INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the most common types of malignancies in Southeast Asia and southern China. Despite significant advances in the screening and medical management of NPC, the prognosis for patients with locally advanced recurrence remains bleak. Accordingly, there is an urgent need to determine the mechanism of NPC progression and identify new therapeutic targets for patients with NPC.
Cancer cells alter their cellular metabolism by metabolic reprogramming to adapt to the high energy demand under rapid growth conditions. Aerobic glycolysis is a classical metabolic adaptation, which is referred to as the "Warburg effect," with high glycolysis even in the presence of sufficient oxygen. Numerous cancer types, including NPC, use this metabolic pathway to meet their energy demands during progression. However, little is known about the mechanisms of regulating metabolism at the post-transcriptional level in NPC, such as the effects of microRNAs (miRNAs).

MiRNAs are endogenous small single-stranded noncoding RNAs that repress gene expression by binding to a target mRNA in the 3′-untranslated region (3′-UTR). Accumulating evidence indicates that aberrant regulation (such as CpG island hypermethylation in miRNA coding regions) of miRNAs play critical roles in the post-transcriptional inhibition in miRNA coding regions) of miRNAs play critical roles in the post-transcriptional inhibition of tumor proliferation, growth, and metastasis. Based on our previous genome-wide methylation microarray study (GSE52068), we discovered that the coding region of miR-9-1, a member of the miR-9 family, was significantly hypermethylated in NPC tissues. A recent study by Pinheva et al predicted miRNAs that regulate the expression of metabolic enzymes through bioinformatic analyses, and among these miRNAs, miR-9-1 was postulated to have a binding site on the 3′-UTR of HK2 (encoding hexokinase 2). HK2 acts as the initial and rate-limiting enzyme, which is mainly involved in aerobic glycolysis, is highly expressed in some tumors, and promotes tumor growth by maintaining a high rate of glycolysis in fast-growing tumors. However, to date, little is known about the downstream target genes and metabolism-related functional mechanisms of miR-9-1 in NPC.

In the present study, we aimed to explore the mechanism of miR-9-1 in NPC cell proliferation and tumor progression, and to determine whether it regulates NPC glycolytic metabolism by targeting HK2, thus leading to tumor development. Furthermore, the relationship between the miR-9-1 expression levels and clinical features of patients with NPC was analyzed.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens and cell culture

In the present study, 156 formalin-fixed, paraffin embedded (FFPE) NPC tissue samples were obtained from the Wuzhou Red Cross Hospital (Wuzhou, China), and 11 normal nasopharyngeal tissue samples and 16 NPC tissue samples were obtained from the Affiliated Hospital of Guilin Medical College (Guilin, China). All patients were diagnosed pathologically with NPC and were restaged according to the eighth American Joint Committee on Cancer (AJCC) TNM staging system. The institutional ethical review committees of two hospitals approved this study, and informed consent was signed by all patients.
MGFD1L, 5'- CGCA CACCTGAATGCTCACTCC-3' (forward) and 5'- CGTGGATCTCAGCGTCAAG TG-3' (reverse); and GAPDH, 5'-CCATGAGAAATGTAGACCAACGC-3' (forward) and 5'-ATGGACTGTGGTCATGAGTC-3' (reverse). Primers for miR-9-1 and U6 were designed and synthesized by RiboBio. GAPDH were the internal control of HK2, MTHFD2, and MTHFD1L, and U6 was the internal control of miR-9-1. Relative expression values were calculated using the 2ΔΔCt method. All the samples were analyzed in triplicate.

### 2.5 Methylation-specific PCR (MSP)

Genomic DNA from NPC cell lines and clinical tissue samples was extracted by the phenol-chloroform method. According to the EZ DNA Methylation-Direct™ Kit (Zymo Research Corporation) protocol, 1 x 10⁸ cells were sequentially treated with proteinase K, CT Conversion Reagent solution (converting unmethylated cytosine residues to uracil), and MDesulphonation Buffer. The bisulfite-modified DNA was finally eluted with 10 μL of M-Elution Buffer.

DNA concentrations were evaluated by absorbance at 260 nm using a UV spectrophotometer (Bio-Rad Inc). The 20-μL mixture prepared for each reaction included 750 ng of sodium bisulfite–treated DNA, 10×MSP PCR buffer, 2.5 mmol/L deoxyribonucleotide triphosphates (dNTPs), and 1 U Polymerase (Roche). MSP DNA amplification was performed under the following condition: 5 minutes at 95°C for initial denaturation; 35 cycles consisting of 30 seconds at 95°C for denaturation, 60 seconds at 60°C for annealing, and 5 minutes at 72°C for final extension. PCR reactions used human GAPDH as an internal control. MSP products were analyzed using a 2% agarose gel electrophoresis and visualized using a chemiluminescence imaging system (Bio-Rad). Primer sequences for methylated DNA were 5'-TTTTAGAGAAGGGTAGGAGATTTC-3' (forward) and 5'-GCTAATCCCAAATAAAAAAAGC-3' (reverse). Primer sequences for unmethylated DNA were 5'-TTTTAGAAGGGTAGGAGATTTC-3' (forward) and 5'-CCACTAATCCCAAATAAAAAAACA-3' (reverse).

### 2.6 Methylation analysis by Sequenom MassARRAY

Genomic DNA was extracted from NPC cells using a QiAamp DNA Blood Mini Kit (BioTeKe Corporation). The Sequenom MassARRAY platform (Agena Bioscience) was applied to examine the promoter methylation level of miR-9-1 quantitatively. The genomic DNA was converted with bisulfite using the EpiTect Bisulfite kit (BioTeKe Corporation) according to the manufacturer’s instructions. PCR was performed using the following cycle conditions: 4 minutes at 94°C, followed by 45 cycles of 20 seconds at 94°C for denaturation, 30 seconds at 56°C for annealing, and 60 seconds at 72°C for extension. The PCR products were tagged with a T7 promoter sequence, and then transcribed in vitro to a single-stranded RNA copy, and processed by base-specific (U-specific) cleavage. The generated small RNA fragments and cleavage products were detected using time-of-flight mass spectrometry. The methylation ratios were outputted using the EpiTyper software (Agena Bioscience). The primers were designed using sequenom® EpiDesigner program (Agena Bioscience). Primer sequences were: 5'- AGGAAG AGAGGATT TTTT TTTAGA GAAGGGTAGGAGAGA (forward) and 5'- AAAAA CCAAATAATCA CATACACTCACCTCGGAAGGAGGATATCATG (reverse).

### 2.7 Luciferase reporter assay

The binding of miR-9-1 to HK2 mRNA in HNE1 and SUNE1 cells was verified using a luciferase reporter assay. The putative miR-9-1 complementary site in the 3' UTR of HK2 mRNA or its mutant sequence was cloned into the p-MIR-reporter vector (Ambion). For the luciferase reporter assay, HNE1 and SUNE1 cells were seeded in 96-well plates and then cotransfected with p-MIR-reporter vectors with miR-9-1 mimics or control using Lipofectamine 2000 (Invitrogen). At 48 hours after transfection, the relative luciferase activity was detected using a dual luciferase reporter assay system (Promega).

### 2.8 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell suspension at the density of 1 x 10⁶ cells/ml was inoculated into 96-well plates, and each well was added with 100 μL cell suspension. Subsequently, we supplemented 5 mg/mL MTT solution (20 µL) for 4-hour culture, and 150 µL dimethyl sulfoxide was afterwards added before 10 minutes vibration. After shaking for 10 minutes at room temperature, absorbance values were measured at 490 nm.

### 2.9 Colony formation assay

Cells were plated at a density of 400 cells per well in 6-well plates after transfection and then cultured for 10 days at 37°C in an atmosphere of 5% CO₂. Formed colonies were washed with phosphate-buffered saline (PBS) three times, fixed with methanol for 24 hours, and stained with hematoxylin for 5 minutes. Thereafter, the plates were washed and air dried. Colonies containing more than 50 cells were counted, and surviving fractions were calculated under a microscope.

### 2.10 Western blotting analysis

Western blot analysis was performed according to standard procedures using a primary antibody and secondary antibody (Cell Signaling Technology); an α-tubulin antibody (Cell Signaling Technology) was used as a loading control. Signals were detected using an enhanced chemiluminescence substrate kit (Abcam).
2.11 | Detection of glucose uptake, lactate generation, cellular ATP level, and G6P levels

The cell medium was collected after treatment with miR-9-1. The intracellular glucose uptake, lactate generation, cellular ATP level, and G6P level were assessed using a glucose assay kit, a D-lactate colorimetric assay kit, an ATP colorimetric/fluorometric assay kit, and a glucose-6-phosphate assay kit, respectively (all Sigma-Aldrich Corporation), according to the manufacturer’s instructions.

2.12 | Xenograft studies

Sixteen male Balb/c nude mice at 4 to 6 weeks old were purchased from Guangdong Experimental Animal Center and divided randomly into two groups. SUNE1 cells (1 × 10⁶) expressing miR-9-1 or its control were injected subcutaneously into either side of the nude mice (n = 8 per group). The mice were euthanized at 28 days after administration of miR-9-1 or vector control. Tumors were dissected, fixed, and imaged using a high-definition digital camera. The tumor volume (mm³) was calculated using

\[ V = \frac{4}{3} \pi (\frac{width}{2})^2 (\frac{length}{2})/6 \]

All animals received care in accordance with institutional policies on the care and use of laboratory animals and with the approval of the ethics committee of Sun Yat-sen University Cancer Center.

2.13 | MiRNA fluorescence in situ hybridization (FISH)

FISH was performed using 3 μm sections of FFPE NPC tissue with a fluorescein-tagged locked nucleic acid (LNA) oligonucleotide probe complementary to the entire has-miR-9-1 sequence, the 5’ end of which was labeled with digoxygenin (DIG). During the LNA-FISH procedure, the paraffin sections were dewaxed, fixed with fresh 4% paraformaldehyde, acetylated, and prehybridized. This was followed by a 1-hour hybridization step at 48 °C using a 5’-DIG-labeled LNA oligonucleotide probe for has-miR-9-1-5p (5’-CATACAGCTAGATAACCAAA-GA-3’) (Exiqon) complementary to miR-9-1. The signal was detected by incubation with horseradish peroxidase (HRP)-conjugated anti-DIG antibodies (1:300; #6212; Abcam). The signal was then amplified using Alexa Fluor 555 TM Tyramide Reagent (Thermo Fisher Scientific). Nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Solarbio). For imaging and processing, a fluorescence inversion microscope system equipped with DIG and DAPI filters (Nikon) and the Image-Pro Plus software (Media Cybernetics) were used, respectively. Semiquantitative assessment of the miR-9-1 fluorescence intensity for each specimen was obtained using the ImageJ software (NIH). The “LNA-FISH score” was based on the staining intensity as 0 (negative), 1 (weak), or 2 (strong), and the percentage area of positive cells as 0 (0%), 1 (interspersed or 0% to 10%), 2 (focal or 10% to 50%), or 3 (diffuse or >50%). An “LNA-FISH score” was generated as the result of the product between the intensity score (0-2) and area score (0-3), similar to that described previously. We selected five horizons in a slice randomly, calculated their scores, and then averaged them. High miR-9-1 expression in cells was defined as an averaged score >3.

2.14 | Statistical analysis

Student’s t-test was used for comparisons between groups. One-way analysis of variance (ANOVA) for multiple comparisons was used to detect differences among the various treatments. Fisher’s exact and the χ² test were used to analyze the association between the baseline characteristics and patients’ outcomes. Kaplan-Meier analyses were used for survival analysis. The Cox regression model was used to estimate univariate and multivariate hazard ratios (HRs) to predict poor prognosis. We reported the HRs with 95% confidence interval (CI) and two-sided P-values. P <.05 was considered statistically significant. All data from three separate experiments are presented as the mean ± SD.

3 | RESULT

3.1 | The miR-9-1 promoter is hypermethylated and miR-9-1 expression is downregulated in NPC cells lines and tissues

Hypermethylation of miR-9-1 occurs mainly in the promoter region (Figure 1A). First, we performed quantitative real-time reverse transcription PCR (qRT-PCR) to compare the expression of miR-9-1 between the immortalized nasopharyngeal epithelial cell line NP69 and NPC cell lines, as well as in normal nasopharyngeal tissues and NPC tissue. The expression of miR-9-1 was significantly lower in NPC cells than in the normal nasopharynx cells (P < .0001 by Student’s t-test) (Figure 1B) and lower in NPC tissues than in normal nasopharyngeal tissue (P = .0124 by Student’s t-test) (Figure 1C). Subsequently, to further investigate the relationship between DNA methylation level and
miR-9-1 expression in NPC cells, we used the Sequenom MassARRAY platform to measure DNA methylation patterns in normal tissues, NPC tissues, NP69 cell lines, and NPC cell lines (Figure 1C). The results showed that the miR-9-1 promoter region was relatively hypermethylated in NPC cell lines and NPC tissues, and hypomethylated in NP69 cells. After all cell lines were treated with the demethylation agent DAC, the expression level of miR-9-1 was restored in all NPC cell lines (Figure 1D-F), and the expression recovery of SUNE1
and HNE1 cell lines was the most significant (P < .0001 by Student’s t-test). These results showed that the miR-9-1 promoter was significantly hypermethylated in NPC cell lines and NPC tissues, which likely accounted for its low expression in these tissues and cells.

3.2 | MiR-9-1 suppressed cell growth in NPC cells

To ascertain the antitumor role of miR-9-1 in NPC cells in vitro, we performed MTT and colony formation assays. The cell viability was assessed by MTT assay, which showed that NPC cell viability was markedly decreased when NPC cells were transfected with miR-9-1 for 72 hours, compared with the control group, implying that miR-9-1 acts as a suppressive miRNA in NPC cells (Figure 2A,B). Colony formation assays (Figure 2C) showed inhibited growth of NPC cells overexpressing miR-9-1, which was consistent with the results of the MTT assays. Taken together, these results indicated that miR-9-1 inhibited cancer cell growth.

3.3 | MiR-9-1 suppresses glycolysis in NPC cells

Glycolysis involves the conversion of glucose to pyruvate and the production of two adenosine triphosphate (ATP) molecules from

![Figure 2](image-url)
each glucose molecule. Cancer cells consume glucose and produce lactate even in the presence of oxygen.

Next, we investigated whether miR-9-1 influences glycolysis in NPC cells by detected related indicators of glycolysis metabolism in NPC cells treated with miR-9-1. After transfection with miR-9-1 mimics for 24 hours, SUNE1 (Figure 3A) and HNE1 (Figure 3B) cells showed significantly decreased glucose uptake, lactate production, ATP levels, and cellular G6P levels compared with those in the control cells. These results supported the hypothesis that the effects of miR-9-1 lead to inhibited glycolytic metabolism in NPC cells.

3.4 HK2 mRNA is a direct target of miR-9-1 in NPC

To characterize the molecular mechanisms underlying the inhibitory effect of miR-9-1 on the growth of NPC cells, the downstream targets of miR-9-1 were predicted using the TargetScan (www.targetscan.org/vert_71/) and miRDB databases (http://mirdb.org/). Target sites for the binding of miR-9-1 were found in the 3′-UTRs of HK2, MTHFD2 (encoding methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2), and MTHFD1L (encoding methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1 like).13 Preliminary screening by qRT-PCR showed relatively high and significant expression of HK2 in NPC cells (Figure 4A). Western blotting results also confirmed high levels of HK2 in NPC cell lines (Figure 4B). Therefore, we selected HK2 for further study. Next, to determine whether the negative regulatory effects of miR-9-1 on HK2 mRNA levels were caused by its direct binding to HK2, we transfected HNE1 and SUNE1 cells with the wild-type HK2 3′-UTR or mutated HK2 3′-UTR luciferase reporters and miR-9-1. miR-9-1 reduced the activity from the wild-type HK2 3′-UTR reporter but not from the luciferase reporter in which the binding sites for miR-9-1 were mutated (Figure 4C). As expected, miR-9-1 mimics reduced the HK level in NPC cells, which could be reversed by HK2 re-expression in the miR-9-1-transfected cells (Figure 4D). Similar results were shown in MTT and colony formation assays (Figure 4E,F). We then detected related indicators of glycolysis metabolism in miR-9-1-transfected cells with HK2 re-expression, which showed that the glucose uptake, lactate production, ATP levels, and cellular G6P levels increased (Figure S1). These enhancement effects were eliminated after treatment with miR-9-1 mimics for 24 hours. Taken together, these results suggested that miR-9-1 targets HK2, which inhibits glycolytic metabolism in NPC cells.

3.5 MiR-9-1 inhibits the tumor growth of NPC in vivo

Next, we used xenograft models to further verify the role of miR-9-1 in retarding NPC cell growth in vivo. Nude mice were injected subcutaneously with HNE1 cells transfected with the vector-control or miR-9-1. At 28 days post injection of cells, nude mice were euthanized to compare the tumor burden between the control group and the miR-9-1 group. As shown in Figure 5A-D, the tumors with miR-9-1 overexpression grew slower than those resulting from cells transfected with the empty control vector. These data suggested that miR-9-1 suppresses tumor growth in vivo.

3.6 Relationship between the miR-9-1 expression level and clinicopathological characteristics and prognosis of patients with NPC

MiRNA-FISH score analysis was performed in NPC paraffin sections (Figure 6A). MiR-9-1 expression levels (0-2: low expression;
3-6: high expression) in NPC tissues were determined according to the available scoring rules. As shown in Table 1, the expression levels of miR-9-1 correlated significantly with sex, T stage, and TNM stage ($\chi^2$ test, $P = .002, .010$, and .020, respectively). However, the miR-9-1 expression level was not associated significantly with age, N stage, tobacco smoking, alcohol drinking, and distant metastasis ($\chi^2$ test, $P = .589, .336, .742, .366$, and .317, respectively).
Kaplan-Meier survival curves suggested that patients in the miR-9-1-low-expression group (n = 92) had a shorter overall survival (OS) and locoregional relapse–free survival (LRRFS) than those in the miR-9-1-high-expression group (n = 64) (log-rank test, \( P < .0001 \), \( P < .0488 \), Figure 6B,D) according to miRNA-FISH score results. However, there was no significant differential trend for distant metastasis–free survival (DMFS) (log-rank test, \( P = .2475 \), Figure 6C).

Furthermore, multivariate Cox regression analysis also confirmed that miR-9-1 expression status (HR = 0.315, 95% CI: 0.140–0.711, \( P = .005 \)) was associated significantly with patient OS (Table S1).

**DISCUSSION**

In the present study, we found that miR-9-1 was hypermethylated in NPC cell lines and clinical tissues and showed relatively low expression in NPC cell lines. Ectopic expression of miR-9-1 inhibited the growth and glycolytic metabolism of NPC cells in vitro and tumor proliferation in vivo. Subsequently, HK2 was identified as a direct target and functional mediator of miR-9-1’s effects. Clinically, the FISH score results indicated that miR-9-1 was an independent prognostic factor in patients with NPC. Thus, these results suggested that hypermethylation of miR-9-1 plays an important role in the development of NPC.

To further unravel the mechanism of miR-9-1 in the progression of NPC, we explored the biological function of miR-9-1 through functional assays. Ectopic expression of miR-9-1 significantly inhibited the in vitro activity, glycolytic metabolism, and proliferative capacity of NPC cells and suppressed the growth of xenograft tumors in vivo. This was consistent with the results observed in breast, prostate, and non–small cell lung cancers.\(^{23-25}\) Moreover, methylation-associated miR-9 downregulation ameliorated its tumor suppressive potential in the progression of oral and oropharyngeal squamous cell carcinoma\(^ {26} \) and gastric cancer.\(^ {27} \) These are consistent with our findings. Furthermore, miR-9-5p has also been reported to play the opposite role in different types of cancer.\(^ {28} \)

In cervical cancer, the differential expression of miR-9-5p in two tissue types (squamous cell carcinoma and adenocarcinoma) suggested a dual function of miR-9-5p in the same cancer type.\(^ {29} \) Previously, it was reported that miR-9 regulates cancer progression and metastasis in NPC by targeting CXCR4.\(^ {30} \) However, even within the same tumor, miRNAs perform different functions due to the diversity of downstream targets. Thus, the downstream regulatory network of miRNAs is complex and tissue specific. Our current findings suggest that miR-9-1 may inhibit the growth of NPC cells by suppressing glycolytic metabolism and that hypermethylation of the miR-9-1 promoter region disrupts its inhibitory effect, leading to cancer cell growth and tumor proliferation. Taken together with previous studies,\(^ {30} \) our results suggest that miR-9-1 has an antitumor effect in NPC.

MiRNAs have been revealed as important regulators of cancer progression and metabolic reprogramming, in which they regulate gene expression negatively and are widely involved in regulating cell proliferation, differentiation, apoptosis, migration, and transformation.\(^ {31,32} \) Aerobic glycolysis is a hallmark of cancer.\(^ {3} \) Song et al revealed that FOXC2 promoted glycolysis in the progression of NPC by activating YAP signaling,\(^ {33} \) and Su et al revealed that JMJD2A regulates aerobic glycolysis in NPC by modulating LDHA expression.\(^ {34} \) The abnormal glycolysis in NPC cells was associated with poor prognosis in patients with NPC.\(^ {35,36} \) Nevertheless, few studies have been reported on the effects of miRNAs on the glycolytic metabolism of NPC. In the present study, bioinformatic analysis and experimental validation confirmed that the mRNA encoding the glycolytic rate-limiting enzyme HK2 is a key cellular target of miR-9-1 in NPC. We discovered that HK2 expression promoted cell proliferation and glycolytic metabolism in cultured NPC cells. Moreover, reintroduction of HK2 rescued the inhibited glycolytic metabolism mediated by miR-9-1 in NPC cells. Similarly, overexpression of HK2 has been observed in some cancers and was associated with increased glucose consumption.
FIGURE 6 Localization of miR-9-1 expression in nasopharyngeal carcinoma (NPC) tissue by fluorescence in situ hybridization and correlation with prognosis in patients with NPC. A, The probe for miR-9-1 was locked nucleic acid (LNA) modified and labeled with digoxigenin at the 5’ end. (a–c) Strong expression of miR-9-1 in NPC. (d–f) Moderate expression of miR-9-1 in NPC. (g–i) Weak expression of miR-9-1 in NPC. Red, miR-9-1; Blue, 4’,6-diamidino-2-phenylindole (DAPI) nuclear staining. Scale bars are 10 µm. Original magnification 200×. B, Kaplan-Meier analysis comparing overall survival, distant metastasis–free survival, and locoregional relapse–free survival between the miR-9-1–high- and miR-9-1–low-expression groups. Patients with low miR-9-1 expression had shorter overall survival and locoregional relapse–free survival. C, Proposed model for miR-9-1 modulation of hexokinase 2 (HK2) expression as well as glycolysis-related tumor growth.
metabolism and poor outcome. As such, HK2 is considered a key factor in the Warburg effect and has been proposed as a target for the treatment of cancer. Recently, studies also have shown that several miRNAs, including miR-143, miR-199a-5p, miR-145, and the newly identified miR-532-3p, are HK2 suppressors. Therefore, our data are consistent with the view that increased glycolysis contributes to the maintenance of the malignant phenotype of cancer cells, and suggests that miR-9-1 exerts an inhibitory effect on NPC, primarily or partially through inhibition of glycolysis. However, the specific molecular mechanisms by which miR-9-1 regulates HK2 expression require further investigation.

In summary, our findings revealed that miR-9-1 is a tumor suppressor miRNA, the coding region of which is aberrantly hypermethylated in NPC. Moreover, the FISH score results indicated miR-9-1 is an independent prognostic factor in NPC. These results confirmed that the miR-9-1/HK2 axis is a novel regulatory mechanism for the Warburg effect in NPC (Figure 6E), which correlates with prognosis and is thus worthy of further study to develop novel treatment strategies for NPC.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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