# Original Article circMYC promotes cell proliferation, metastasis, and glycolysis in cervical cancer by up-regulating MET and sponging miR-577

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**Abstract:** Objective: To analyze the role of circMYC in cervical cancer. Methods: Protein and RNA expression was detected by RT-qPCR and western blotting. Transwell, CCK8, and colony formation assays were used for measuring metastasis, cell viability, and proliferation, respectively. Lactate production, glucose uptake, and ATP generation were examined to evaluate cell glycolysis. Interactions between circMYC, miR-577, and MET were determined by RNA pull-down and immunoprecipitation, and dual-luciferase reporter assays. Xenografts were established in mice to evaluate the functions of circMYC *in vivo*. Results: circMYC was overexpressed in tumor tissue, which was related to poor prognosis. CircMYC knockdown reduced proliferation, colony formation, metastasis, and glycolysis in cervical cancer cells as well as inhibiting tumor growth *in vivo*. Mechanistically, circMYC targeted miR-577, and the effects of circMYC knockdown could be reversed by miR-577 inhibition. Moreover, miR-577 downregulated the expression of MET. Therefore, the oncogenic role of circMYC in cervical cancer was achieved by sponging miR-577 and maintaining MET expression. Conclusion: circMYC promotes cervical cancer progression through regulation of the miR-577/ MET axis. circMYC may thus be a potential target for diagnosing and treating cervical cancer.

Keywords: Cervical cancer, cancer progression, metastasis, glycolysis, circMYC, miR-577, MET

#### Introduction

Cervical cancer is common and mainly caused by infection of human papillomavirus (HPV). Even with the introduction of the vaccine, the mortality of cervical cancer is still high [1]. Cervical cancer has poor prognosis and therapeutic outcomes in patients with tumor metastasis [2], showing a five-year survival rate of 16.5% in patients with metastases as compared with 91.5% of those without [3]. Therefore, the prevention of metastasis would improve both the clinical outcomes and survival of cervical cancer patients.

The Warburg effect is a remarkable characteristic of tumor cells. This aerobic glycolysis involves upregulation of glucose utilization and increased production of lactate [4]. The enhancement of glycolysis facilitates cancer cells to overcome nutrient and energy deficiency and thus has a strong association with cancer progression and metastasis [5, 6]. For example, CARM1-induced PKM2 methylation activated aerobic glycolysis to promote cancer progression [7]. Therefore, interfering glycolytic metabolism in cancer cells may be an effective strategy to inhibit tumor invasion and metastasis.

Nowadays, RNA-sequencing techniques have greatly accelerated the discovery of numerous non-coding RNAs with previously unknown functions [8]. MicroRNAs (miRNAs) and circular RNAs (circRNAs) are small non-coding RNA molecules with regulatory roles on gene expression and can thus influence tumor progression. Numerous reports have demonstrated pivotal roles of miRNAs in cervical cancer metastasis [2]. For example, miR-1246 and miR-221-3p

facilitate the metastasis of cervical cancer [9]. On the other hand, miR-494 and miR-145 are reported to suppress cervical cancer metastasis [10, 11]. CircRNAs usually regulate mRNA expression by serving as miRNA sponges [12]. The regulatory network, as well as mechanisms underlying interactions between these three RNA species and their roles in human cancers, have been emphasized in numerous works [13]. For example, circ 101996, circ 0023404, and circRNA 0000285 can enhance the metastasis of cervical cancer [14-16]. Therefore, circRNAs also can be biomarkers in cancer prognosis [17]. Herein, in our study, we used the bioinformatics approaches and focused on the potential functions of circMYC, and evaluated its role in tumor progression and downstream mechanism of cervical cancer.

# Materials and methods

#### Cells and tissues

The cervical cancer cell lines SiHa, C-4 I, HeLa, and C-33A, and the normal endocervical cell line End1/E6E7 were purchased from the ATCC, and incubated with DMEM medium (Gibco, Grand Island, USA) containing 11% fetal bovine serum (FBS) at 37°C with 5%  $CO_2$ . The tissue samples were from patients with newly diagnosed cervical cancer who had not yet received treatment and adjoining normal tissue samples were collected at the same time (n = 68). The study was approved by the ethics committee of our institute.

# Cell transfection

For circMYC knockdown, circMYC shRNA was used. miRNA mimics and inhibitors were purchased from GenePharma Company (Shanghai, China). Cells (2-5×10<sup>4</sup> per well) were plated in 12-well plates with FBS-free DMEM medium. The transfections were performed in FBS-free DMEM mixed with Lipofectamine<sup>™</sup> 2000 reagent (Thermo Fisher Scientific, USA); the plasmids were also diluted with FBS-free DMEM medium. The Lipofectamine<sup>™</sup> 2000 reagent and plasmids were mixed and allowed to stand for 20 min, then incubated with the cells for 48 h.

# Xenografts

BALB/c nude mice were obtained from the Institutional Animal Care Committee of Beijing

Institute of Biotechnology (Beijing, China). SiHa cells transfected with shRNA NC (sh-NC) or circ-MYC shRNA were subcutaneously injected in the right front legs of the mice  $(5 \times 10^6$  cells per mouse). Tumor sizes were measured every seven days using a vernier caliper (Hanqun, Suzhou, China). After sacrifice, the tumor volumes were determined by the formula V (mm<sup>3</sup>) =  $(L \times D^2)/2$ .

# Bioinformatics analysis and dual-luciferase reporter (DLR) assay

StarBase 2.0 (http://starbase.sysu.edu.cn) was used for the prediction of miR-577 binding to circMYC and MET. To examine whether miR-577 targeted MET or circMYC directly, DLR assay was used. circMYC WT reporter plasmid, mutated (mut) circMYC reporter plasmid (mut circMYC), MET WT reporter plasmid, and mutated MET reporter plasmid (mut MET) were incorporated into the pmiR-GLO dual-luciferase target expression vector (Promega, Madison, WI, USA) and assays were performed as previously described [18].

# CCK8 assay

The CCK8 assay was used to measure cell proliferation. Five thousand cells per well in a 100  $\mu$ L volume were seeded and incubated in 96-well plates for 24, 48, and 72 h. Fifteen microliters of CCK8 solution (Dojindo, Shanghai, China) were added and reacted for 2 h before measuring the OD450 in a microplate reader (BioTek, Winooski, VT, USA).

# Colony formation assay

Cells were digested, inoculated into a culture plate, and incubated for 2-3 weeks. After visible colony formation, 4% paraformaldehyde (Sigma-Aldrich) was used to fix the cells for 15 min. Two milliliters of 0.1% crystalline violet solution (Abcam, Cambridge, UK) was then added to the fixed cells for 10 min. After washing, plates were inverted and covered with a transparent grid, and allowed to air-dry. The number of colonies on the plates was counted either by the naked eye or under 10× magnification under a light microscope.

# RNA-pull down

Biotinylated circMYC (Tsingke, Wuhan, China) was added to streptavidin-magnetic beads (Life

 Table 1. The primer sequences

Primer	Sequence
GAPDH-F	5'-TCCGTGGTCCACGAGAACT-3'
GAPDH-R	5'-GAAGCATTTGCGGTGGACGAT-3'
U6-F	5'-CTCGCTTCGGCAGCACA-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'
miR-577-F	5'-ACCGCGCGCGCGTAGATAAAATATTGG-3'
miR-577-R	5'-ATCCAGTGCAGGGTCCGAGG-3'
MET-F	5'-AGCAATGGGGAGTGTAAAGAGG-3'
MET-R	5'-CCCAGTCTTGTACTCAGCAAC-3'
CircMYC-F	5'-CTCACAGCCCACTGGTCCTC-3'
CircMYC-R	5'-TCCAGCAGAAGGTGATCCAG-3'

Technologies, USA), left to stand at room temperature for 2 h followed by incubation with the lysate of  $1 \times 10^7$  cells overnight at 4°C. After washing, the miRNAs bound to the beads were purified using TRIzol (Takara, Otsu, Japan) and analyzed by RT-qPCR.

# RNA immunoprecipitation (RIP) assay

The EZ-Magna RIP kit (Millipore, USA) was used for the RIP assay. Cell lysates were incubated in RIP buffer with magnetic beads (coated with either anti-Ago2 or anti-IgG). After proteinase K treatment, the precipitated RNA was purified and analyzed as described above.

# Measurement of glucose uptake, ATP, and lactate

Assay kits for glucose uptake (Glucose Uptake Colorimetric Assay kit), ATP (ATP Colorimetric Assay kit), and lactate (Lactate Assay Kit II) were obtained from Biovision (USA) and used according to the manufacturers' instructions [19].

# Transwell assay

Cells (2×10<sup>5</sup>/well) were added to the upper chamber of the apparatus pre-coated with Matrigel (BD Biosciences, USA). The lower chamber contained medium with 12% FBS. After migration, cells were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet, and counted. Cell invasion was measured in the same way, except that Matrigel was added in a 1:7 ratio with serum-free medium to the Transwell chamber. Quantitative reverse transcription PCR (RTqPCR)

RNA extraction, cDNA synthesis (RevertAid cDNA Synthesis Kit, ThermoFisher Scientific), and amplification (ABI Prism 7900HT platform, Applied Biosystems, Foster City, CA, USA) were performed as previously described [20]. The Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada) was used according to instructions [21]. The mRNA reference was GAPDH mRNA and the miRNA reference was U6 snRNA. The primer sequences are shown in **Table 1**.

# Western blot

The protein extraction (RIPA buffer, Thermo-Fisher Scientific, Waltham, MA, USA), concentration determination (BCA protein assay kit, ThermoFisher Scientific), separation and transfer to nitrocellulose filter membrane (Millipore, Boston, USA), and incubation with BSA and antibodies were performed as described previously [15]. The primary antibodies including Hexokinase 2 (HK2) (diluted 1:1,000), Lactate dehydrogenase A (LDHA) (diluted 1:1,000), and MET (diluted 1:1,000) were purchased from HuaBio Company (Hangzhou, China) and β-actin (diluted 1:5,000) was obtained from ZSGB-BIO (Beijing, China). The secondary antibodies were obtained from Proteintech (USA). Protein expression was detected by ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

# Statistical analysis

SPSS 18.0 software was used for analyses. Continuous data were expressed as mean  $\pm$  standard deviation ( $\overline{x} \pm$  sd) from at least three independent experiments. Between-group differences were determined by Student's t-test or one-way ANOVA. Significant differences are indicated by \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. The graphic software used in the study included Adobe Photoshop CC and Adobe Illustrator CS6.

# Results

circMYC expression in tissues and cells

circMYC expression was investigated in cervical cancer patients using 68 pairs of tumor tis-



**Figure 1.** circMYC expression in normal and cervical cancer cells. A: circMYC expression in cancerous and adjacent normal tissue (n-68) detected by RT-qPCR; B: Survival curves of cervical cancer patients in relation to circMYC expression; C: circMYC expression in SiHa, C-4 I, HeLa, and C-33A cells, and normal endocervical cell line End1/E6E7 as detected by RT-qPCR. \*\*\*P<0.001.

Table 2. Correlation bet	etween circMYC expression and the	
clinical pathological fea	atures of 68 cervical cancer patien	ts

		circMYC expression		
Characteristic	Cases	High (n =	Low (n =	Р
		34)	34)	
Tumor size (n)				0.808
<4 cm	31	16	15	
≥4 cm	37	18	19	
Age (years)				0.618
<50 years	32	20	22	
≥50 years	26	14	12	
Histologic grade (n)				0.302
G1 + G2	50	24	26	
G3	18	10	8	
FIGO stage (n)				0.028*
lb-lla	31	11	20	
llb-llla	37	23	14	
Lymph node metastasis (n)				0.004*
No	32	10	22	
Yes	36	24	12	

Note. FIGO: Federation of Gynecology and Obstetrics. \*P<0.05.

sues and normal adjacent tissues. The expression was significantly higher in tumor tissues and was associated with poor patient outcome (**Figure 1A, 1B**). Analysis of circMYC expression in relation to patients' clinical features showed a positive correlation between circMYC expression and both cancer staging and lymph node metastasis (**Table 2**). Moreover, circMYC expression was increased in the four cancer cell lines compared to normal End/E6E7 cells, particularly in SiHa and HeLa cells (**Figure 1C**). Therefore, circMYC expression was enhanced in both tumor cell lines and tissues.

# Knockdown of circMYC decreased cell proliferation and metastasis

circMYC expression was silenced in SiHa and HeLa cells by circMYC shRNA transfection (Figure 2A), while the expression of MYC mRNA was unaffected (Figure 2B). CCK8 assay showed that knockdown of circMYC decreased cell growth (Figure 2C) and colony formation, suggesting the pro-proliferative role of circMYC (Figure 2D). Moreover, the Transwell assay showed that both the migration and invasion of cells were diminished after knockdown of circMYC (Figure 2E, 2F). In the xenograft mouse model, circMYC knockdown significantly reduced both tumor growth (Figure 2G) and weight (Figure 2H).

# Knockdown of circMYC inhibited glycolysis

Inhibition of glycolysis has been found to reduce the malignant behaviors of tumor cells [22]. Here, we evaluated whether circMYC affected the glycolysis of cervical cancer cells. ATP generation, lactate production and glucose uptake were decreased significantly in circ-MYC-knockdown SiHa and HeLa cells (Figure **3A-C**). In addition, the protein expressions of hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA) were also reduced (Figure 3D) [4]. Taken together, these results indicate that circMYC downregulation suppresses glycolysis.

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**Figure 2.** Downregulated circMYC expression suppresses cancer progression in vitro. A: Knockdown efficiency of circMYC shRNA in SiHa and HeLa cells as assessed by RT-qPCR; B: MYC mRNA in the cells transfected with circMYC shRNA; C: Cell viability of transfected cells measured by the CCK8 assay; D: Cell proliferation measured by the colony formation assay; E, F: Transwell assay showing cell invasion and migration (amplification: ×200); G: The effects of circMYC on tumor growth in xenografts; H: The effects of circMYC on tumor weight in xenografts. \*\*P<0.01, \*\*\*P<0.001. OD: optical density.



Figure 3. The decreased expression of circMYC suppresses glycolysis of cervical cancer cells. A-C: Glucose uptake, ATP generation, and lactate production were examined in circMYC shRNA-transfected cells; D: Protein expression shown by western blot;  $\beta$ -actin used as loading control. \*\*P<0.01, \*\*\*P<0.001. HK2: Hexokinase 2; LDHA: Lactate dehydrogenase A.

#### miR-577 targeted circMYC directly

We next investigated the downstream effects of circMYC. First, the components of cytoplasm/membrane and nuclei were separated to detect circMYC expression, and the results indicated that circMYC expression was mainly cytoplasmic (Figure 4A). StarBase predicted the binding of miR-577 to circMYC (Figure 4B). In the DLR assay, miR-577 overexpression inhibited luciferase activity while this inhibitory effect was abolished after the binding site mutation (Figure 4C). RNA pull-down also indicated that miR-577 could interact with circMYC (Figure 4D). Furthermore, the RIP assay further confirmed the interaction between circMYC and miR-577 (Figure 4E). These data confirmed that miR-577 targeted circMYC. Moreover, knockdown of circMYC enhanced the expression of miR-577 (Figure 4F). In clinical specimens, we found that miR-577 expression was reduced in tumor tissues and was negatively correlated with circMYC (Figure 4G, 4H). The reduced miR-577 expression was also detected in cell lines (Figure 4I). These results indicate that miR-577 expression is negatively regulated by circMYC.

# MET was targeted directly by miR-577

StarBase predicted the binding between miR-577 and the 3'-UTR of MET mRNA (**Figure 5A**). After transfection of cells with a miR-577 mimic (Figure 5B), the DLR assay confirmed that miR-577 targeted MET. Moreover, the miR-577 mimics reduced luciferase activity in wild-type MET 3'-UTR-transfected cells while the inhibitory effects were blocked after the mutation of this binding site (Figure 5C). Moreover, MET protein expression of MET was decreased by the mimics (Figure 5D) but increased in cells transfected with the inhibitor (Figure 5E, 5F). MET expression was also increased in tumor tissues (Figure 5G), and correlated positively with circMYC and negatively with miR-577 expression (Figure 5H, 5I), suggesting that miR-577 targeted and negatively regulated MET expression.

# CircMYC regulated cell proliferation, metastasis, and glycolysis via miR-577/MET in cervical cancer

Finally, we investigated whether the oncogenic role of circMYC was dependent on its regulatory effects on miR-577 and MET. SiHa and HeLa cells were transfected with circMYC shRNA with/without the miR-577 inhibitor. This showed that circMYC knockdown reduced MET expression which was increased by the co-transfection of the miR-577 inhibitor (**Figure 6A**). As shown in **Figure 6B**, **6C**, circMYC shRNA reduced cell viability and colony formation. How-



**Figure 4.** miR-577 targets circMYC directly. A: circMYC expression in cytoplasm, membrane, and nuclei measured by RT-qPCR; B: StarBase prediction of circMYC and miR-577 interaction; C: circMYC and miR-577 interaction shown by DLR; D: RNA pull-down assay; E: RIP assay; F: miR-577 expression in circMYC knockdown cells measured by RT-qPCR; G: miR-577 expression in tumor and normal tissues (n = 68) measured by RT-qPCR; H: Correlation between circMYC and miR-577 expression in tumor and normal tissues; I: miR-577 expression in cell lines. \*\*\*P<0.001.

ever, knockdown of miR-577 enhanced cellular proliferation, alleviating the effects of circMYC knockdown. Moreover, the role of circMYC on cervical cancer cell metastasis was also antagonized by miR-577 (**Figure 6D**, **6E**). At last, the role of circMYC/miR-577/MET in cell glycolysis was determined. CircMYC shRNA significantly suppressed ATP generation, lactate production, and glucose uptake, all of which were reversed by the knockdown of miR-577 (**Figure 6F-H**). MiR-577 knockdown also rescued HK2 and LDHA expression in circMYC knockdown cells (**Figure 6I, 6J**). In conclusion, circMYC modulated cell proliferation, metastasis, and glycolysis via the miR-577/MET axis in cervical cancer.

# Discussion

Metastasis of cervical cancer results in poor outcomes [3]. Here, we revealed the oncogenic

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Figure 5. MET is a direct target of miR-577. A: StarBase prediction of miR-577 and MET binding; B: miR-577 expression in SiHa and HeLa cells; C: The DLR assay; D: Western blot showing MET expression in miR-577-overexpressing cells; E: miR-577 expression in cells measured by RT-qPCR; F: Western blot showing MET expression in miR-577-knockdown cells; G: MET expression in tumor and normal tissues (n = 68) measured by RT-qPCR; H, I: The correlation between expression of circMYC or miR-577 with MET in tumor and normal tissues. \*\*P<0.01, \*\*\*P<0.001.

activity of circMYC in cervical cancer, including its functions in promoting cell proliferation, metastasis, and glycolysis. circMYC exerts its effect via sponging miR-577 and up-regulating MET expression. We investigated the effects of circMYC overexpression in cervical cancer tissues, observing an association with poor prognosis and outcomes. CircMYC is a novel circRNA with oncogenic activity. It has been shown to promote proliferation in breast cancer cells [23]. In multiple myeloma, circMYC derived from exosomes correlates with high relapse and mortality rates, and enhances the drug resistance [24]. In nasopharyngeal carcinoma, circMYC reduces radiosensitivity [25]. Here, we found that circMYC promoted both metastasis

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**Figure 6.** CircMYC/miR-577/MET axis regulates cell proliferation, metastasis, and glycolysis. A: Western blot showing MET expression; B: CCK8 assay showing cell viability; C: Colony formation assay demonstrating proliferation; D, E: Transwell assay demonstrating invasion and migration (amplification: ×200); F-H: Glucose uptake, ATP generation, and lactate production in cells; I, J: Western blot of HK2 and LHDA expression. \*\*P<0.01, \*\*\*P<0.001. HK2: Hexokinase 2; LDHA: Lactate dehydrogenase A.

and glycolysis. In contrast, it has been reported that circMYC reduced glycolysis in melanoma [26]. This discrepancy might result from the usage of different cancer cell types.

Our results indicated that the oncogenic activity functions via the miR-577/MET axis. miR-577, a miRNA, can suppress the development of many kinds of cancers by reducing cell proliferation, metastasis, or inducing cell apoptosis [27-29]. In addition, some non-coding RNAs sponge miR-577 to promote tumor progression. For example, circCSPP1 facilities hepatocellular carcinoma growth through sponging miR-577 [30] while the long non-coding RNA SNHG3 promotes migration and the epithelialmesenchymal transition through the same mechanism [31]. Our results also indicated that cervical cancer cells have reduced miR-577 expression, and knockdown of miR-577 reversed the antitumor effects of circMYC silencing. This evidence suggests that rescuing the expression of miR-577 might be an approach to inhibit cancer progression.

The predictive tools and our results showed that circMYC and MET protein were the targets of miR-577. MET, as an oncogene, is a target in anticancer drug discovery [32]. It has been shown that MET derived from exosomes accelerates melanoma metastasis [33]. MET activation promotes drug resistance [34]. In addition, MET is recognized as a cancer biomarker [32]. To date, there are few efficient MET inhibitors used in clinical practice [32]. Therefore, decreasing MET expression by upstream regulation is a feasible means of inhibiting METcontrolled cancers. Our research found that miR-577 targeted MET directly and negatively regulated its expression. miR-577 overexpression reduced MET expression and exerted antitumor effects, confirming the findings of a previous study that showed that miR-449a could suppress cell growth of hepatocellular carcinoma via targeting MET [35]. Thus, it is feasible to reduce MET expression by regulating miRNA to suppress tumor progression.

In this paper, we explored the relationship between circMYC and miR-577 in cervical cancer. There are, however, other miRNAs that are downstream targets of circMYC. Although few studies have focused on circMYC-miRNA interactions, it has been found that circMYC can directly target miR-1233 to regulate the glycolysis process in melanoma [26]. Therefore, further investigation is required to determine whether miR-577 is the sole target of circMYC in cervical cancer progression. This study cannot rule out the possibility that circMYC may also regulate other miRNAs in cervical cancer progression. In addition, according to the different downstream target genes of miRNA, circ-MYC might have other mechanisms of action on cervical cancer. These questions require further investigation.

In conclusion, we have revealed the modulatory role of circMYC/miR-577/MET in the proliferation, metastasis, and glycolysis of cervical cancer, and thus identified circMYC upregulation as a potential biomarker and target for cervical cancer therapy.

# Disclosure of conflict of interest

# None.

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