MiR-139-5p is associated with poor prognosis and regulates glycolysis by repressing PKM2 in gallbladder carcinoma

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Abstract

Objectives: Gallbladder carcinoma (GBC) is the most highly aggressive cancer of biliary tract, but effective therapeutics are lacking. Emerging evidence has unveiled that miR-139-5p is aberrantly downregulated in cancers, including GBC. However, the functions and mechanisms of miR-139-5p in GBC remain unclear.

Materials and methods: MiR-139-5p-overexpression was established in GBC cell lines, after which cell proliferation, migration, invasion, colony formation, and glucose metabolism were assayed in vitro. Subsequently, bioinformatics prediction and dual-luciferase reporter were performed to confirm that pyruvate kinase M2 (PKM2) was a direct target of miRNA-139-5p. Xenograft mouse models were applied to investigate the role of miR-139-5p in GBC tumorigenicity in vivo. In situ hybridization and immunohistochemical assays were performed to determine the relationships among miR-139-5p, PKM2 expression and clinical malignancies in GBC samples.

Results: We found that miR-139-5p was substantially downregulated in GBC tissues. Low expression of miR-139-5p was significantly associated with poor clinical outcomes. GBC cell proliferation, migration, and invasion could be inhibited by overexpression of miR-139-5p either in vitro or in vivo. In addition, miR-139-5p overexpression could directly inhibit PKM2 expression and lead to suppression of glucose consumption, lactate production, and cellular ATP levels. Moreover, PKM2 was frequently upregulated in GBC and correlated with poor prognosis. Mechanistically, miRNA-139-5p inhibited cell proliferation, migration, and glycolysis in GBC, at least in part, by repressing PKM2.

Conclusions: These results demonstrated a novel role for miR-139-5p/PKM2 in GBC progression and provided potential prognostic predictors for GBC patients.

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1 | INTRODUCTION

Gallbladder carcinoma (GBC) is the seventh malignant tumour in the gastrointestinal tract, as well as the most highly aggressive cancer in the biliary tract.¹ Although significant efforts have been made in basic research on GBC, the clinical outcomes of GBC patients have not improved due to late diagnosis, early metastasis, unsatisfactory treatment, and poor prognosis.^{2,3} In this regard, elucidating the molecular mechanisms underlying progression of GBC, which may have a critical role in the development of targeted GBC treatment, is extremely urgent.

Over the past few decades, numerous studies have demonstrated that the Warburg effect is a key hallmark of cancer cells.⁴ Pyruvate kinase isozyme M2 (PKM2) is one of the isoforms of pyruvate kinase and a pivotal rate-limiting enzyme of glycolysis.^{5,6} Robust expression of PKM2 has been confirmed among diverse human cancers, such as gastric cancer,⁷ colon cancer,⁸ and GBC.⁹ In addition, overexpressed PKM2 is associated with unfavourable prognosis in those patients.¹⁰ High PKM2 expression is correlated with increased glucose uptake, upregulated lactate production, and reduced oxygen consumption which contribute to cancer cell proliferation and tumour growth.¹¹ However, the underlying causative mechanism of aberrant PKM2

expression in GBC is not clear. Several studies have confirmed that microRNAs (miRNAs) can regulate the expression of PKM2.^{12,13}

A growing body of evidence suggests that miRNAs carry out critical function in the progression of many human cancers.¹⁴ MiRNAs are a set of small, noncoding RNAs of ~22 nucleotides which play a pivotal part in regulating gene expression by reducing posttranscriptional translation of target mRNAs.¹⁵ The global alteration of their expression is a hallmark of tumorigenesis and can serve as a diagnostic and prognostic cancer signature. MiR-139-5p, with a length of 23 nt, is located within the second intron of the phosphodiesterase 2A gene on chromosome 11q13.4 and is a common type of mature miRNA generated from miR-139 precursor.^{15,16} MiR-139-5p has been discovered to be downregulated in various human tumours including glioblastoma multiforme,¹⁷ bladder cancer,¹⁸ hepatocellular carcinoma (HCC),¹⁹ and breast cancer.²⁰ Overexpression of miR-139-5p inhibits the capacity of proliferation, invasion or metastasis of cancer cells.²¹ Moreover, Pablo et al²² had reported that the expression of miR-139-5p was significantly decreased in GBC tissues. However, the functions and mechanisms of miR-139-5p in human GBC remain unclear.

Further study on the molecular mechanisms underpinning GBC energy metabolism and progression may provide novel targets and

TABLE 1 The relationship between miR-139-5p expression and clinicopathological features of GBC

Clinicopathological features	No. of cases (n)	miR-139-5p expression		P	PKM2 expression		P
		High (n = 30)	Low (n = 36)		High (n = 35)	Low (n = 31)	
Age(years)							
≤median	33	13	20	0.652	20	13	0.477
>median	33	17	16		15	18	
Gender							
Male	19	9	10	0.625	11	8	0.389
Female	47	21	26		24	23	
Tumour size							
≤5 cm	35	16	19	0.964	18	17	0.782
>5 cm	31	14	17		17	14	
TNM stage							
Stage I and II	31	22	9	<0.001**	21	10	0.001**
Stage III and IV	35	8	27		14	21	
Distant metastasis							
Absent	37	23	14	0.190	20	17	0.780
Present	29	7	22		15	14	
Pathological type							
Squamous cell carcinoma	58	27	31	0.561	30	28	0.484
Adenocarcinoma	2	1	1		1	1	
Adenosquamous carcinoma	6	2	4		4	2	
Differentiation grade							
Stage I–II	28	18	10	0.012*	12	16	0.010*
Stage III–IV	38	12	26		23	15	

TNM: tumour-node-metastasis; PKM2: pyruvate kinase isoenzyme type M2.

*P < 0.05. **P < 0.01.

therapies to treat advanced GBC patients. In this study, we found that miR-139-5p played a pivotal role in GBC progression and metastasis by regulating PKM2. These results unravelled a novel and important mechanism for the regulation of anaerobic glycolysis and the development of GBC.

2 | MATERIALS AND METHODS

2.1 | Tissues and patients

Fifty-three paraffin-embedded tissues of GBC and 27 adjacent non-tumour tissues (Zhengzhou cohort) were acquired between April 2009 and April 2014 from the First Affiliated Hospital of Zhengzhou University, China. All enrolled patients met the following criteria: (a) definite pathological diagnosis of GBC and (b) signed informed consent form before surgery. Tissue microarrays (TMA) using Zhengzhou cohort GBC tissues were constructed using 1.5-mm-diameter cores. TMA containing 79 GBC specimens and 20 nontumour tissues (Outdo cohort) were purchased from Outdo Biotech (Shanghai, China). The clinicopathological data for these patients were presented in Table 1. The study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University.

2.1.1 | Cell lines and culture

The cell lines used in this study were shown in Table S1. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) mixing with 100 U/mL penicillin/streptomycin (Corning, New York, NY, USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. All cell lines used in this study were passaged for less than 6 months when the experiments were carried out.

2.1.2 | Transfection of miRNAs and siRNAs

Synthesized RNA duplexes of miR-139-5p mimics (miR-139-5p), inhibitors (anti-miR-139-5p) and PKM2 siRNAs (P-siRNA) as well as respective negative controls (NC) were commercially purchased from GenePharma (Shanghai, China). For cell transfection, 1×10^5 cells per well were seeded into the 12-well plates, and subsequently transfected with PKM2-siRNA, miR-139-5p mimics and inhibitor or negative control using LipoFiter (Hanbio, Shanghai, China) based on the manufacturer's protocol. RNA and protein were harvested 48 hours after transfection. The siRNA sequences used in this study were provided in Table S2.

2.1.3 | RNA isolation and quantitative real-time PCR

Total RNA from GBC-SD and NOZ cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the standard RNA isolation protocol and the concentration of RNA was determined by NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA,

USA). Total RNA was reverse-transcribed with TransScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). Quantitative real-time PCR (qRT-PCR) was performed using TransScript Top Green qPCR SuperMix (TransGen, Beijing, China) on 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). β -actin was used as the endogenous controls. Data were analysed using the comparative Ct method ($2^{-\Delta\Delta C_t}$). Each group included three repeated wells. The primer sequences used in the study were showed in Table S3.

2.1.4 | Western blotting

The procedure of Western blotting was performed as described previously.²³ Briefly, cells were collected and lysed using radio immunoprecipitation assay protein extraction reagent (Beyotime, Beijing, China) with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) to obtain cell lysates. Equal amount of protein was electrophoresed on 12% SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with phosphate buffer saline (PBS) containing 5% skim milk and then incubated with the primary antibody (β -actin and PKM2; Proteintech Group, Wuhan, China) for 12 hours at 4°C. After secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) incubation, the membranes were used to expose the photographic film. The antibodies used in this study are listed in Table S4.

2.1.5 | Cell proliferation and colony formation assays

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays were performed as follows. GBC-SD and NOZ cells (5×10^3 in 100 μ L) were seeded in a 96-well plate. Each group included three repeated wells. At 1, 2, 3, 4 and 5 days after incubation, proliferation was assessed using sterile MTT solution (Beyotime), following the standard protocol. The absorbance at a wavelength of 490 nm (A₄₉₀) was measured on a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The DNA synthesized rate was assayed with EdU assay kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. The number of EdU-positive cells were counted in three random images per well by microscopy using a 100 objective (Olympus, Tokyo, Japan). The ratio of EdU-positive cells (with red fluorescence) to Hoechst-stained cells (with blue fluorescence) was used to evaluate the cell proliferation activity. For the plate colony formation assay, 1000 cells per well were plated in six-well plates and routinely cultured. Fourteen days later, the cells were then fixed with 30% formaldehyde for 15 minutes and stained with 0.1% crystal violet. The colony (defined as more than 50 cells/colony) number was counted using an optical microscope.

2.1.6 | Wound-healing assay and transwell invasion assay

For the wound-healing assay, GBC-SD and NOZ cells (5×10^4) were seeded in six-well plates. When they had reached 80% confluence, a

200- μ L sterile micropipette tip was used to form a 1 mm wide strip across the well. The cells migrating into the wounded areas were visualized and photographed at 0 and 48 hours after washing the well with PBS.

For the cell invasion assay, transwell matrigel chambers coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used. 1×10^5 cells were seeded into the upper chamber, which was pre-coated with matrigel in serum-free DMEM. The lower chamber contained DMEM with 20% FBS. The cells that invaded into the lower chamber were collected after 48 hours. The invasive cells were stained with 0.5% crystal violet and then counted under a microscope.

2.1.7 | Immunohistochemical staining and in situ hybridization on TMA

Immunohistochemical (IHC) staining was performed according to the method described in a previous study.²³ Briefly, TMA sections were deparaffinized, rehydrated, and antigen retrieval was performed with Retrieval Solution (Dako, San Francisco, CA, USA). The slides were incubated with primary antibody at 4°C overnight and then probed with secondary antibody. Reactions were detected with Envision peroxidase kit O2 (Dako).

Expression of miR-139-5p in GBC TMA was detected by in situ hybridization (ISH) with probes for miR-139-5p. ISH was performed using digoxigenin (DIG)-labelled miRNA-139-5p ISH probes (BOSTER, Wuhan, China) for TMA on the basis of the protocol provided by the manufacturer. Briefly, TMA slides were fixed in 4% paraformaldehyde and then incubated with proteinase-K for 20 minutes at 37°C. Subsequently, the slides were hybridized with miR-139-5p probe (200 nmol/L) for 40 minutes at 50°C. The slides were incubated with anti-DIG reagent, and the probe signal was visualized with diaminobenzidine (DAB) solution (BOSTER). Two pathologists evaluated the IHC and ISH scores in a blinded manner. The intensity of PKM2 or miR-139-5p staining was scored on a scale of 1-4 as follows: 1 (no staining), 2 (weak staining), 3 (moderate staining), and 4 (strong staining). Tissues with scores of 3 and 4 were defined as exhibiting high expression, and those with scores of 1 and 2 were classified as exhibiting low expression. The information of antibodies used in this study is listed in Table S4.

2.1.8 | Immunofluorescence analysis

Immunofluorescence (IF) staining was performed according to the method described in a previous study.²³ Briefly, cells were washed with 1 $\times$ PBS, fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Solarbio, Shanghai, China). After blocking with 5% goat serum, the cells were incubated with primary antibody (PKM2 and β -actin; Proteintech Group) at 4°C overnight and then incubated with the appropriate secondary antibodies (Jackson ImmunoResearch Inc., USA). The nuclei were counterstained with DAPI (Beyotime, China), and the IF signals were detected using a Zeiss-AX10 microscope (Zeiss, Oberkochen, Germany). The information of antibodies used in this study is listed in Table S4.

2.1.9 | Luciferase activity assay

The 3'UTR (3' untranslated region) sequence of PKM2 containing the predicted miR-139-5p-binding site, was subcloned into psiCHECK-2 luciferase reporter vector (Promega, Madison, WI, USA). The mutant vector was created through mutating the binding site region of the miR-139-5p. Cells were then seeded in triplicate in 24-well plates and transfected with 200 ng of wild-type or mutated PKM2 3'UTR. Then, cells were cotransfected with 45 nmol/L miR-139-5p or NC oligo using LipoFiter (Hanbio, Shanghai, China) according to the manufacturer's instructions. After transfection for 48 hours, the relative luciferase enzyme activity was determined with the Dual-Luciferase Reporter Assay System (Promega) by the manufacturer's protocols. The primer sequence used in this study provided in Table S3.

2.1.10 | Vector construction and lentiviral transduction

The coding sequences of human miR-139-5p were amplified and cloned into pcDNA3.1 (+) to generate miR-139-5p expression vectors (GenePharma, Shanghai, China). An empty pcDNA3.1 vector was used as the negative control. GBC-SD cells (1×10^5) were infected with a lentivirus encoding miR-139-5p mimics or an empty lentivirus for 96 hours and then selected by treatment with puromycin (Santa Cruz Biotechnology) for 4 weeks.

2.1.11 | Glucose, oxygen consumption, and lactic acid assays

Glucose consumption was accessed using a glucose oxidase-peroxidase (Sigma, St. Louis, MO, USA) reaction coupled to oxidation of Amplex Red reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Glucose consumption by cells was quantified by deducting the amount of glucose present in cell culture medium without cells. Oxygen consumption was examined using the Oxygen Consumption Rate Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. The product of lactic acid in the medium was measured with lactic acid assay kit (Sigma) according to the manufacturer's protocol. The optical density value was measured and used as a standard curve to measure the concentration in the test samples.

2.1.12 | Glycolysis stress test

Measurements of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were performed to assess the effect of miR-139-5p overexpression on glycolysis stress and cell mitochondrial stress using the Seahorse XF96 Glycolysis Analyser (Seahorse Bioscience, Billerica, MA, USA). For ECAR analysis, glucose, oligomycin, and 2-deoxyglucose were sequentially added in special medium. First, glucose was injected into the medium, catabolized to lactate and ATP, with a corresponding increased ECAR value. Then, oligomycin was injected, which inhibited mitochondrial ATP

production and shifted the energy production to glycolysis, with the corresponding increase in ECAR. The ECAR is reported in milli-pH (mpH) units per minute.

For the OCR analysis, oligomycin, an ATP synthase inhibitor, was first injected into the medium, and the induced decrease in OCR was associated with the proton current resulting from ATP synthase. Subsequently, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was injected, which leads to rapid oxygen consumption. Compared with basal respiration, the induced increased respiratory capacity indicates the spare respiratory capacity. Finally, rotenone and antimycin A, electron transport chain inhibitors, were injected. Residual respiration corresponds to the nonmitochondrial respiration. The OCR is reported in units of picomoles per minute.

2.1.13 | Tumour xenograft experiments

A total of 12 male nude mice (4 weeks old) were obtained from Vital River Laboratory Animal Technology (Beijing, China) and maintained under pathogen-free conditions (12 h light-dark cycle, 25°C, 60%-70% humidity). For the tumour xenograft model, nude mice were injected subcutaneously into bilateral flanks with either the stably miR-139-5p overexpressing (Lenti-miR-139-5p) or empty lentivirus control (Lenti-NC) cell lines to establish tumours at a density of 2×10^6 cells/100 μ L ($n = 6$ per group). Tumour growth was examined after 1 week, and the volumes of tumour were calculated by the following formula: volume (cm^3) = (length $\times$ width²)/2. In 4 weeks, tumour-carrying mice were euthanized, the tumours were weighed and photographed, and tissue sections were obtained for further IHC staining. All procedures for the

FIGURE 1 (A) miR-139-5p expression levels in GBC tissues and adjacent normal tissue of Gene Expression Omnibus (GEO) database. (B) miR-139-5p expression was significantly lower in GBC tissues ($n = 123$) compared with that in nontumour tissues ($n = 43$) based on ISH assays. (C) Representative miR-139-5p staining patterns. (D-F) The inverse correlation of miR-139-5p expression level with TNM classification (D), metastasis (E), and OS rate (F; red, low miR-139-5p expression [$n = 36$]; blue, high miR-139-5p expression [$n = 30$]). (G) Univariate analysis of the association between miR-139-5p expression and clinicopathological features ($n = 66$). The X-axis represents months, and the Y-axis indicates OS probability. Mean $\pm$ SD, unpaired Student's t test, or Kaplan-Meier analysis

mouse experiments were approved by the Animal Care Committee of the First Affiliated Hospital of Zhengzhou University.

2.1.14 | Statistical analysis

Statistical analyses were performed using SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). All experiments were repeated at least three times to calculate the mean and standard deviation (SD). Clinicopathological variables were analysed by chi-square tests. Kaplan-Meier curves and log-rank tests were used to analyse the OS of GBC patients. To identify the factors that had a significant influence on survival, univariate and multivariate Cox regression analyses were performed. Student's *t* test was used for comparison between two groups. *P* (two-sided) values less than 0.05 were statistically significant. Data were presented as the mean $\pm$ standard deviation (SD).

3 | RESULTS

3.1 | MiR-139-5p was inversely associated with clinicopathological features in patients with GBC

To explore whether miR-139-5p was dysregulated in GBC, we first analysed miR-139-5p expression levels in an independent GBC

datasets (GSE90001, four GBC tissues and four normal tissues) from the Gene Expression Omnibus (GEO) database. The results showed that miR-139-5p expression was downregulated in GBC tissues (Figure 1A). We further used ISH to measure miR-139-5p status in 123 GBC tissues and 43 adjacent normal gallbladder samples from GBC patients. We observed that the expression level of miR-139-5p was downregulated in GBC tissues compared with that in nontumour gallbladder tissues ($P = 0.0212$; Figure 1B). According to staining intensity and the percentage of positive cells in the tissue sections, we categorized miR-139-5p staining patterns into four groups, as shown in Figure 1C. We found the expression level of miR-139-5p was negatively correlated with tumour stages ($P < 0.0001$; Figure 1D) and the presence of metastasis ($P = 0.0468$; Figure 1E). Moreover, Kaplan-Meier analysis demonstrated a statistically significant negative relationship between overall survival (OS) and miR-139-5p expression levels (Figure 1F). A detailed summary of the relationships between miR-139-5p expression and the clinicopathological features of GBC patients is shown in Table 1.

In addition, univariate Cox proportional hazards analysis of OS exhibited that miR-139-5p expression, distant metastasis, pathological grade, and TNM stage were important prognostic factors (Figure 1G and Table 2), and multivariate analysis showed that miR-139-5p and TNM stage were independent prognostic indicators for OS in patients

TABLE 2 Univariate and multivariate analyses of overall survival of gallbladder carcinoma

Clinicopathological features	Univariate analyses			Multivariate analyses		
	HR	95% (CI)	<i>P</i> value	HR	95% (CI)	<i>P</i> value
Age(years)						
≤Median	1.000		0.750			0.750
>Median	1.102	0.607-1.998			0.607-1.998	
Gender						
Male	1.000		0.454			0.454
Female	1.289	0.663-2.507			0.663-2.507	
Tumour size						
≤5 cm	1.000		0.547			0.547
>5 cm	1.199	0.664-2.168			0.664-2.168	
TNM stage						
Stage I and II	1.000		0.000**	1.000		0.040*
Stage III and IV	4.525	2.296-8.919		2.319	1.038-5.180	
Differentiation grade						
Stage I-II	1.000		0.001**	1.000		0.155
Stage III-IV	3.026	1.616-5.666		1.674	0.822-3.409	
Distant metastasis						
Absent	1.000		0.003**	1.000		0.052
Present	2.894	1.448-5.783		2.102	0.992-4.454	
miR-139 expression						
Low	1.000		0.000**	1.000		0.042*
High	3.637	1.869-7.075		2.659	1.036-6.825	

TNM: tumour-node-metastasis; HR: hazard ratio; CI: confidential interval; PKM2: pyruvate kinase isoenzyme type M2.

* $P < 0.05$, ** $P < 0.01$.

with GBC. In conclusion, these data suggested that miR-139-5p might play a pivotal role in the progression and development of human GBC.

3.2 | Overexpression of miR-139-5p inhibited in vitro GBC cell proliferation, migration, and invasion

Considering the notable association between miR-139-5p and aggressive GBC features, we postulated that re-expression of miR-139-5p in GBC cells could impede the proliferation and invasiveness of GBC cells. To investigate this possibility, we first detected the role of miR-139-5p in GBC cell proliferation. MTT assays presented that overexpression of miR-139-5p significantly reduced the viability of GBC-SD and NOZ cells (Figure 2A,B). Consistently, the colony formation assay showed that overexpression of miR-139-5p promisingly suppressed the growth capacity of GBC cells (Figure 2C,D).

Additionally, EdU assays indicated that miR-139-5p-overexpressing GBC cells had lower proliferative activity than the control cells (Figure 2E,F). Next, we further evaluated the role of miR-139-5p in the migration and invasion of GBC cells and found that migration and invasion of GBC cells were significantly inhibited after miR-139-5p overexpression (Figure 2G-J). Thus, these data implied that miR-139-5p served as a tumour suppressor which could inhibit the proliferation, migration, and invasion of GBC cells.

3.3 | PKM2 as the functional target of miRNA-139-5p

We next investigated the potential gene targets of miR-139-5p that contributed to its tumour suppressor function. Online miRNA database analysis (TargetScan, miRanda and PicTar) indicated

FIGURE 2 (A, B) Overexpressing miR-139-5p dramatically inhibited GBC-SD and NOZ cell viability. Cell growth rates were evaluated with a MTT assay. (C, D) Overexpressing miR-139-5p suppressed clone formation in GBC-SD and NOZ cells. (E, F) Overexpression of miR-139-5p inhibited GBC cell growth as evaluated by the 5-ethynyl-20-deoxyuridine (EdU) assay. (G, H) miR-139 dramatically inhibited the invasiveness of GBC-SD and NOZ cells, as determined by transwell assay. (I, J) Wound-healing assays indicated that miR-139-5p overexpression reduced the migration distance of GBC-SD and NOZ cells at 0 and 48 h. *P < 0.05; **P < 0.01. Data represent the mean $\pm$ SD

that PKM2 could be a target of miR-139-5p according to the putative target sequence in the PKM2 3'UTR (Figure 3A). Western blot and qRT-PCR analysis were conducted to further identify whether PKM2 expression was indeed regulated by miR-139-5p. The results showed that the protein and mRNA levels of PKM2 were significantly reduced after transfecting miR-139-5p mimics in GBC cells (Figure 3B,C). Subsequently, luciferase reporter assay was conducted, and we found that the signal from the luciferase reporter fused with the wild-type 3'UTR fragments of the PKM2 mRNA was significantly decreased compared with that from the mutant vector, demonstrating the specificity of the interaction between miR-139-5p and the mRNA 3'-UTR of PKM2 (Figure 3D).

These results suggested that miR-139-5p could directly target PKM2 and negatively regulate PKM2 expression.

3.4 | PKM2 silencing inhibited the proliferation and invasion of GBC cells by repressing aerobic glycolysis

To investigate the role of PKM2 in the progression of GBC, we first used IF to determine the location of PKM2 in GBC cells and found that PKM2 were mostly distributed in cell cytoplasm of GBC cells (Figure 3E). We then examined the relative protein levels of PKM2 in four GBC cell lines by Western blot (Figure 3F) and chose GBC-SD and NOZ cell lines for following

FIGURE 3 (A) Bioinformatic analysis identified a potential miR-139-5p target site in the 3'-UTR of PKM2. (B, C) The protein and mRNA expression levels of PKM2 were measured in GBC-SD and NOZ cells transfected with miR-139-5p or NC. (D) Relative luciferase activity based on a dual-luciferase reporter assay. Values represent the mean $\pm$ SD ($n = 5$, each). (E) Fluorescent micrographs of PKM2 expression in gallbladder carcinoma GBC-SD and NOZ cells. DAPI (blue) and PKM2 (red). (F) The expression level of PKM2 in different GBC cell lines (OCUG1, GBC-SD, SGC996 and NOZ). (G, H) Relative protein and mRNA expression level of PKM2 in siRNA-transfected GBC cells. (I, J) PKM2-siRNA significantly inhibited GBC-SD and NOZ cell viability, and the cell growth rate was evaluated by an MTT assay. (K, L) The invasiveness of GBC-SD and NOZ cells was significantly suppressed according to a cell invasion assay. (M-O) Glucose consumption (M), lactate production (N) and cellular ATP level (O) in GBC cells with NC or PKM2 inhibition. * $P < 0.05$; ** $P < 0.01$. Data represent the mean $\pm$ SD

FIGURE 4 (A) Representative PKM2 staining in GBC tissues and paired normal tissues. (B) PKM2 expression was significantly increased in GBC tissues ($n = 123$) compared with that in nontumour tissues ($n = 43$) according to IHC assays. (C) Representative PKM2 and Ki-67 staining patterns. (D–F) The inverse correlation of PKM2 expression level with TNM classification (D), metastasis (E), and OS rate (F; red, high PKM2 expression [$n = 31$]; blue, low miR-139-5p expression [$n = 35$]). (G) The association between miR-139-5p expression and PKM2 expression. The X-axis represents months, and the Y-axis indicates OS probability. Mean $\pm$ SD, unpaired Student's *t* test, or Kaplan-Meier analysis

experiments, owing to their relatively high PKM2 expression levels. Subsequently, GBC-SD and NOZ cell lines were transfected with siRNA targeting PKM2 (P-siRNA) or with siRNA negative control (NC), and the results showed that PKM2 protein and mRNA expression were significantly reduced by P-siRNA transfection (Figure 3G,H). In addition, the tumourigenic capacity of PKM2 was assessed by MTT and transwell assays. The MTT assay showed that PKM2 knockdown cells grew more slowly than the control cells, and the difference was more notable after day 4 or 5 (Figure 3I,J). Silencing PKM2 in GBC-SD and NOZ cells resulted in reduced invasive potential when compared to the control group (Figure 3K,L). Besides, given the critical function of PKM2 in the reprogramming of cell metabolism, we confirmed the effect of PKM2 silencing on GBC cell glucose metabolism. As shown in Figure 3M–O, significantly lower glucose consumption, cellular lactate production and ATP levels were observed after

PKM2 knockdown, indicating a critical role of PKM2 in GBC glycometabolism. Collectively, our data demonstrated that PKM2 contributed to proliferation and invasion, possibly mediated by promotion of the “Warburg effect” in GBC cells.

3.5 | PKM2 was overexpressed in GBC tissues and negatively correlated with OS

To further confirm PKM2 expression in GBC, we examined PKM2 expression in GBC samples and normal tissues using IHC. Brown cytoplasmic PKM2 staining was relatively high positive in GBC tissues but nearly absent from normal epithelia ($P = 0.0059$; Figure 4A,B). Additionally, there was a strong positive relationship between PKM2 and the tumour proliferation marker Ki-67 staining score, as shown in Figure 4C. The expression levels of PKM2 were significantly higher in the late TNM stage ($P = 0.0019$; Figure 4D) and in tumours in

which metastasis was present ($P = 0.0468$; Figure 4E). Moreover, in GBC patients, increased PKM2 immunoreactivity was accompanied by significantly poor postoperative survival ($P = 0.0002$; Figure 4F). Additionally, there was a notable negative correlation between the expression level of PKM2 and miR-139-5p (Figure 4G). These results indicated that GBC patients with high PKM2 expression possessed poorer prognosis than those with low PKM2 expression.

3.6 | The miR-139-5p/PKM2 axis regulated glucose metabolism in GBC cells

Considering the important roles of PKM2 in GBC cell metabolism, we further determined the function of miR-139-5p in glycolysis. We observed that miR-139-5p-overexpressing GBC cells consumed less glucose (Figure 5A) and exhibited lower lactate production

(Figure 5B) and cellular ATP levels (Figure 5C) than the control cells. Inhibition of miR-139-5p caused a dramatic elevation in glucose consumption, lactate production, and cellular ATP level in GBC cells (Figure 5A-C). Furthermore, restoration of PKM2 protein in GBC cells diminished the inhibitory effects of miR-139-5p on glucose consumption, lactate production, and ATP level (Figure 5D-F), implying that miR-139-5p might involve in glycolysis by regulating PKM2 expression in GBC cells.

To further assess whether miR-139-5p modulates glucose metabolism of GBC cells, the glycolytic flux was first analysed based on ECAR using the Seahorse assay. We observed a significant decrease in ECAR, an indicator of lactic acid fermentation, of GBC cells transfected with miR-139-5p mimics compared with NC (Figure 5G-J). Mitochondrial respiration was detected by measurements of the OCR to determine changes in the level of OxPhos-dependent ATP

FIGURE 5 (A) Glucose consumption in GBC-SD and NOZ cells with miR-139-5p overexpression or inhibition. (B) Lactate production in GBC cells with miR-139-5p overexpression or inhibition. (C) Cellular ATP levels in GBC-SD and NOZ cells with miR-139-5p overexpression or inhibition. (D-F) Glucose consumption (D), Lactate production (E), Cellular ATP levels (F) in GBC cells with ectopic expression of PKM2. (G-J) ECAR was measured by the glycolysis stress test in GBC cell lines after the cells were transfected with miR-139-5p mimics or NC. (K-P) OCR was measured by the Cell Mito Stress Test in GBC cell lines after the cells were transfected with miR-139-5p or NC. * $P < 0.05$; ** $P < 0.01$. Data represent the mean $\pm$ SD

FIGURE 6 (A) Mice with established tumours were imaged every 7 d. The images obtained at day 14 are shown. (B) miR-139-5p inhibited GBC cell growth in vivo. Images of the tumours induced by lenti-miR-139-5p (left side) or lenti-NC (right side) are shown. (C) Tumour growth curves of pre-miR-139-5p and control transfected GBC-SD cells in nude mice. Values are the mean $\pm$ SD ($n = 6$ /group). (D) Tumour weight of xenograft tumours. (E) miR-139-5p inhibited not only PKM2 expression but also Ki-67 expression. (F) Schematic representation showing miR-139-5p/PKM2 axis-mediated aggressive behaviours in GBC. ** $P < 0.01$. Data represent the mean $\pm$ SD

generation in GBC cells. The results showed that overexpression of miR-139-5p significantly increased basal mitochondrial OCR as well as caused a substantial increase in the maximal OCR (Figure 5K-P). Impressively, these data suggested that miR-139-5p overexpression caused severe defects in glycolysis and caused an increase in mitochondrial function.

3.7 | miR-139-5p overexpression suppressed GBC tumour proliferation in vivo

To explore the in vivo effects of miR-139-5p on GBC growth, cells (GBC-SD/Lenti-miR-139-5p and GBC-SD/Lenti-NC) were

subcutaneously injected into the nude mice bilateral flanks (Figure 6A). We found that mice injected with miR-139-5p-overexpressing cells showed a significantly decreased tumour growth compared with those injected with control cells (Figure 6C). And xenograft tumours grown from cells overexpressing miR-139-5p had smaller mean volumes and weights than the tumours developed from control cells (Figure 6B,D). In addition, IHC analysis revealed that the tumour tissues of the GBC-SD/Lenti-miR-139-5p group displayed much weaker staining of PKM2 and Ki-67 (Figure 6E). Collectively, these results further verified the antiproliferative role of miR-139-5p through the downregulation of PKM2 in GBC.

4 | DISCUSSION

MiRNAs are involved in multiple tumorigenesis processes, and understanding the biological functions of miRNAs in regulating a series of tumour suppressor genes and oncogenes is a new avenue for research.^{24,25} MiR-139-5p is known to be dysregulated in several cancers.^{16,26,27} However, the biological functions and mechanisms of miR-139-5p in GBC are unknown. Warburg effect has been implicated in gallbladder tumorigenesis.^{28,29} Emerging evidence has also indicated an association between miRNA dysregulation in general, and Warburg effect in particular, during cancer development.^{30,31}

In our study, we identified that miR-139-5p was significantly downregulated in GBC tissues and was negatively associated with malignancies. Further functional analyses revealed that overexpression of miR-139-5p led to significant suppression of cell proliferation, migration, and invasion of GBC cells. Additionally, *in vivo* experiments, upregulated miR-139-5p suppressed the growth rate of GBC in BALB/c nude mice xenografts. These results suggested that miR-139-5p played a tumour suppressor role in GBC, which was consistent with its function in other cancer types. For instance, miR-139-5p could reduce tumour metastasis by targeting BCL2 pathway in colon cancer.³² Wang et al. reported that low miR-139 expression enhanced tumour growth and migration, as well as predicted poor prognosis in HCC.³³ Krishnan et al. also showed that miR-139-5p suppressed the invasive capacity of breast cancer.³⁴ In contrast, Li et al. reported that miR-139-5p overexpression increased pancreatic cancer endothelial cells (CECs) migration and angiogenesis in pancreatic tumour.³⁵ Additionally, miR-139-5p was significantly higher in peripheral blood from prostate cancer (PCa) patients and was positively associated with progression of PCa.³⁶ MiRNAs have been identified to function as either tumour suppressors or oncogenes depending on the cell or cancer type in which they are expressed. For instance, miR-155 might function as a tumour suppressor in pancreatic cancer,³⁷ while it has oncogenic activity in B-cell lymphomas.³⁸ Thus, taking these findings together, miR-139-5p also appears to play a dual role as both a main tumour-suppressing and secondary tumour-promoting miRNA.

miRNAs carry out critical function in diverse biological processes through modulating a variety of target genes. Studies by Qiu et al.¹⁹ demonstrated that miR-139-5p might serve as a suppressor of HCC epithelial-mesenchymal transition and metastasis by targeting ZEB1 and ZEB2. Findings by Zhang et al.³⁹ indicated that miR-139-5p could induce apoptosis, cause cell cycle arrest in S phase, inhibit invasion and migration by directly binding to gene NOTCH1 in breast cancer. In addition, miR-139-5p could suppress glioma cell proliferation by targeting ELTD1 and regulating cell cycle.⁴⁰ However, the mechanisms underlying the functional role of miR-139-5p in GBC were largely unknown.

Bioinformatics analysis and luciferase reporter assays demonstrated that PKM2 was a direct target of miR-139-5p. In addition, an inverse correlation between the expression levels of miR-139-5p and PKM2 was also observed in our GBC TMA cohort. PKM2 is known as a molecular switching for aerobic glycolysis (Warburg effect),

and has a critical role in essential processes of glucose metabolism in cancer cells.⁵ Increased expression of PKM2 has been found in diverse human cancers and leads to metabolic reprogramming in cancer cells.⁴¹ Thus, PKM2 has become a potential target for therapeutics. Accordingly, our data indicated that PKM2 was overexpressed in GBC tissues. PKM2 overexpression was associated with malignancies and poor overall survival, consisting with the results of Li et al. and Lu et al.^{9,42} who both identified that high PKM2 expression was significantly related to poor prognosis in GBC. Additionally, our data indicated that PKM2 knockdown potentially inhibited GBC cell proliferation and invasion through repressing glucose metabolism. This was in agreement with previous studies of PKM2 in other cancer types.^{43,44}

In a subsequent mechanistic experiment, we investigated whether miR-139-5p inhibited the progression of GBC by suppressing PKM2-mediated glycolysis. Our findings exhibited that miR-139-5p could suppress glucose metabolism of GBC cells. Moreover, restoration of PKM2 expression in GBC cells remarkably abolished the inhibitory effects of miR-139-5p on glucose consumption, lactate production, and cellular ATP level. These findings suggested that miR-139-5p might regulate glycolysis to suppress GBC tumorigenesis and metastasis by repressing PKM2 expression. This notion was further supported by Glycolysis Stress and Cell Mito Stress tests. The results showed that upregulated miR-139-5p inhibited glycolysis capacity as well as increased the mitochondrial oxidative phosphorylation ratio, indicating a glucose metabolism shift in GBC cells. In line with our study, two recent studies also found that miR-139-5p could regulate glycolysis through targeting EST1 and PRKAA1, respectively in HCC⁴⁵ and gastric cancer,⁴⁶ which indicated that miR-139-5p might play an important role in modulating aerobic glycolysis. Taking the aforementioned functions of PKM2 and miR-139-5p contributed by our and others' endeavours into account, we hypothesized that the miR-139-5p/PKM2 axis played an important role in the development of GBC (Figure 6F).

In summary, the present study revealed that miR-139-5p was downregulated and negatively associated with malignancies in GBC. MiR-139-5p significantly inhibited GBC proliferation, invasion, and glucose metabolism by targeting PKM2. These results provided a better understanding of the mRNA-miRNA feedback loop function in the glycolysis of GBC. MiR-139-5p may be considered as a biomarker for GBC progression and a potential target for therapeutic intervention for GBC.

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AUTHORS' CONTRIBUTIONS

JAC, YY, and XLC performed all the experimental work. YTH, QYH, and SS participated in data analysis. XLC, FR, JL, JB, and ZGR designed and conducted the animal experiment. GYC and RRS conceived and participated in the design of the study. The manuscript was written by JAC, GYC, and RRS. ZFD offer linguistic assistance for this manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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