Histone lactylation drives hepatocellular carcinoma progression by promoting ASPM expression

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Introduction. Hepatocellular carcinoma (LIHC) is the most common form of primary liver cancer in the world, the sixth most common tumor, and the third leading cause of cancer-related mortality worldwide. In recent years, more than 700,000 people die every year worldwide. Although great progress has been made in the diagnosis and treatment of LIHC, the prognosis of LIHC is still poor due to the complex mechanisms of tumor occurrence and development. Therefore, there is an urgent need to understand the molecular mechanisms of LIHC occurrence and development. In recent years, it is of great significance to study the molecular causes related to LIHC. With the development of tumor metabolism, more and more studies have proven that lactic acid is related to the progression of human cancer, and lactic acid has been shown to mediate histone lactylation. Histone lactylation regulates gene expression to drive tumor progression in ocular melanoma and clear cell renal cell carcinoma. However, the role of histone lactylation in LIHC progression remains unclear. Here, we demonstrate that histone lactylation modification may be involved in the progression of LIHC. We noticed that histone lactylation modification regulates the expression of ASPM and is positively correlated with poor prognosis. ASPM knockdown significantly inhibited the proliferation of LIHC cells in vitro. Therefore, we believe that ASPM can serve as a promising therapeutic target for LIHC.
Keywords. lactylation, gene expression, hepatocellular carcinoma, ASPM

INTRODUCTION

The vast majority of primary liver cancers are hepatocellular carcinoma (LIHC), which accounts for about 75% to 80% of primary liver cancers [1]. In recent years, the incidence and mortality of LIHC have been on the rise, and it is the third most common cause of tumor-related death. In China, the mortality rate of liver cancer is second only to lung cancer, causing about 700,000 deaths each year [2,3]. Surgical resection is the main treatment for patients with early liver cancer. Since most LIHCs occur in the context of liver disease and cirrhosis, liver transplantation is usually also a treatment option for patients with early liver cancer [4]. The best treatment for patients with intermediate-stage liver cancer is transarterial chemoembolization (TACE) [5]. In the past, chemotherapy, radiotherapy and molecular targeted therapy were the main treatments for patients with advanced LIHC. Nowadays, newer clinically effective treatments include kinases and immune checkpoints. Due to the high recurrence rate

and the influence of intrahepatic or distant metastasis, LIHC patients have poor long-term efficacy and their 5-year survival rate is still very low [6,7]. Therefore, it is of great significance to study the molecular etiology of LIHC, which may provide a reference for finding new targets for the treatment of LIHC. Glucose is the body's most important energy source[8]. Under normal circumstances, the primary means of obtaining energy is through oxidative phosphorylation of glucose. Only when oxygen is insufficient do these cells turn to glycolysis to meet their energy needs. Tumor cells use both traditional oxidative metabolism and anaerobic metabolism using glycolysis. However, when oxygen is sufficient, their proliferation is more dependent on increased glycolysis metabolism, a feature known as the Warburg effect[9]. The Warburg effect was first discovered in the early 20th century in studies of rat liver cancer tissue sections[10]. Lysine lactylation (Kla) is a new type of protein post-translational modification discovered in 2019. Aerobic glycolysis leads to increased production of lactate metabolites, which are converted to lactoyl CoA, and finally a lactoyl (La) group is added to the lysine residues on the histone tail[11]. It is worth noting that the level of lactylation is positively correlated with the cellular lactate content. Lactation modification increases when cells are exposed to drugs or conditions that increase lactate levels, such as exogenous addition of lactate and mitochondrial inhibitor rotenone; glycolysis inhibitors such as 2-deoxy-D-glucose (2-DG) and sodium oxamate (Oxamate) reduce lactate production, thereby reducing the level of lactation modification [12]. As a member of post-translational modification of proteins, lactation modification is also regulated by writers and erasers, and recognized by readers to exert its corresponding biological functions. E1A binding protein p300 (EP300) is a classic lactation modification reader, and its corresponding protein product histone acetyltransferase is involved in the enzymatic process of lactation modification [13]. Numerous studies have shown that lactation modification abnormalities are common in various tumors, indicating that this gene may be related to tumorigenesis. Increasing evidence shows that lactation modification is also enhanced in different types of malignant tumors, such as ocular melanoma [14], non-small cell lung cancer [15] and renal clear cell carcinoma [16], leading to tumor progression and associated with poor prognosis. In this study, we focused on the role of lactylation in LIHC cell proliferation and migration and found that ASPM could serve as a new target for combined therapy and a useful biomarker for LIHC prognosis.

MATERIALS AND METHODS

Gene expression analysis and survival analysis

The RNA sequencing data of 369 liver cancer tumor tissues and 160 normal liver tissues from the TCGA and GTEx databases were obtained using the GEPIA database (http://gepia.cancer-pku.cn/). The differential expression of genes in tumor combinations and normal tissues was analyzed using the Boxplot of the ExpressionDIY module. The expression of genes at different stages of the tumor was analyzed using the Stageplot of the ExpressionDIY module. The correlation between

two genes or two features in the tumor was analyzed using the CorrelationAnalysis module. The pan-cancer analysis of target genes regulated by lactylation modification was performed using the Gene_DE module in the TIMER2.0 database (https://cistrome.shinyapps.io/timer/). The distribution of gene expression levels is shown in box plots.

Cell lines and cell cultures

All cells were purchased from Shanghai Cell Bank. Human liver cancer cells Huh-7 and normal human cells L02 were cultured in Zhongqiao Xinzhou high-glucose DMEM medium, and human liver cancer cells HepG2 were cultured in MEM medium. 10% fetal bovine serum and 1% penicillin-streptomycin were added to the above medium. The cell culture conditions were 37°C and 5% CO2 saturated humidity conditions. After 48 hours of cell culture, the cells were digested with 0.25% EDTA-trypsin and passaged. By adding lactate or 2-DG to the above cell culture medium, the level of histone lactylation in cells can be regulated

Western blot analysis

Proteins were extracted for immunoblot analysis. Proteins (20 μg) were separated on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline Tween20 (TBST) and detected with PanKla antibody (1:1000 dilution, PTM-1401RM), anti-ASPM antibody (1:1000 dilution, 26223-1-AP), goat anti-rabbit β-actin (1:1000 dilution, BL003A,), and β-actin was used as an internal loading control. Cell proliferation assay

Cell proliferation was assessed using the Cell Counting Kit 8 (CCK-8) assay according to the manufacturer's instructions (Dojindo Molecular Technologies, Rockville, MD). Huh-7 and HepG2 cells in 96-well plates were incubated with CCK-8 solution at 37°C for 1.5-2 hours. The absorbance of each well was quantified at 450 nm using a microplate reader.

Cell scratch assay

Huh-7 cells and the two transfected cells were seeded into 6-well plates at a seeding cell number of 8x105/well. After culturing for 24 hours, the cells were attached to the wall. A straight line was evenly and stably drawn perpendicularly to the bottom of the 6-well plate with a 100uL pipette tip, and the detached cells were repeatedly washed with PBS. The low-concentration serum medium was replaced and continued to be cultured. Pictures were taken under a microscope at 0h, 12h, 24h, and 48h for preservation and analysis.

RNA sequencing，data analysis

Total RNA was extracted using an RNA extraction kit (RC112) following the manufacturer’s instructions. RNA sequencing samples were then submitted to Annoroad Gene Technology Corporation (Beijing) for transcriptome sequencing and analysis.

RNA isolation and quantitative real-time PCR(RT-qPCR)

Total RNA was isolated from tissue samples and cell lines using an RNA extraction

kit (RC112) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a reverse transcription kit (R333-01). Subsequently, quantitative real-time polymerase chain reaction (RT-qPCR) was performed on an Cobas z480 Fully automatic fluorescent PCR analyzer using a PCR kit (R333-01). The relative expression of related genes was detected using the 2-ΔΔCt method. Primers been used in this study were listed in Table 1. β-actin was selected as the internal control for normalization.

Table1
Primer sequences for RT-qPCR.

Genes	Sequence 5'-3'
ASPM	Primer -F: GAGTTAATGCAGCACTCGTCA
	Primer -R: TCTCCTCCACATAGCCTGAATAA
PPFIA4	Primer -F: GTGAGGCAACATCCATCCAT
	Primer -R: GTGAGGGCTGCAATTCTCTG
SLC38A3	Primer -F: CTGCTTTCTCACGGGTTGA
	Primer -R: CAGACTCAGGGCATTGACAG
AKR1D1	Primer -F: CATGTCCCAGAGATGGTCCG
	Primer -R: ATGGCACTCAACCTCCCAAG
β-actin	Primer -F: CATGTACGTTGCTATCCAGGC
	Primer -R: CTCCTTAATGTCACGCACGAT

Data analysis

GraphPad Prism 8.0 statistical software was used for analysis and processing. Two independent samples were tested by t-test. The comparison of data between more than two groups was analyzed by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Altered expression of metabolic enzymes in HCC is associated with poor prognosis

Our TCGA data analysis of HCC shows that lactate dehydrogenase A (LDHA) (Figure 1A), key enzyme of glycolysis (HK2) (Figure 1B), M2-type pyruvate kinase (PKM2) (Figure 1C), solute carrier The increased expression of family 16 member 3 (SLC16A3) (Figure 1D) was significantly associated with survival prognosis of HCC, and the expression of these metabolic enzymes increased with the progression of

tumor pathological stages (Figure 2A-D).

LDHA directly regulates lactate metabolism;KK2 and PKM2 stimulate glycolysis and transfer glucose metabolism in tumor cells from the normal respiratory chain to lactate production. MCT4 encoded by SLC16A3 can act as a lactate transporter. These results revealed a correlation between increased expression levels of enzymes related to lactate metabolism in liver cancer and survival prognosis and tumor progression, strongly suggesting cellular metabolic disorders and the important role of lactate in liver cancer.

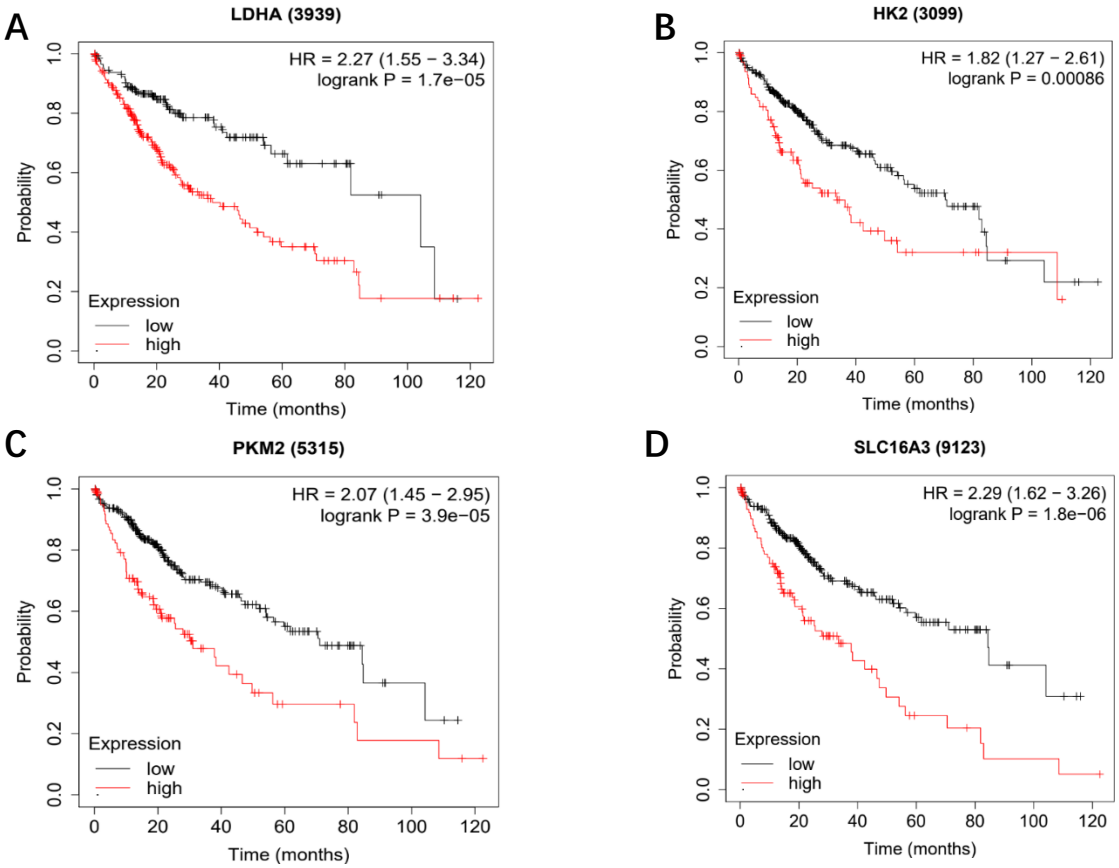


Figure 1. Metabolic enzyme abnormalities may contribute to human hepatocellular carcinoma progression. Elevated expression levels of LDHA (A), HK2 (B), PKM (C), and SLC16A3 (D) are all significantly associated with poor prognosis in human hepatocellular carcinoma.

Hepatocellular carcinoma cells increase lactate secretion and subsequently increase lactation levels

As expected, due to the Warburg effect in tumor cells, the lactate concentration in the culture supernatant of human liver cancer cells Huh-7 and HepG2 cells cultured under normoxic conditions for 48 hours was significantly higher than that of human normal liver cells LO2. increased (Figure 3A); correspondingly, the histone lactylation level of Huh-7 and HepG2 liver cancer cells was also significantly increased compared with the histone lactylation level of normal liver cells LO2 (Figure 3B).

Histone lactylation regulates the proliferation and migration of liver cancer cells

To evaluate whether histone lactylation can regulate the proliferation and migration of

liver cancer, we regulated the overall histone lactylation level in tumor cells. In this paper, the addition of glycolysis inhibitor 2-deoxy-D-glucose (2-DG) to inhibit lactate production reduced the level of histone lactylation (Figure 4A) and the addition of lactate to increase the substrate increased the level of histone lactylation (Figure 4B). Compared with the control group, the histone lactylation level of Huh-7 cells treated with 2-DG was inhibited, while the histone lactylation level of the lactate-treated group was enhanced (Figure 5A). We found that regulating histone lactylation can effectively affect the proliferation and migration of liver cancer cells (Figure 5B-D). In summary, histone lactylation plays a role in regulating the proliferation and migration of liver cancer cells.

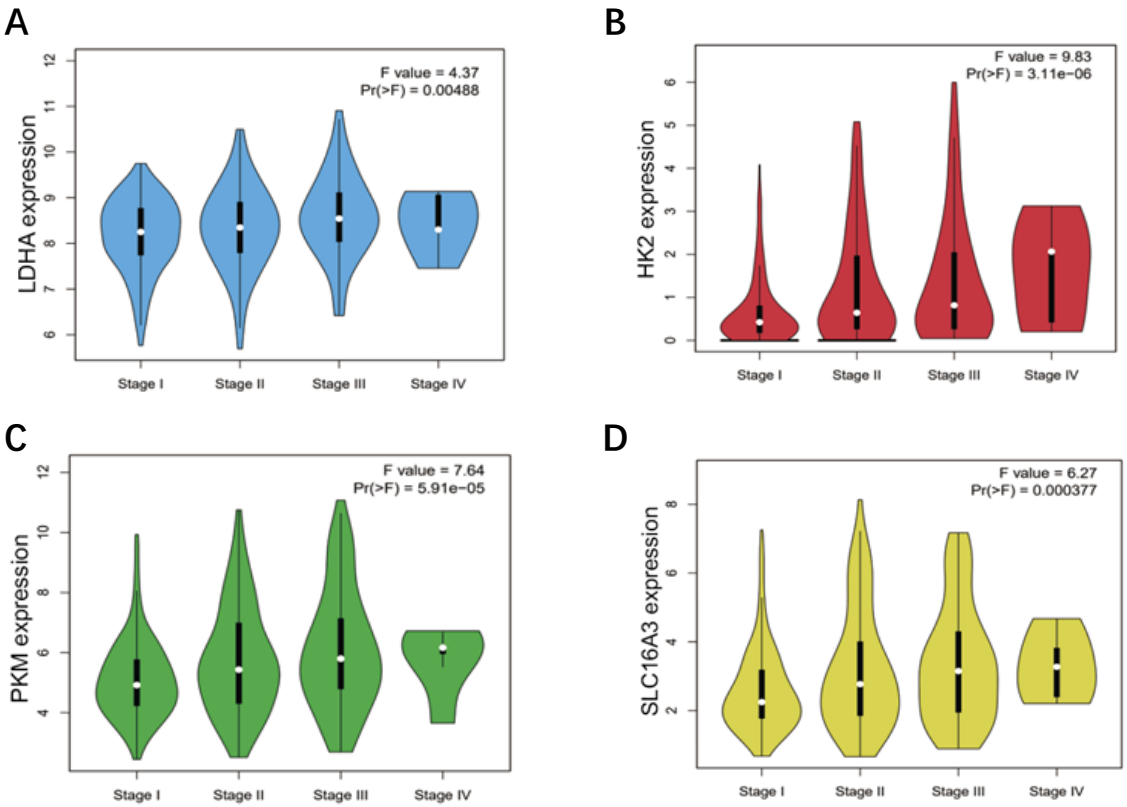
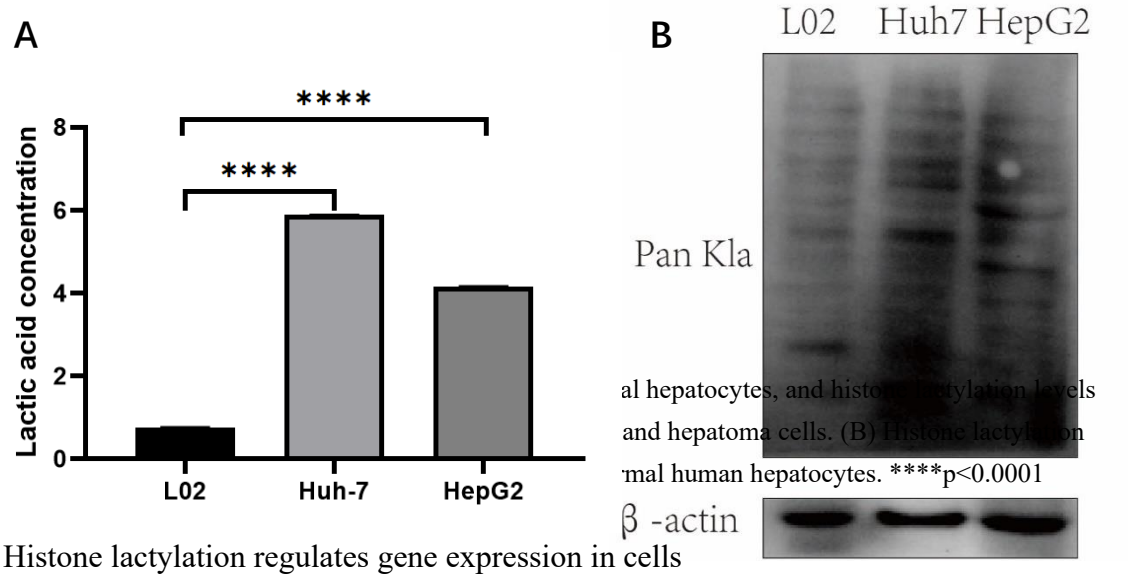


Figure 2. Increased expression of metabolic enzymes during the progression of human hepatocellular carcinoma. The expression levels of LDHA (A), HK2 (B), PKM (C), and SLC16A3 (D) increase with pathological stage.



al hepatocytes, and histone lactylation levels and hepatoma cells. (B) Histone lactylation mal human hepatocytes. ****p<0.0001

Histone lactylation regulates gene expression in cells

Given the latest research reports that lactate regulates gene transcription through histone lactylation, it is speculated that the effects of lactate on the metabolism and biological characteristics of liver cancer cells may be caused by changes in gene expression mediated by histone lactylation. In order to reveal the regulatory role of histone lactylation in gene expression, we extracted RNA from two groups of cells in the untreated control group and the 2-DG-treated group for transcriptome RNA sequencing (RNA-seq) to obtain genes regulated by histone lactylation. We performed KEGG pathway enrichment analysis on the obtained differentially expressed genes, and we found that these differentially expressed genes were enriched in metabolic pathways and tumor-related pathways (Figure 6A). We selected four target genes that may be regulated by lactylation, and the mRNA levels of these genes were significantly reduced in cells treated with 2-DG. Among these candidate genes, RT-qPCR was used to verify that the mRNA level of ASPM in 2-DG-treated cells was significantly reduced, which was consistent with the RNA-seq results. In contrast, the mRNA level of ASPM was significantly increased in lactate-treated cells (Figure 6B). The ASPM gene encodes a protein composed of 3477 amino acids and is closely related to cell mitosis. Therefore, we focused on it.

ASPM is a novel oncogene in hepatocellular carcinoma

As ASPM is regulated by histone lactylation, we next explored its function in hepatocellular carcinoma. The Gene Expression Profiling Interactive Analysis (GEPIA) database confirmed that ASPM was highly expressed in hepatocellular carcinoma (Figure 7A), and higher ASPM levels were associated with poor prognosis (Figure 7B). We then validated the function of ASPM in hepatocellular carcinoma cells by silencing its expression with three small interfering RNAs (named siASPM#1, siASPM#2, and siASPM#3). After successful knockdown of ASPM (Figure 7C, D), we performed CCK-8 assays, and the results showed that cell proliferation was inhibited (Figure 7E). In addition, we also observed that knockdown

of ASPM could inhibit the migration of tumor cells, as measured by cell scratch assay (Figure 7F)

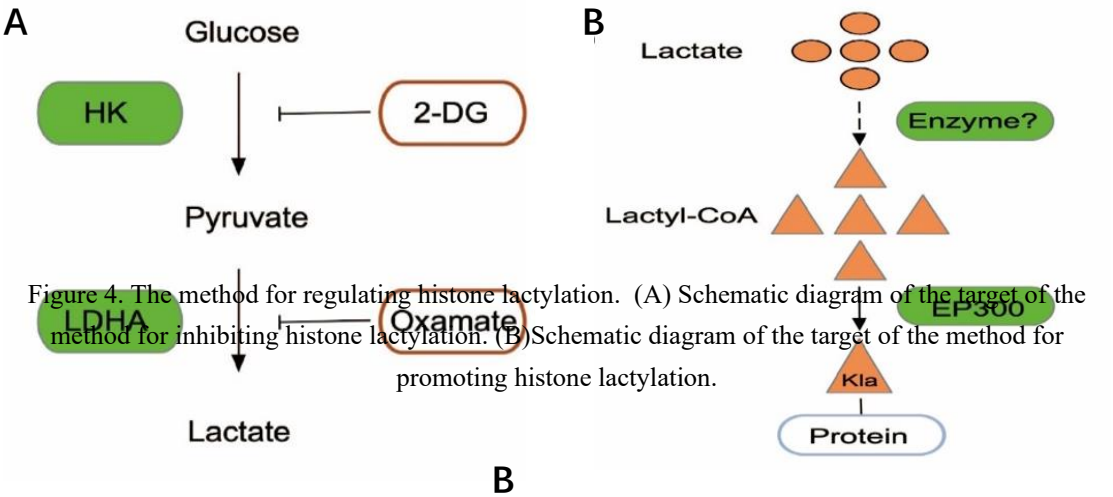


Figure 4. The method for regulating histone lactylation. (A) Schematic diagram of the target of the method for inhibiting histone lactylation. (B)Schematic diagram of the target of the method for promoting histone lactylation.

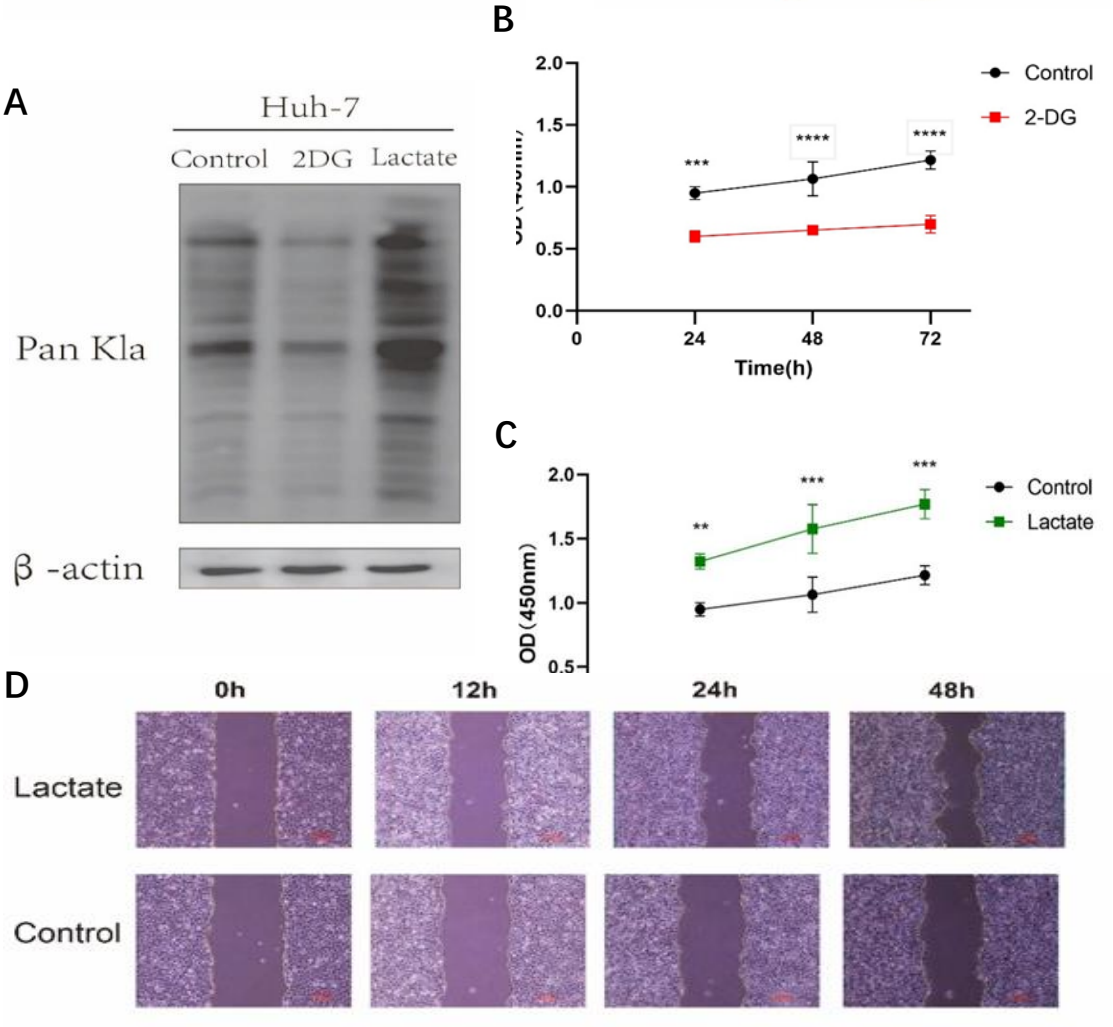


Figure 5. Regulation of histone lactylation affects the proliferation and migration of liver cancer cells. (A)Immunoblotting was used to detect the level of histone lactylation in Huh-7 cells after 24 hours of culture in untreated, 2-DG-treated and lactic acid-treated cells. (B,C) CCK8 assay was used to analyze the proliferation of Huh-7 cells after 2-DG treatment (B) and lactic acid treatment (C). **p<0.01,

***p<0.001, ****p<0.0001. (D) Cell scratch assay was used to detect the migration of Huh-7 cells after untreated, 2-DG-treated and lactic acid-treated cells.

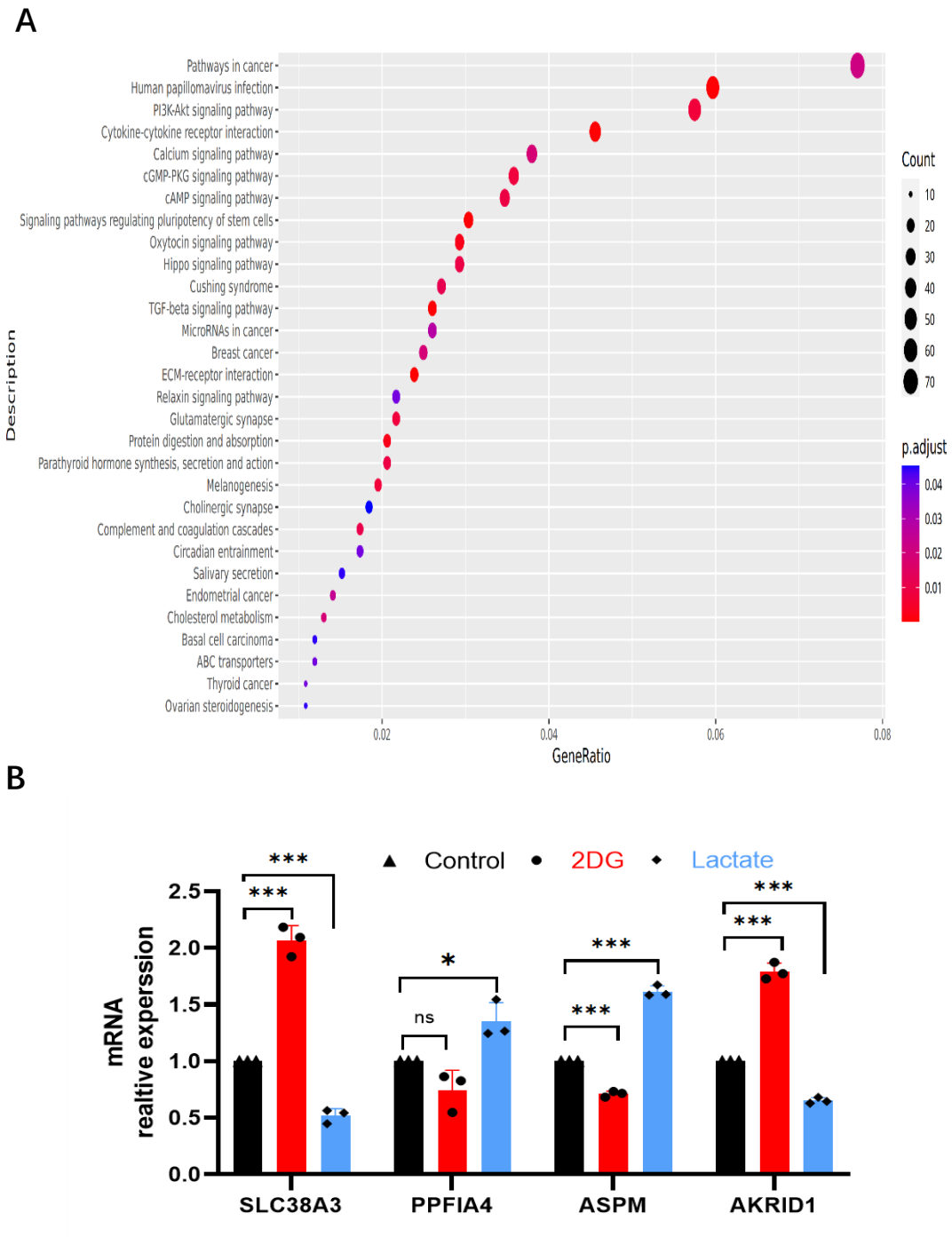


Figure 6. Histone lactylation promotes the transcription of ASPM (A) KEGG pathway enrichment analysis bubble diagram. (B) mRNA expression levels of the four identified genes in the experimental group and the control group. ns no statistical significance, *p<0.01, ***p<0.001

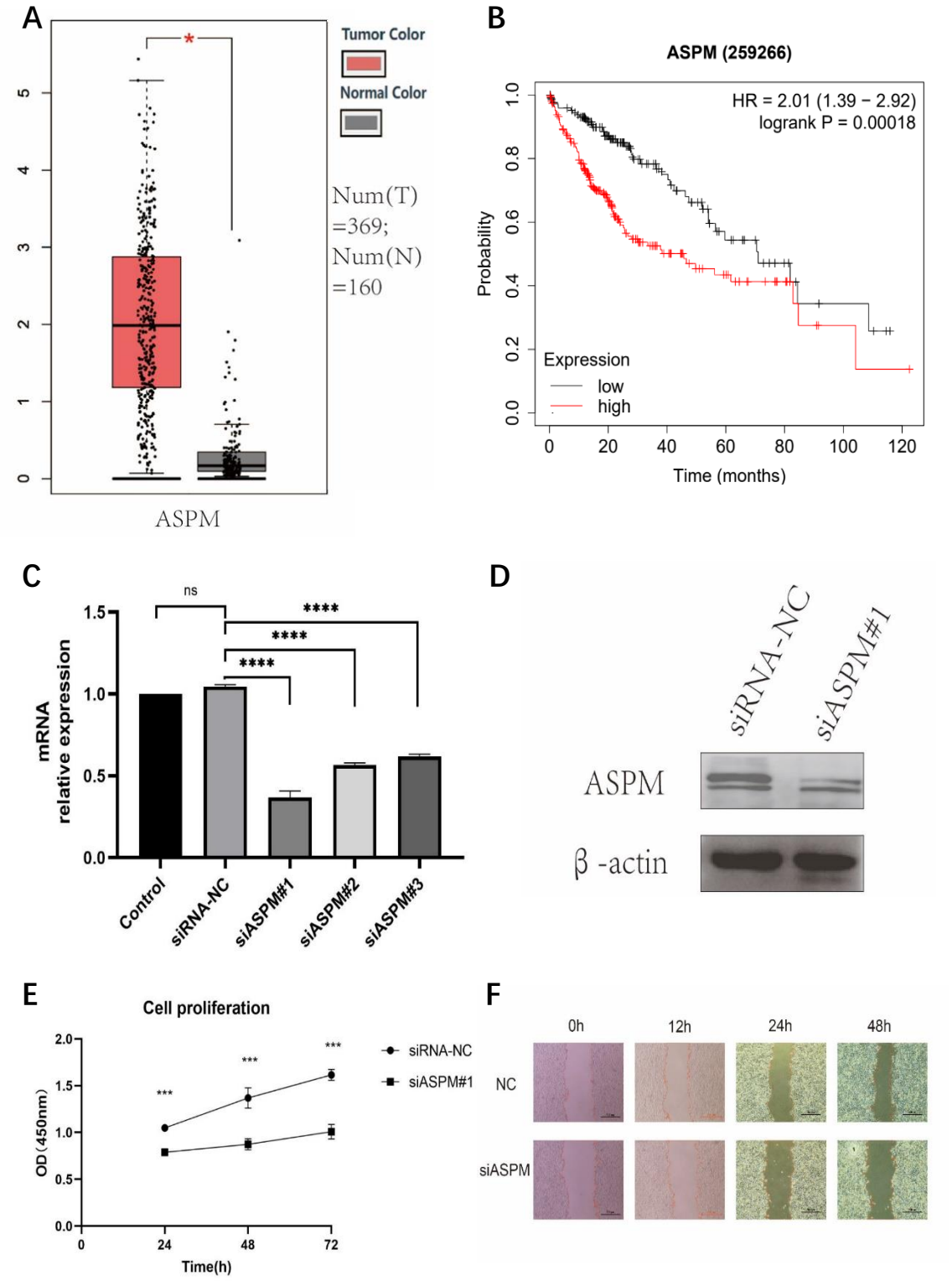


Figure 7. ASPM acts as an oncogene in liver cancer (A) ASPM is highly expressed in liver cancer tissues.(B)The relationship between ASPM transcription level and liver cancer prognosis. (C,D) RT-qPCR (C) and Western blot (D) showed that ASPM was silenced after siRNA transfection in liver

cancer cells. (E)CCK8 assay analyzed the proliferation of Huh-7 cells in the control group and after ASPM silencing. ***p<0.001. (F)Cell scratch assay detected the migration of Huh-7 cells in the control group and after ASPM silencing.

DISCUSSION

Lactic acid is both the end point of glycolysis and a universal metabolic fuel for energy. The emerging concept of "lactate shuttle" promotes the movement and signaling of lactate between cells[17,18,19].The prominence of glycolysis-dependent metabolism in tumors and rapidly proliferating cells makes lactate a key player in the reprogramming of energy metabolism, allowing cells to efficiently acquire sufficient energy within a limited time period[20,21]. In addition, lactate has the potential to create a favorable environment for tumor development by affecting the tumor's acidic microenvironment, recruitment of immune cells, and other mechanisms. In addition to playing an important role in accelerating tumorigenesis, histone lactylation can also serve as an important diagnostic marker for various cancers, such as ocular melanoma[14] and colorectal cancer[22,23]. Notably, elevated levels of global lactylation (pan-lactylation) are specifically upregulated in tumor tissue, indicating a higher likelihood of early and more aggressive recurrence of ocular melanoma. Furthermore, there was a significant correlation between pan-lactylation and elevated H3K18la levels and decreased overall survival in colorectal cancer patients. Positive feedback regulation between glycolysis and histone lactylation drives oncogenesis in pancreatic ductal adenocarcinoma[24]. Taken together, these findings corroborate the notion that aberrant histone lactylation is significantly elevated in different tumor categories, thereby exerting a critical influence on the development of malignant tumors.

Abnormal spindle-like microcephaly-associated protein (ASPM) is the human homolog of the Drosophila mitotic spindle protein. The gene spans 65 kb of genomic DNA at chromosome 1q31 and consists of 28 exons. Contains a 10,434 bp long coding sequence[25]. ASPM is a spindle pole/intermediate-associated protein that is a component of the mitotic spindle and regulates and maintains cell proliferation and symmetric division[26]. Because ASPM can regulate cell division and proliferation, its correlation with tumor occurrence and development has also attracted the attention of many scholars. In glioblastoma research, it was found that patients with high expression of ASPM have significantly worse prognosis, and downregulation of ASPM can arrest the cell cycle of glioblastoma cells and weaken Wnt/β-catenin signaling in glioblastoma. conductive activity[27]. A large number of studies have found that ASPM is also abnormally expressed in other cancers and is used to predict the prognosis of various types of tumors, including endometrial adenocarcinoma[28], pancreatic cancer[29] and prostate adenocarcinoma[30]. In addition, ASPM genes have been found to be overexpressed in various types of tumors, such as lung cancer[31], bladder cancer[32], gastric cancer[33], and colon adenocarcinoma [34]. The overexpression of ASPM affects the occurrence and development of various types of tumors.

There are not many reports on the role of this new type of histone modification, histone lactylation, in tumors. Since cancers exhibit active glycolysis and produce a

large amount of lactic acid as a substrate for histone lactylation, we explored for the first time the potential role of histone lactylation in the occurrence and development of liver cancer. We found that the expression of lactate metabolism and glycolysis-related enzymes was elevated in hepatocellular carcinoma, and targeted correction of abnormal histone lactylation could effectively inhibit tumorigenesis. Increased histone lactylation promotes the expression of ASPM. The TIMER and GEPIA databases were used to analyze the mRNA levels of ASPM in hepatocellular carcinoma and its corresponding normal tissues, as well as the effect of ASPM gene expression on prognosis. The results showed that ASPM was significantly upregulated in hepatocellular carcinoma tissues, and high ASPM expression was associated with poor prognosis in patients. Targeted knockdown of ASPM can effectively inhibit the occurrence of hepatocellular carcinoma.

AUTHOR CONTRIBUTIONS

Conception and design: Qin Zhu, Qin Zheng. Financial support: Qin Zheng. Data collection and compilation: Yu Sun, Zhicheng Cao. Data analysis and interpretation: Qin Zhu. Manuscript writing: all authors. Final approval of the manuscript: all authors. Responsible for all aspects of the work: all authors. All authors have read and approved the manuscript.

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