar results. In rectal cancer, expression of *PFKFB4*, *SIX1* and *MACC1* was significantly increased. It has recently been shown that *SIX1* overexpression promotes HCC progression through downregulation of *p53*.[25] *SIX1* may increase resistance to chemotherapy in liver cancer.[16,26]

Under hypoxic conditions, *PIM1* is upregulated, resulting in increased glucose uptake and facilitated glycolysis, which promotes tumor progression and metastasis.[19] Downregulation of *PIM1* suppressed tumor growth in HCC cells.[27] We observed *PIM1* expression was significantly decreased in gastric and liver cancer. It has been reported that inhibition of PIM kinases causes excessive mitochondrial fission or increased intracellular ROS production and apoptosis.[28] Also, *PIM1* knockdown significantly accelerated apoptosis in myoblasts.[29] Therefore, *PIM1* may have different roles in different cancer types.

Since *BRD7* assists in the assembly of the *p53* transcriptional complex, *BRD7* is downregulated in several types of cancer. In contrast, we observed that *BRD7* was upregulated in GI cancers, including liver cancer, which may be due to the wide range of mutations found in the GI cancers analyzed in this study. A previous study showed that poly-ubiquitinated HIF1A was upregulated in BRD7-overexpressing MCF-7 breast cancer cells, indicating that BRD7 promotes the degradation of HIF1A in an ubiquitination-dependent manner in breast cancer.[30] Similarly, according to our data, *HIF1A* mRNA expression was upregulated, possibly revealing why *BRD7* mRNA was highly expressed.

Furthermore, we found that *MACC1*, first reported to be a crucial biomarker of metastasis in colon cancer, was upregulated in GI cancers. In agreement with our findings, some studies have reported that *MACC1* interacts with the ERK and AKT pathways in pancreatic cancer.[31] It has also been reported that *MACC1* is strongly associated with MET signaling in liver metastases of resected CRC and is involved in EMT in colorectal cancer.[32,33] However, our analysis showed that it was downregulated in liver cancer. Until now, no studies have been conducted to try to understand the differential expression of *MACC1* between primary and metastatic liver tumors.

Expression of Genes Related to the TCA Cycle

In addition to studying Warburg effect-associated genes, we also compared the expression of TCA-associated genes between normal and tumor tissues. As expected, expression of most genes was significantly higher in normal tissues than in GI tumor cells. Some genes showed high expression in colon and rectal cancers, but others showed an inverse pattern, including *CS*, *SDHB*, *FH*, and *IDH1*. Some studies have reported that *CS* activity is higher in human pancreatic ductal carcinoma than in adjacent, non-cancerous tissues.[34] Next, we analyzed the genetic alterations found in the *IDH1*, *FH* and *SDHB* genes upregulated in GI cancers by means of cBioPortal (Supplementary Figure). Based on recent studies, mutations in the *SDHB*, *IDH1*, and *FH* genes are common and are associated with different kinds of tumors.[35–37] Overall, these results probably explain why these genes are expressed at higher levels in tumors than in normal tissues.

Influence of the Warburg Effect on Clinical Progression

We analyzed the relationship between mRNA expression and clinical stages in GI cancer (Figure 3). The cancer stage codes were based on the American Joint Committee on Cancer definitions. Our results showed that high *CUEDC2*, *HMGB2*, *MACC1*, *PFKFB4*, and *PFKP* mRNA expression was associated with the worst stages of liver cancer. We also detected higher expression of *MACC1*, *PFKFB4* and *PIM1*

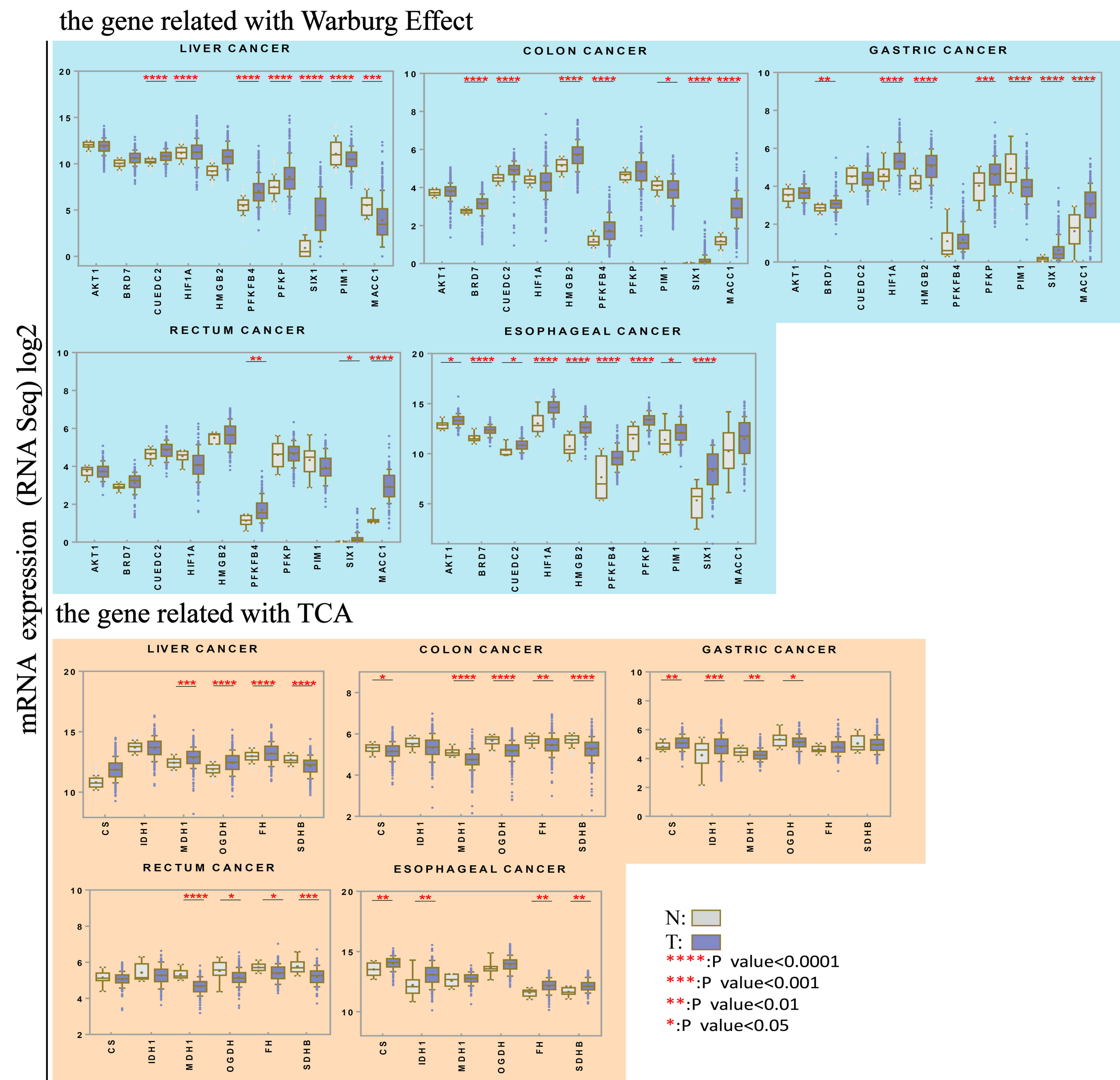

Figure 2 Expression of genes associated with the Warburg effect and the TCA cycle in GI cancers and normal tissues. Upper panel: Expression of genes associated with the Warburg effect. *AKT1*, *BRD7*, *CUEDC2*, *HIF1A*, *HMGB2*, *PFKFB4*, *PFKP*, *PIM1*, *SIX1* and *MACC1* expression in six different types of gastrointestinal cancers. TCGA RNA-Seq data was analyzed to compare expression between tumors (grey) and normal tissues (white). Lower panel: Expression of genes associated with the TCA cycle in tumoral and normal tissues. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.001.

in colon cancer patients with the worst stages. In contrast, expression of genes involved in the Warburg effect was not significantly associated with stages in other cancers. However, *PIM1* expression was significantly increased in the more advanced stages of rectum cancer.

Furthermore, we analyzed the relationship between high or low expression of different genes in GI cancers and overall survival (OS) (Figure 4). Based on Kaplan–Meier curves and log-rank analysis, we found that high expression of *CUEDC2*, *HMGB2*, *PFKFB4*, *PFKP*, and *SIX1* Warburg effect-associated genes was significantly associated with worse OS in patients with liver cancer. Collectively, these results suggest that the Warburg effect may play a role in the progression and outcome of GI and liver cancers. In agreement with our results, *PFKP* was previously reported as a marker of tumor progression and OS in liver cancer.[38,39] The main problem encountered in the treatment of cancer is resistance to chemotherapy,

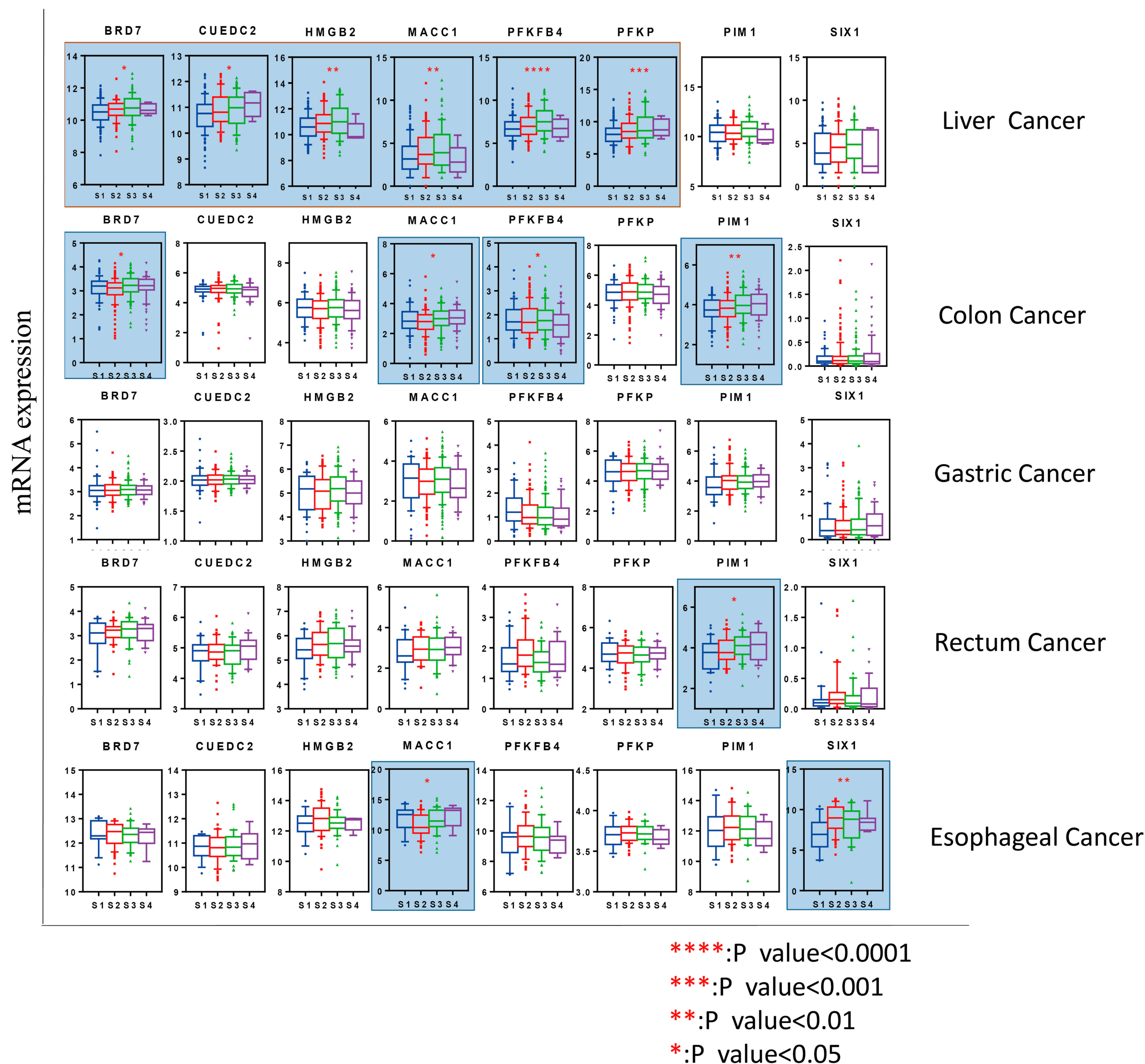

Figure 3 Association of genes linked to the Warburg effect with pathological stages. In cancers of the liver, esophagus, colon and rectum, expression of some genes was significantly associated with worse pathological stages. However, in gastric cancer, the correlation was not obvious. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.
Abbreviations: S1, Stage-I; S2, Stage-II; S3, Stage-III; S4, Stage-IV.

which is crucially linked to cancer stem cells. We found that *HMGB2* was strongly upregulated in all the cancers analyzed. Similarly, increased expression of *HMGB2* has been reported in liver cancer.[17] In addition, HMG members, including HMGB2, interact with hepatitis viruses (HBV and HCV) to modulate HCC progression.[40,41]

In addition to studying mRNA expression, we also investigated the influence of the Warburg effect on clinical parameters. The Warburg effect was identified as an essential factor associated with the proliferation, growth and metastasis of different tumors and with the tumor stage and OS. Patients with high expression of Warburg effect-associated genes showed more advanced stages and a poorer prognosis. This indicates that the Warburg effect may enhance tumor aggressiveness and worsen patient prognosis, particularly in liver cancer, and this result also has been demonstrated in other studies.[42,43] Although the mechanism has not been investigated, increased expression of genes linked to the Warburg effect may be considered as a prognostic marker.

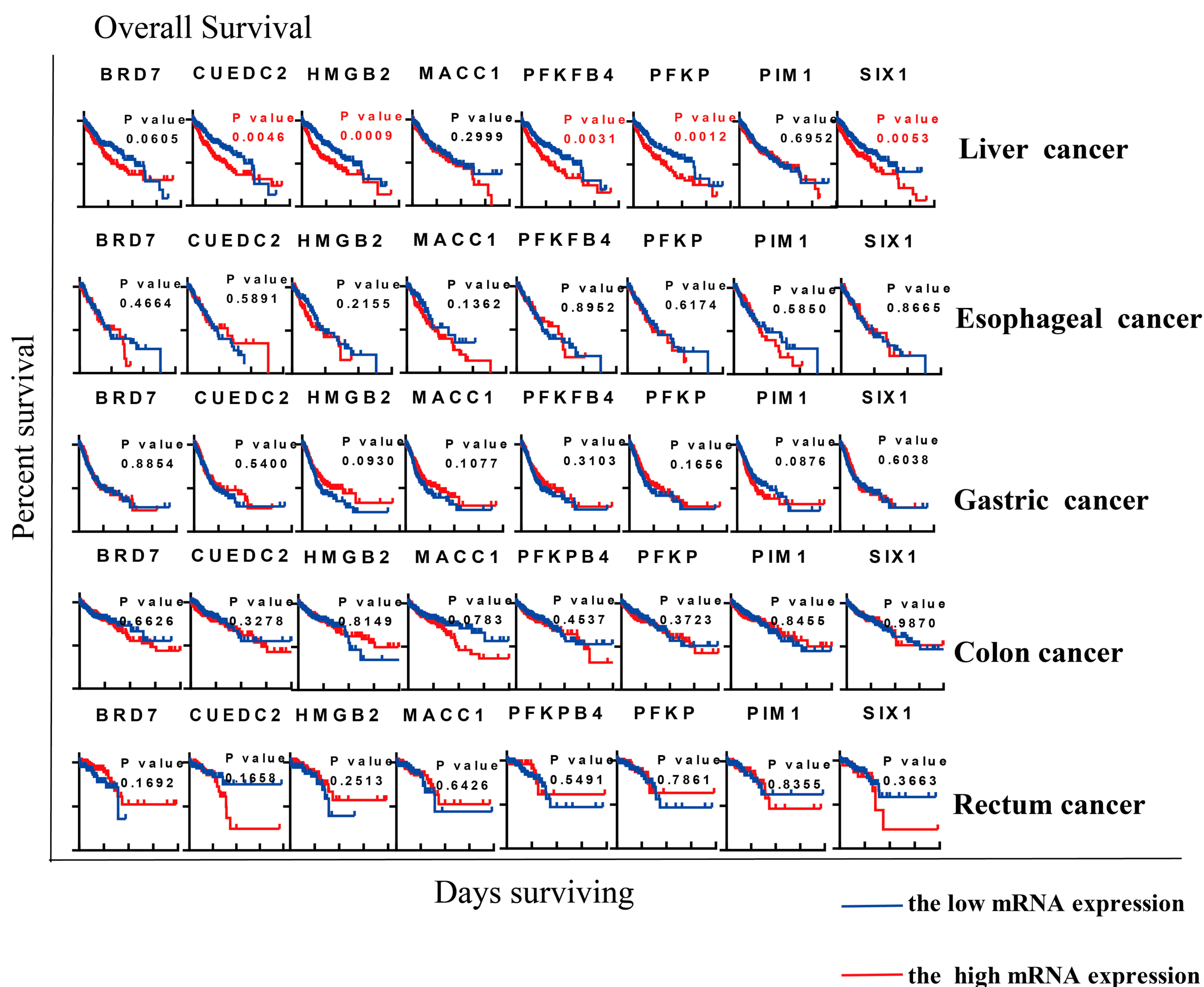

Figure 4 Association of genes linked to the Warburg effect with overall survival. The Warburg effect seemed to affect overall survival in liver cancer more than in any other type of cancer. High expression of genes was significantly associated with worse overall survival. High expression of *CUEDC2*, *HMGB2*, *PFKFB4*, *PFKP* and *SIX1* was significantly associated with poor prognosis in liver cancer. Statistical analysis was carried out based on Kaplan–Meier curves.

Inhibition of the Warburg Effect in Liver Cancer Cells

GAPDH promotes higher glycolysis in cells and KA has been shown to be an irreversible and selective inhibitor of GAPDH. Therefore, KA significantly reduces glycolysis and targets the Warburg effect in cancer cells[44] (Figure 5A). Based on our bioinformatics results, we determined that liver cancer was more likely to be affected by key regulators of the Warburg effect than other GI cancers. In order to understand the influence of the Warburg effect in liver cancer cells, we used KA to suppress the Warburg effect by inhibiting GADPH. Based on the MTT assay, KA (5 µM and 10 µM) significantly inhibited the viability of Hep3B, Huh-7 and Bel7407 HCC cells (Figure 5B). Consistent with these results, migration of HepG2, Huh-7, Bel7407 and Hep3B cells was inhibited by treatment with 1 µM KA for 24 hours (Figure 5C). As shown in Figure 5D, GAPDH activity was strongly inhibited in liver cancer cells. We hypothesize that the explanation for these results is that KA inhibited the Warburg effect in liver cancer cells.

As expected, migration and growth of hepatocellular carcinoma cell lines were significantly inhibited after blocking the Warburg effect with the GAPDH inhibitor. According to some studies, the occurrence of liver cancer is indeed closely related to glycolysis.[45,46] Since the liver is one of the three main metabolic centers in our body, the occurrence and development of malignant tumors in this organ may be closely linked to glucose metabolism. However, the influence of the Warburg effect in patients with liver cancer needs further investigation.

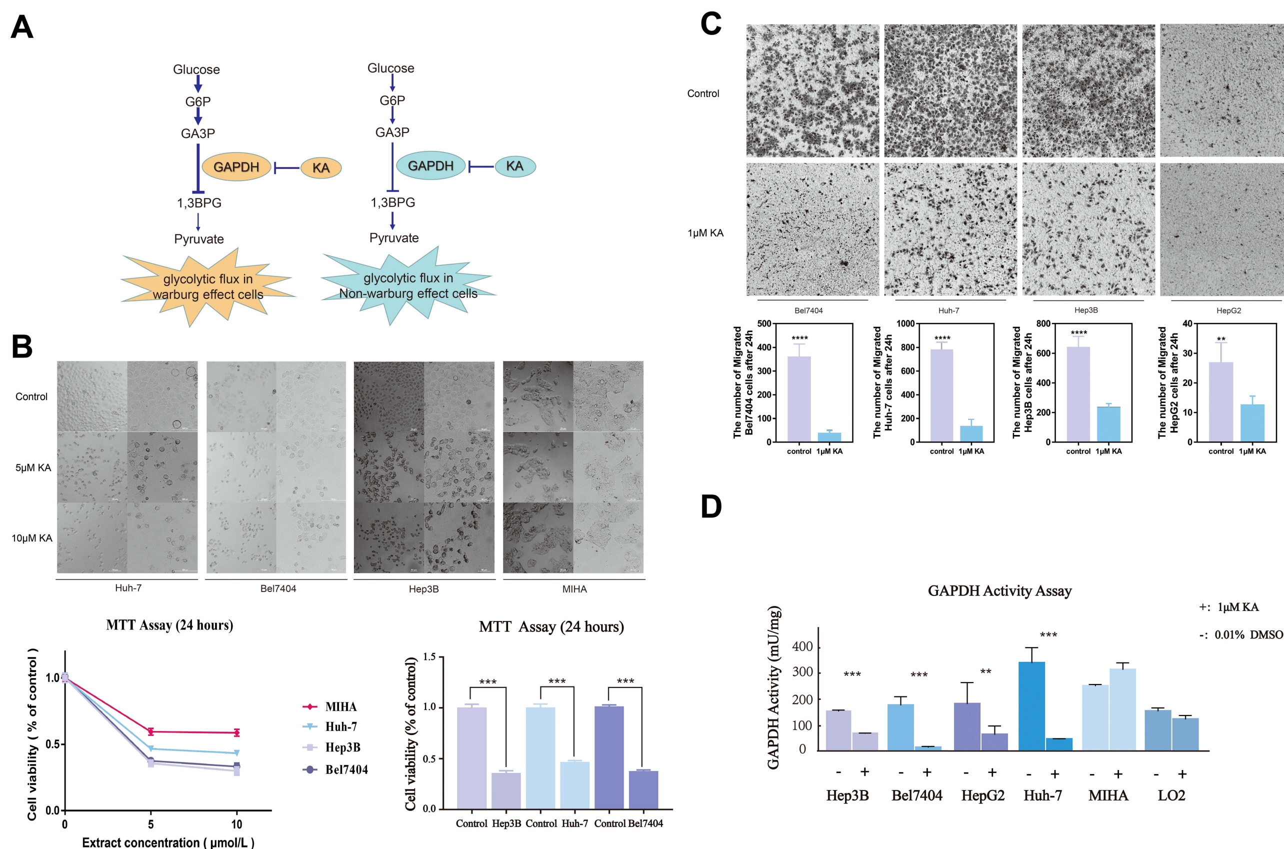

Figure 5 GAPDH inhibition suppresses the Warburg effect in HCC cells. (**A**) Diagram showing how KA-mediated inhibition of GAPDH interferes with glycolytic flux. (**B**) MIHA, Huh-7, Hep3B, and Bel7404 cell proliferation after GAPDH inhibition, measured with the MTT assay. Cellular viability in the treated groups (5 or 10 μmol/L KA for 24 h) was significantly decreased with respect to controls. (**C**) Representative microscopic fields showing migration of control cells and cells treated with 1μM KA. KA significantly suppressed migration of the four types of liver cancer cells. (**D**) GAPDH activity after treatment with 1μM KA or 0.01% DMSO (control). **$P < 0.01$, ***$P < 0.001$, ****$P < 0.001$.

Conclusions

In conclusion, our analysis showed that genes associated with the Warburg effect were overexpressed in GI cancers. Our results also provided insight into the association between the Warburg effect and overall survival and clinical characteristics. Our study suggests that blocking the Warburg effect may be a promising approach to treat liver cancer.

Abbreviations

CS, citrate synthase; *FH*, fumarate hydratase; *IDH1*, isocitrate dehydrogenase (NADP (+))1, cytosolic; *MDH1*, malate dehydrogenase 1; *OGDH*, oxoglutarate dehydrogenase; *SDHB*, succinate dehydrogenase complex iron sulfur subunit B; *ATK1*, serine/threonine kinase 1; *BRD7*, bromodomain-containing 7; *CUEDC2*, CUE domain-containing protein 2; *HIF1A*, hypoxia-inducible factor 1 subunit alpha; *HMGB2*, high mobility group box protein 2; *MACC1*, metastasis associated in colon cancer 1; *PFKFB4*, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; *PFKP*, platelet isoform of phosphofructokinase 1; *PIM1*, Pim-1 proto-oncogene, serine/threonine kinase; *SIX1*, sine oculis homeobox 1 transcription factor.

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Disclosure

The authors have no conflicts of interest to declare.

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