Hsa_circ_0001806 promotes glycolysis and cell progression in hepatocellular carcinoma through miR-125b/HK2

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Abstract

Objective: Hepatocellular carcinoma (HCC) is one of the most common malignant tumours and a leading cause of cancer death. Circular RNA (circRNA) has been demonstrated to play an important role in regulating tumour development. The current study aims to explore the specific role of hsa_circ_0001806 during HCC progression.

Methods: The expression of hsa_circ_0001806 in HCC tissues and cells was measured through qRT-PCR. Cell proliferation, apoptosis and migration were measured using CCK-8 and Annexin V/PI staining kits, and Transwell assay. Bioinformatics prediction and dual-luciferase reporter assay were adopted to explore the mechanism underlying the cell function of hsa_circ_0001806 in HCC cells. In addition, glycolysis was assessed by measuring the glucose uptake, lactate production and ATP level using a glucose assay kit, fluorometric lactate assay kit and ATP detection assay kit.

Results: Hsa_circ_0001806 was up-regulated in HCC tissues and cells and positively associated with the advanced TNM stage, metastasis and poor overall survival. The overexpression of hsa_circ_0001806 promoted HCC cell proliferation, migration and glycolysis and inhibited cell apoptosis, while the silence of hsa_circ_0001806 showed an opposite effect. Furthermore, hsa_circ_0001806 acted as a sponge of miR-125b to up-regulate hexokinase II (HK2) expression. In addition, the inhibition of miR-125b and HK2 overexpression partly reversed the inhibitory effect of hsa_circ_0001806 silencing on HCC cell proliferation, migration and glycolysis.

Conclusion: The inhibition of hsa_circ_0001806 suppressed HCC cell proliferation, migration and glycolysis through mediating miR-125b/HK2 axis.
1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant primary tumours occurring in liver and the third leading cause of cancer death. There were about 782,000 cases diagnosed and 746,000 deaths in 2012. Most patients were diagnosed as HCC at an advanced stage with no obvious symptoms found in the early stages. According to statistics, the 5 years survival rates of patients with early stage of HCC reached 40%–70% following radical surgery, while the survival time for patients with advanced liver cancer was only 6–18 months. In addition, a high recurrence rate was also presented in HCC patients. Chronic hepatitis B and C, cirrhosis, non-alcoholic fatty liver disease, excessive alcohol intake and diabetes were reported to be crucial factors leading to HCC.

Hence, it is necessary to determine the underlying mechanism by which HCC tumorigenesis is regulated and identify new biomarkers and targets for HCC diagnosis and therapy.

First, circular RNAs (circRNAs) are discovered in 1976 and found to be more stable than long non-coding RNA. A covalently closed continuous loop structure without 5′-caps or 3′-tails is formed within circRNAs by back-splicing at exon or intron regions. Increasing evidence indicated that circRNAs played an important role in tumour evolution and progression. The biological regulatory mechanisms of circRNAs include working as sponges of miRNAs, interacting with RNA-binding proteins (RBPs), serving as transcriptional and translational regulators, affecting the splicing of pre-miRNAs and being translated into peptides. Based on that, large amount of circRNAs had been regarded as essential potential biomarkers of various tumour diseases.

In this study, we verified the alteration of hsa_circ_0001806 in HCC tissues and cells, and its relevance with the survival outcomes of HCC patients. Additionally, the role of hsa_circ_0001806 in HCC cell viability, apoptosis, migration and glycolysis was explored. A potential mechanism in which hsa_circ_0001806 exerted on HCC cells was investigated. Overall, our study indicated that hsa_circ_0001806 may act as a potential target for HCC prognosis and therapy.

2 | MATERIALS AND METHODS

2.1 | Collection of human HCC tissues

Sixty-three pairs of HCC tumour tissues and corresponding adjacent normal tissues were collected from HCC patients in Affiliated Hospital of Northwest University Xian NO.3 Hospital, from 2012 to 2015. All experiments involving human participants were approved by the Ethics Committee of the Affiliated Hospital of Northwest University Xian NO.3 Hospital. All patients provided the written informed consents before tissue collection. The overall survival curve was drawn using the Kaplan-Meier method and analysed by the log-rank test according to hsa_circ_0001806 expression (values were expressed as median).

2.2 | Cell culture

Human normal liver cells, LO2 and HCC cells lines (HepG2, Hep3B and Huh7) were acquired from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. Cells were cultured in DMEM (HyClone, Salt Lake City, UT) supplemented with 10% foetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Gibco). Cells were incubated in a cell culture incubator at 37°C in 5% CO₂ atmosphere.

2.3 | Plasmid construction and stable transfection

Overexpression vector (ov) of hsa_circ_0001806 and HK2, small interfering RNA (siRNA) of hsa_circ_0001806, miR-125b mimic, miR-125b inhibitor and negative controls (NC) were synthesized and constructed by GenePharma. Cell transfection was performed by using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocol. Transfection efficiency was confirmed by qRT-PCR and Western blot at 48 h after transfection.

2.4 | RNA preparation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). The obtained RNA samples were quantified on a Nanodrop platform (Thermo Scientific Fisher) and purified using miRNAeasy spin columns (QIAGEN). To detect hsa_circ_0001806 expression, the RNA extract was incubated with RNase R (Epicenter, Madison) before RNA purification. Then, RNA was reverse-transcribed using HiScript II Q RT SuperMix for qPCR.
genes was performed using 2
verse:	5′- CCAGTGCAGGGTCCGAGGT- 3′. The final quantization of target
target:	5′- TGAGACCCTAACTTGTGA- 3′ and reverse:	5′- AGTCTCAGGGTCCGAGG
verse:	5′- ACACCATGTATTCCGGGTCAAT- 3’;	miR-	125b, forward:	5′- T	CC	C
TATTC- 3′;	U6, forward:	5′- TGCGGGTGCTCGCTTCGGCAGC- 3′ and re

Cell migration was evaluated through the Transwell assay. The cul-
tured cells (5 × 10^4 cells) in 300 ml serum free medium were planted
onto the upper chamber (8 μm pore size, Corning). The lower chamber
was supplemented with 600 ml medium and 10% FBS. After being
cultured for 48 h, cells migrated to the lower chamber were fixed
with 4% paraformaldehyde for 15 min and stained with 0.1% crystal
violet. The representative images were obtained under an upright mi-
croscope, and the migrated cells were counted in 5 randomly selected
fields per chamber. This experiment was repeated in triplicate.

western blot analysis
Cells were harvested and lysed by the treatment with RAPI lysis buffer at 4°C
for 30 min. After being centrifuged at 4°C with a speed of 25,764 g for 10 min,
the supernatant was obtained and the protein concentration was analysed
using a BCA Protein Assay Kit (Beyotime). Then, the equal amount of protein
samples was separated by 10% sodium dodecyl sulphate-polyacrylamide gel
electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride
(PVDF) membranes (Millipore). After blocking with 5% non-fat milk, the mem-
branes were incubated with primary rabbit anti-HK2 antibody (ab209847,
1:1000, Abcam, Inc. Cambridge) at 4°C overnight. Then, membranes were
probed with goat anti-rabbit secondary antibody (ab7090, 1:2000, Abcam).
Tubulin (AB6160, 1:2000, Abcam) was used as normalization control. The pro-
tein signals were detected under a chemiluminescence system and analysed
by FluorChem FC3 Software (ProteinSimple) for quantification.

Glucose uptake, lactate production, and
ATP levels
The glucose uptake, lactate production and ATP levels were quanti-
ﬁed by using the glucose assay kit (Sigma-Aldrich), the fluoromet-
ric lactate assay kit (Abcam) and Luminescent ATP Detection Assay
(Abcam) following the manufacturers’ instructions.

Statistical analysis
Statistical analyses were carried out using GraphPad Prism
(GraphPad). Student’s t test was used to compare the statistical sig-
nificance of differences between two groups. Survival curves for
HCC patients were analysed using the Kaplan-Meier method and log-
rank test. Data were presented as mean ± standard deviation (SD).
P < 0.05 indicated that the difference was statistically significant.

RESULTS
Hsa_circ_0001806 was up-regulated in
HCC tissues and cells and correlated with the poor
prognosis of patients
As shown in Figure 1A,B, hsa_circ_0001806 was up-regulated in
HCC tissues compared with paired adjacent normal tissues. The clin-
icopathological characteristics of HCC patients suggested that the
high expression of hsa_circ_0001806 was associated with advanced TNM stage (p = 0.0273), lymph gland metastasis (p = 0.0109) and organ metastasis (p = 0.0142) in HCC patients (Table 1). Meanwhile, prognosis analysis calculation suggested that the high level of hsa_circ_0001806 expression was positively associated with lower survival rate (p = 0.0291) (Figure 1C). In addition, we found that hsa_circ_0001806 was notably up-regulated in HCC cells (HepG2, Hep3B and Huh7), compared with LO2 cells (Figure 1D).

### TABLE 1 Correlation between hsa_circ_0001806 levels in HCC patients and their clinicopathologic characteristics

| Clinicopathologic parameters | Low hsa_circ_0001806 (n = 32) | High hsa_circ_0001806 (n = 31) | p-value |
|------------------------------|-------------------------------|-------------------------------|---------|
| Age (year)                   |                               |                               | 0.5126  |
| ≤40                          | 15                            | 12                            |         |
| >40                          | 17                            | 19                            |         |
| Gender                       |                               |                               | 0.3002  |
| Female                       | 11                            | 7                             |         |
| Male                         | 21                            | 24                            |         |
| Tumour size                  |                               |                               | 0.111   |
| ≤5                           | 19                            | 11                            |         |
| >5                           | 13                            | 20                            |         |
| TNM stage                    |                               |                               | 0.0273* |
| Stage I + II                 | 25                            | 16                            |         |
| Stage III + IV               | 7                             | 15                            |         |
| Lymph gland metastasis       |                               |                               | 0.0109* |
| No                           | 28                            | 19                            |         |
| Yes                          | 4                             | 13                            |         |
| Organ metastasis             |                               |                               | 0.0142* |
| No                           | 25                            | 15                            |         |
| Yes                          | 7                             | 16                            |         |

The significance level (p) was determined by chi-square, *p < 0.05.

#### Figure 1
Hsa_circ_0001806 was up-regulated in HCC tissues and correlated with the poor prognosis. (A and B) The expression of hsa_circ_0001806 in HCC tissues and adjacent normal tissues (n = 63). (C) Kaplan-Meier analysis of a correlation between hsa_circ_0001806 expression and the five-year survival of HCC patients (Values are expressed as median ± D). *** p < 0.001. (D) The expression levels of hsa_circ_0001806 in normal LO2 cells and HCC cells (HepG2, Hep3B and Huh7) were measured through RT-PCR. ***p < 0.001

### 3.2 Effect of hsa_circ_0001806 on cell viability, apoptosis, migration, and glycolysis in Hep3B cells

To detect the functional effects of hsa_circ_0001806 on the cell processes of HCC, hsa_circ_0001806 expression was significantly depressed by siRNA (si-hsa_circ_0001806) and up-regulated by hsa_circ_0001806 overexpression vector (ov-hsa_circ_0001806) in Hep3B cells (Figure 2A). The results of CCK-8 assay indicated...
that hsa_circ_0001806 overexpression promoted cell viability of Hep3B cells, while the knockdown of hsa_circ_0001806 suppressed the cell viability of Hep3B cells (Figure 2B). Subsequently, we found that hsa_circ_0001806 inhibition significantly induced cell apoptosis in Hep3B cells (Figure 2C). In addition, the result of the Transwell assay showed that the overexpression of hsa_circ_0001806 facilitated Hep3B cell migration, while the silencing of hsa_circ_0001806 suppressed cell migration (Figure 2D,E). What is more, glucose uptake, lactate production and ATP level were significantly elevated by hsa_circ_0001806 overexpression and inhibited by hsa_circ_0001806 inhibition in Hep3B cells (Figure 2F–H).

3.3 Hsa_circ_0001806 served as a sponge of miR-125b

The online bioinformatics prediction tools, CircBank (http://www.circbank.cn/index.html) and BiBiServ2-RNAhybrid (https://bibiserv.cbi.ict.ac.cn/rnahybrid), indicated that miR-125b may serve as a target of hsa_circ_0001806. MiR-125b was down-regulated in HCC cells (HepG2, Hep3B and Huh7), compared with LO2 cells (Figure 3A). Mimic and inhibitor of miR-125b were constructed and transfected into Hep3B cells to promote or suppress miR-125b expression (Figure 3B). Furthermore, the dual-luciferase reporter assay verified the direct binding between miR-125b and hsa_circ_0001806 (Figure 3C,D).
Meanwhile, we found that miR-125b expression was suppressed by hsa_circ_0001806 overexpression and up-regulated by hsa_circ_0001806 inhibition in Hep3B cells (Figure 3E). Previous studies have reported that HK2 was a key target of miR-125b and was involved in tumour progression.19,20 Our study verified that HK2 expression was increased in HCC cells, compared with LO2 cells (Figure 3F,G). Also, HK2 expression was promoted by hsa_circ_0001806 overexpression and the transfection of miR-125b inhibitor and suppressed by hsa_circ_0001806 inhibition and miR-125b overexpression in Hep3B cells (Figure 3H–K).

### 3.4 Hsa_circ_0001806 regulated the progression of HCC by regulating miR-125b/HK2

Overexpression vector of HK2 (ov-HK2) was constructed and transfected into Hep3B cells. The results of qRT-PCR and Western blotting indicated that transfection of ov-HK2 markedly up-regulated the expressions of HK2 mRNA and protein in Hep3B cells, compared with the ov-NC group (Figure 4A,B). Moreover, to explore the role of miR-125b/HK2 axis in the anti-tumour effect of hsa_circ_0001806 inhibition on HCC cells, miR-125b inhibitor or HK2 overexpression vector was transfected into hsa_circ_0001806 silenced Hep3B cells. The results indicated that the inhibition of miR-125b and the overexpression of HK2 both partly reversed the inhibitory effect of si-hsa_circ_0001806 on cell viability and migration in Hep3B cells, and abated si-hsa_circ_0001806 induced cell apoptosis (Figure 4C–F). Moreover, miR-125b inhibition and HK2 up-regulation increased glucose uptake, lactate production and ATP level in si-hsa_circ_0001806-transfected Hep3B cells (Figure 4G–I).
In this study, we found that hsa_circ_0001806 was highly expressed in HCC tissues and cells, and the increased expression of hsa_circ_0001806 was related to advanced TNM stage, lymph gland and organ metastasis, as well as poor survival of HCC patients. This discovery was consistent with previous reports that hsa_circ_0001806 was up-regulated in colorectal carcinoma and HCC tissues.\textsuperscript{16,18} This suggested that hsa_circ_0001806 may serve as a molecular marker for HCC. In addition, our study indicated that the overexpression of hsa_circ_0001806 promoted cell proliferation and migration in HCC cells, while hsa_circ_0001806 inhibition...
suppressed cell proliferation, migration and induced cell apoptosis in HCC cells. Consistently, Cheng and colleagues discovered the elevated expression of circ_0016788 which was closely related to aggravated performance and poor outcome of HCC patients.\(^2\) Another study presented that circ_0004913 was dysregulated during HCC progression and the alteration of circ_0004913 could be used as an evaluation factor for HCC severity and prognosis.\(^2\) Liu et al. demonstrated the down-regulation of circ_5692 in HCC tissues and cells, and circ_5692 overexpression inhibited malignance of HCC cells and the tumour growth of HCC xenograft mouse model.\(^2\) Accumulating evidence indicates that circRNAs functions as miRNA sponges to regulate target gene expression. In our study, we found that hsa_circ_0001806 served as a sponge of miR-125b in HCC cells. Studies discovered that miR-125b was down-regulated in bladder and liver cancer and was associated with a high risk of bladder cancer, as well as poor survival rates.\(^19\)–\(^25\) MiR-125b overexpression suppressed cell proliferation and migration, induced cell apoptosis in bladder and liver cancer cells.\(^25\) It has been reported that HK2 served as a target gene of miR-125b, which was negatively associated with miR-125b expression in bladder cancer cells and laryngeal squamous cell carcinoma cells.\(^19\)–\(^20\) In our study, we found that hsa_circ_0001806 positively regulated HK2 expression in HCC cells. HK2 was highly expressed in several tumours, including breast cancer, ovarian cancer, gastric cancer, bladder cancer, lung cancer and hepatocellular carcinoma.\(^19\)–\(^26\)–\(^28\) Also, HK2 was required for tumour initiation and maintenance in a mouse model; its inhibition suppressed the malignant behaviour of breast and lung cancer, both in vitro and in vivo.\(^29\) Our study identified that HK2 served as a direct target of miR-125b in HCC cells and participated in hsa_circ_0001806-mediated proliferation, apoptosis and migration in HCC cells. Glycolysis provides the major energy for HCC tumour progression and is involved in cell proliferation, migration and invasion.\(^30\)–\(^31\) It is known that cancer cells exhibit aberrant metabolism and are in preference to glycolysis rather than mitochondrial oxidative phosphorylation to produce glycolytic intermediates and glucose-dependent ATP for macromolecular biosynthesis, even under an oxygen abundant environment.\(^31\) HK2 is the first and initial rate-limiting enzyme in the glycolytic pathway\(^27\) and catalyses glucose transforming to glucose-6-phosphate.\(^32\) The inhibition of HK2 suppressed glycolysis in HCC tumours. In addition, miR-125b targeted to HK2 and suppressed glycolysis in laryngeal squamous cell carcinoma.\(^20\) The overexpression of miR-125b promoted the dysregulation of cellular glucose metabolism by directly targeting to HK2 in human retinal pigment epithelium cells.\(^33\) A previous study found that astragalin suppressed HK2 through up-regulating miR-125b to inhibit HCC cell proliferation in vitro and in vivo.\(^24\) In this study, oncogenic hsa_circ_0001806 promoted glycolysis in HCC cells, while the overexpression of HK2 and inhibition of miR-125b suppressed the inhibitory effect of hsa_circ_0001806 silencing on the glycolysis process in HCC cells.

5 | CONCLUSION

In summary, this study investigated the role and mechanism of hsa_circ_0001806 in HCC tumours. The results verified that hsa_circ_0001806 was significantly up-regulated in HCC tissues and cells and associated with an advanced TNM stage, lymph gland and organ metastasis and the poor prognosis of HCC patients. Moreover, hsa_circ_0001806 promoted cellular malignant behaviour and glycolysis by regulating the miR-125b/HK2 axis, which may provide a potential therapeutic target for HCC.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data used to support the findings of this study are available from the corresponding author upon request.

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