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ORIGINAL RESEARCH

circ-MYBL2 Serves As A Sponge For miR-361-3p Promoting Cervical Cancer Cells Proliferation And Invasion

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Background: Circular RNAs (circRNAs) have been considered as a key regulator in tumor carcinogenesis. However, the roles and underlying mechanisms of circRNAs in cervical cancer (CC) remain largely unknown. In this study, we explored the effects of circ-MYBL2 (hsa_circ_0060467) on CC progression.

Methods: Levels of circ-MYBL2 and miR-361-3p were examined by qRT-PCR. CCK-8 assay, colony formation assay and transwell invasion assay were used to determine the roles of circ-MYBL2 in CC. Dual-luciferase reporter and RNA pull down assays were employed to verify the relationship between circ-MYBL2 and miR-361-3p.

Results: We showed that the expression of circ-MYBL2 was significantly upregulated and positively associated with advanced FIGO stage, larger tumor size, lymph node metastasis, and poor prognosis in CC patients. Function assays revealed that circ-MYBL2 inhibition suppressed CC cells' proliferation, invasion and epithelial–mesenchymal transition (EMT) processes. In mechanism, miR-361-3p was identified as a direct target of circ-MYBL2, rescue assays showed that miR-361-3p suppression reversed the effects of si-circ-MYBL2 on CC cells' progression.

Conclusion: Our findings suggested that circ-MYBL2 promoted CC progression by regulating miR-361-3p expression, which provided a novel therapeutic target for the treatment of CC patients.

Keywords: circ-MYBL2, miR-361-3p, cervical cancer, proliferation, invasion

Introduction

Cervical cancer (CC) is the most common gynecological malignant tumor worldwide, with a global incidence of 530,000 cases and nearly 275,000 deaths per year.[1,2] The number of CC cases in developing countries accounts for about 85% of global incidence.[3] In recent decades, owing to advances in CC screening, as well as surgery, radiotherapy, and chemotherapy, the clinical outcomes of patients were significantly improved. However, the prognosis for advanced CC patients is still unsatisfactory.[4,5] Therefore, it is urgently necessary to elucidate the underlying mechanisms for CC treatment.

Circular RNAs (circRNAs) are a novel class of endogenous RNA that has a covalent closed loop structure.[6] It is highly evolutionarily conserved and stable and particularly resistant to RNases activity.[7] Accumulating evidence showed that circRNAs were widely involved in diverse physiological and pathological processes, especially in tumor progression.[8,9] For example, Zong et al found that circRNA_102231 expression was

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significantly upregulated lung cancer patients.[10] Li et al found that circRBMS3 promoted gastric cancer tumorigenesis by regulating miR-153-SNAI1 axis.[11] Zhou et al revealed that circPCNXL2 sponged miR-153 to promote the proliferation and invasion through upregulating ZEB2 in renal cancer.[12]

Recently, increasing evidence showed that circRNAs play vital roles in CC progression. For example, Zhang et al showed that hsa_circ_0023404 exerted an oncogenic circRNA in CC progression by modulating the miR-136-TFCP2/YAP axis.[13] Liu et al found that circRNA8924 acted as a ceRNA of the miR-518d-5p/519-5p family to promote CC progression.[14] Recently, Li et al used microarray identifed that has_circ_0060467, has_circ_0060458, and has_circ_0090531 was increased in CC tissues.[15] However, the roles and underlying mechanisms remain unclear in CC progression. In the present study, we showed that circ-MYBL2 (hsa_circ_0060467) was significantly upregulated and associated with advanced clinical features and poor prognosis in CC patients. In mechanism, we found that circ-MYBL2 might serve as a sponge for miR-361-3p to promote CC progression. Thus, we suggested that circ-MYBL2 might act as an effective therapeutic target for CC treatment.

Materials And Methods

Tissue Samples

Primary CC tissues (cervical squamous cell carcinoma) and adjacent normal tissues (ANT; at least 3 cm away from the edge of the tumor and no tumor cells were observed) from 49 patients were obtained in Linfen People's Hospital from 2009 to 2014. The fresh samples were immediately frozen in liquid nitrogen and stored until total RNA extraction. All patients read and signed the informed consent forms and the study was approved by the Ethic Committee of Linfen People's Hospital. No patient received chemotherapy or radiotherapy before surgery.

Cell Culture And Transfection

The normal cervical epithelium cell line (HCvEpC) and CC cell lines (C33A, HeLa, SiHa, CaSki, and C4-1 cells) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), all cells were maintained in DMEM (Gibco, USA), supplemented with 10% FBS (Invitrogen, USA) in a humidified incubator containing 5% CO_2 at 37 °C.

Small interfering RNA targeting circ-MYBL2 (si-circ-MYBL2-1, 5′- CTCTTGTTTGTAACCCCAGAT-3′; si-circ-MYBL2-2, 5′-TCTCTTGTTTGTAACCCCAGA-3′), miR-361-3p mimics and inhibitors were purchased from Genepharma (Shanghai, China). All oligonucleotides and vectors were transfected into cells by using Lipofectamine 3000 (Invitrogen, MA, USA). After 48 h, the transfection efficiency was determined by qRT-PCR.

CCK-8 Assay

Transfected cells were inoculated into 96-well plates (5000 cells/well) for routine culture at 37°C, 5% CO_2. At 24, 48 and 72 h, 10 μL of CCK-8 solution was added to each well. Then, a microplate reader was used to detect the optical density (OD) value of each well at 450 nm according to the manufacturer's instructions

Colony Formation Assay

Colony formation assay was performed as previous study [13]

Transwell Invasion Assay

Cell invasion ability was explored by transwell invasion assay. Briefly, transfected cells (1×10^5 cells/mL) were seeded in upper chambers with 100 μL of serum-free medium. The lower chamber was supplied with 500 μL of DMEM medium supplemented with 10% FBS. After 24 h, cells invaded into the lower side were fixed and stained by 0.1% crystal violet. Then, the number of invaded cells was counted under a light microscope (Nikon, Japan).

RNA Extraction And Quantitative RT-PCR

Total RNA was extracted from tissues and cell lines using Trizol reagents (TaKaRa, Dalian, China), then 500ng total RNA was reverse transcribed into cDNA by PrimeScript RT Master Mix (TaKaRa). Quantification of circ-MYBL2 and miR-361-3p was performed by using a SYBR Green PCR Kit (TaKaRa) through StepOnePlus system (Applied Biosystems, CA, USA). Fold changes were calculated by using the $2^{-\Delta\Delta Ct}$ method. GAPDH and U6 were used as references for mRNA and miRNA, respectively.

Luciferase Reporter Assay

The recombination luciferase plasmids containing circ-MYBL2 full-length sequences with wild-type (WT) or mutant (MUT) miR-361-3p binding site were constructed from Genecopoeia company (Rockville, Md, USA). Subsequently, luciferase plasmids were co-transfected with miR-361-3p mimics or miR-NC into cells by Lipofectamine 3000 reagents (Invitrogen). After 48 h, the luciferase activity was detected by

the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Pull-Down Assay

Biotinylated-labeled probe was synthesized by RiboBio (Guangzhou, China). The sequence of the probe was Bio-5′-GTGGCCAGGGGTGCTCCTGTGCTCACCCTCTC-3′. Pull-down assay was carried out as previous study.[11]

Western Blot Assay

Cells were lysed in RIPA lysis buffer (Beyotime, Shanghai). Then, proteins were separated on 12% SDS-PAGE gels and then transferred onto a PVDF membrane (Millipore). The membrane was incubated with primary antibodies at 4°C overnight. Then the prepared membranes were incubated with secondary antibody for 2 h. Finally, the blots were detected by enhanced chemiluminescence kit (Pierce, MA, USA).

Statistical Analysis

All statistical analysis was performed by SPSS 19.0 software. Data were expressed as mean± SD at least three experiments. The differences between groups were calculated using Student's *t*-test or Chi-square test. Overall survival rate was evaluated by Kaplan-Meier analysis and log rank test. $P < 0.05$ was considered statistically significant.

Results

circ-MYBL2 Is Up-Regulated In CC

In the present study, we determined has_circ_0060467, has_circ_0060458 and has_circ_0090531 expression in 18 paired CC tissues by qRT-PCR. Results showed that the expression of has_circ_0060467, has_circ_0060458, and has_circ_0090531 was significantly increased in CC compared to ANT tissues, and has_circ_0060467 displayed the greatest upregulation (Figure 1A–C). According to the human reference genome, we termed has_circ_0060467 (located at chr20:42338602-42345122, is derived from gene MYBL2) as "circ-MYBL2".

To further confirm the results, we explored circ-MYBL2 expression in 49 paired CC tissues, qRT-PCR showed that circ-MYBL2 was significantly upregulated and associated with advanced FIGO stage, larger tumor size, and lymph node metastasis in CC patients (Figure 2A–E). Moreover, circ-MYBL2 expression in normal cervical epithelium cell line (HCvEpC) and CC cell lines (C33A, HeLa, SiHa, CaSki, and C4-1) was also examined by qRT-PCR. The relative expression of circ-MYBL2 in CC cell lines was obviously elevated compared with HCvEpC cells (Figure 2F).

circ-MYBL2 Inhibition Suppressed CC Cells Proliferation And Invasion

To explore the effects of circ-MYBL2 on CC progression, we firstly knockdown circ-MYBL2 expression in HeLa and CaSki cells, the efficiency was determined by qRT-PCR (Figure 3A). CCK8 and colony formation assays showed that circ-MYBL2 knockdown significantly inhibited HeLa and CaSki cells viability in vitro (Figure 3B and C). Transwell invasion assay showed that si-circ-MYBL2 reduced HeLa and CaSki cells invasion ability in vitro (Figure 3D). Next, we explored whether circ-MYBL2 mediated epithelial–mesenchymal transition (EMT) processes in CC, qRT-PCR and Western blot assays showed that circ-MYBL2 inhibition significantly reduced expression of N-cadherin (mesenchymal marker) and increased expression of E-cadherin (epithelial marker) in HeLa and CaSki cells (Figure 3E and F).

circ-MYBL2 Directly Interacted With miR-361-3p

We further explored miR-361-3p expression in CC. Results revealed that the expression of miR-361-3p was

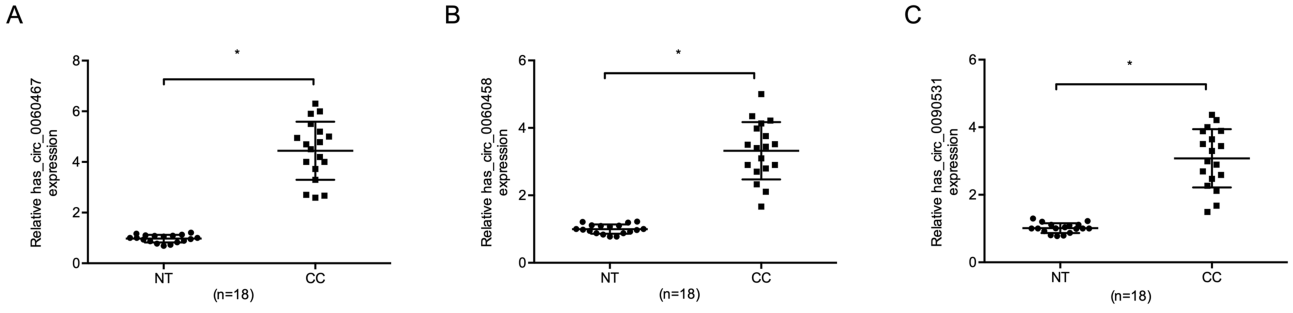

Figure 1 Dysregulated circRNAs in CC tissues. Relative expression in CC tissues and adjacent non-tumor tissues of (**A**) has_circ_0060467 (**B**) has_circ_0060458 and (**C**) has_circ_0090531. *P < 0.05.

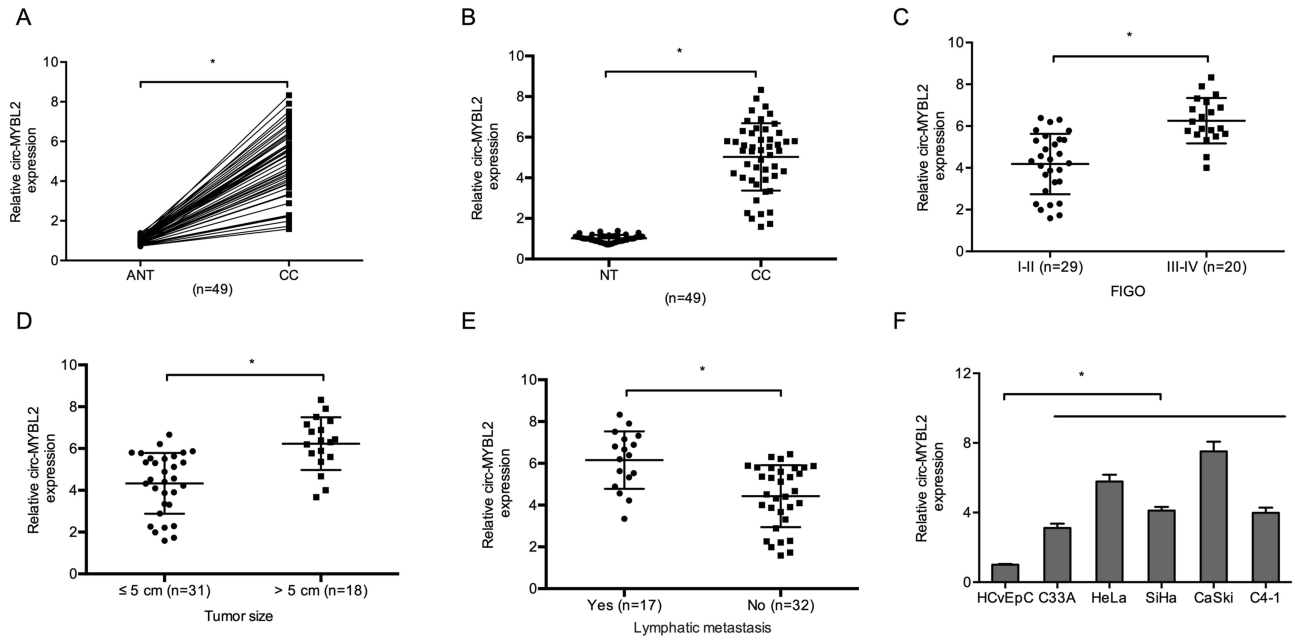

Figure 2 circ-MYBL2 was up-regulated in CC. (**A**, B) circ-MYBL2 expression was significantly increased in CC tissues. (**C**–**E**) High circ-MYBL2 expression was positively associated with advanced FIGO stage, larger tumor size, and lymph node metastasis in CC patients. (**F**) circ-MYBL2 expression was upregulated in CC cells. *P < 0.05.

significantly decreased in CC tissues and cell lines (Figure 4A and B). Low miR-361-3p expression was associated with advanced FIGO stage and lymph node metastasis in CC patients (Figure 4C and D). Furthermore, Kaplan-Meier assay revealed that low miR-361-3p expression was associated with poor overall survival rate in CC (Figure 4E).

Next, we explored whether circ-MYBL2 shared complementary binding sites with miR-361-3p by online software circinteractome. (Figure 5A). Luciferase reporter assay indicated that miR-361-3p expression dramatically reduced the luciferase activity of WT-circ-MYBL2 luciferase vector (Figure 5B). QRT-PCR showed that circ-MYBL2 inhibition induced miR-361-3p expression in CC cells (Figure 5C). RNA pull-down assay showed that miR-361-3p was enriched in cells by the circ-MYBL2 probe as compared to the control oligo probe (Figure 5D). In addition, we found that low miR-361-3p expression was negatively correlated with circ-MYBL2 expression in CC tissues (Figure 5E).

miR-361-3p Inhibitors Reversed The Effects Of circ-MYBL2 On CC Progression

Next, we explored the roles of miR-361-3p during the regulation of circ-MYBL2 in CC. CCK8 assay showed that miR-361-3p inhibitors abolished the proliferation capability caused by circ-MYBL2 inhibition in HeLa cells (Figure 6A). Transwell invasion assay suggested that the effects caused by circ-MYBL2 inhibition could be reversed by miR-361-3p inhibitors in HeLa cells (Figure 6B). Moreover, qRT-PCR and Western blot assays showed that down-regulation of miR-361-3p could reverse the anti-EMT effects of silencing circ-MYBL2 in HeLa cells (Figure 6C and D). Thus, we suggested that circ-MYBL2 could serve as a sponge for miR-361-3p promoting CC progression.

Discussion

Cervical cancer is a frequent gynecologic malignancy worldwide, thus it is necessary to find out the molecular targets so as to unearth the in-depth mechanisms for the improvement of CC.[16,17] Recently, increasing evidence showed that circRNAs play vital roles in the cancer biology,[18] including cervical cancer. For example, Hu et al showed that overexpressed circ_0067934 acted as an oncogene to facilitate CC progression via the miR-545/EIF3C axis.[19] Mao et al found that circEIF4G2 modulated the malignant features of CC via the miR-218/HOXA1 pathway.[20] In the present study, we validated that circ-MYBL2 was increased in CC tissues. Then, we focused on the correlation between circ-MYBL2 expression and the clinical features of CC patients, results showed that high circ-MYBL2 expression was associated

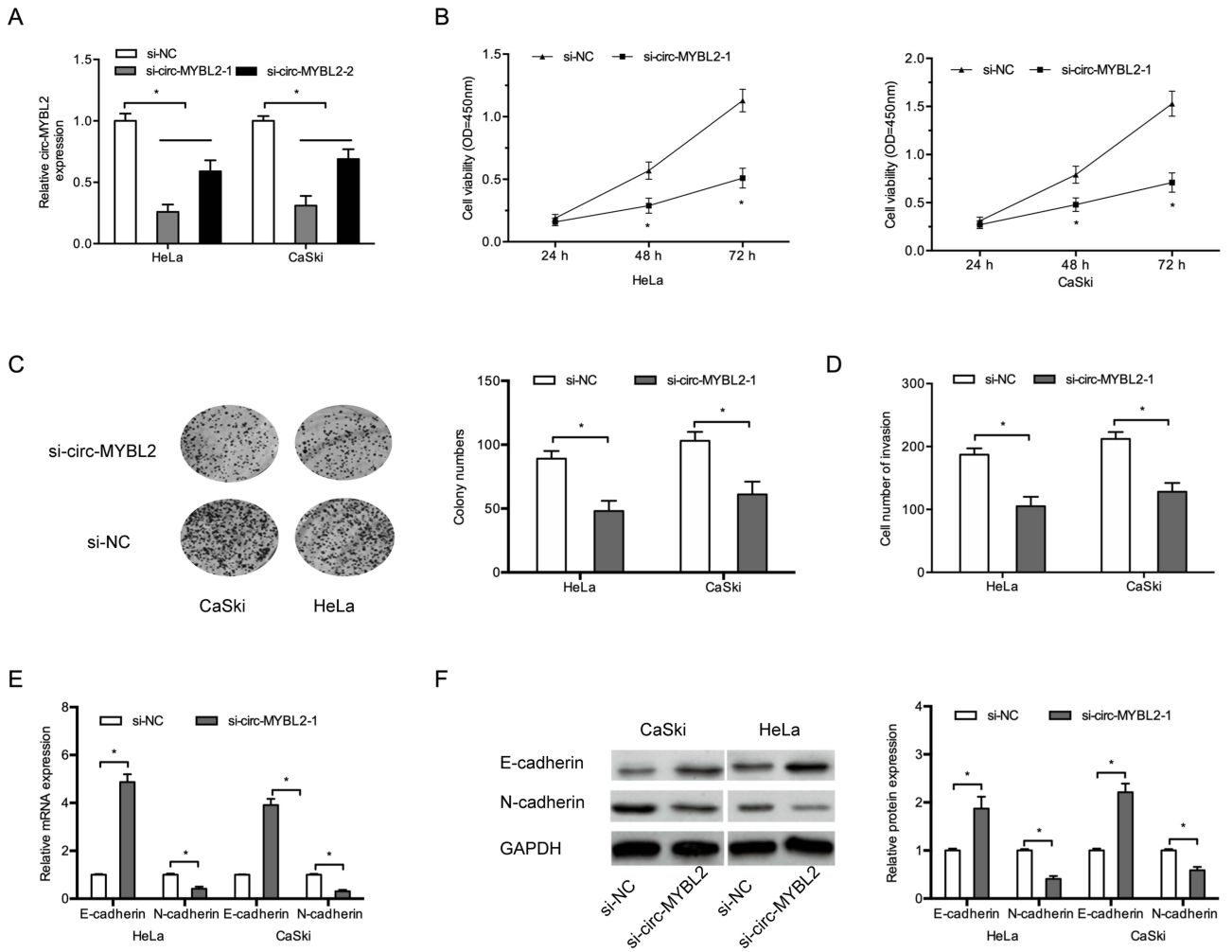

Figure 3 circ-MYBL2 inhibition reduced CC cells proliferation and invasion. (**A**) circ-MYBL2 expression in HeLa and CaSki cells transfected with si-circ-MYBL2 or si-NC. (**B, C**) CCK8 and colony formation assays showed that circ-MYBL2 inhibition significantly reduced CC cells viability. (**D**) Transwell invasion assay revealed that circ-MYBL2 inhibition suppressed CC cells invasion ability in vitro. (**E, F**) Relative expression of E-cadherin and N-cadherin in CC cells was detected by qRT-PCR and Western blot assay. *P < 0.05.

with advanced tumor stage, larger tumor size, lymph node metastasis in CC patients. In function assays, we showed that circ-MYBL2 depletion suppressed CC cells proliferation, invasion abilities and reduced EMT processes in vitro.

MicroRNAs (miRNAs) are a class of non-coding small RNAs of about 19–23 nucleotides in length, which are widely involved in various biological behaviors of cancer cells.[21,22] Studies showed that miRNAs were closely related to cell biological processes of CC cells, such as proliferation, apoptosis, invasion and angiogenesis.[23,24] Recently, miR-361-3p was found to play critical roles in tumor progression. For example, Chen et al showed that miR-361-3p reduced lung cancer cells proliferation and metastasis by targeting SH2B1.[25] Zhao et al suggested that miR-361-3p might function as a tumor suppressor in retinoblastoma by targeting hedgehog signaling.[26] Recently, Liu et al showed that miR-361-3p expression was significantly reduced and associated with advanced clinical features and poor prognosis in CC.[27] In the present study, we found that miR-361-3p expression was significantly decreased in CC tissues and cell lines. Low miR-361-3p expression was associated with advanced FIGO stage, lymph node metastasis and poor overall survival rate in CC patients, which consistent with the previous study.[27] In function assays, we showed that miR-361-3p inhibitors promoted CC cells proliferation, invasion and EMT processes.

Emerging evidence established that circRNAs could serve act as miRNA "sponge" affecting miRNA activity in tumor progression.[28] In the present study, we explored the regulatory functions of circ-MYBL2 at post-transcriptional level. Bioinformatic algorithms predicted that circ-MYBL2 shared complementary binding sites with miR-361-3p. Luciferase reporter assay showed that miR-361-3p mimics reduced the luciferase activity of WT-circ-MYBL2 group. circ-MYBL2

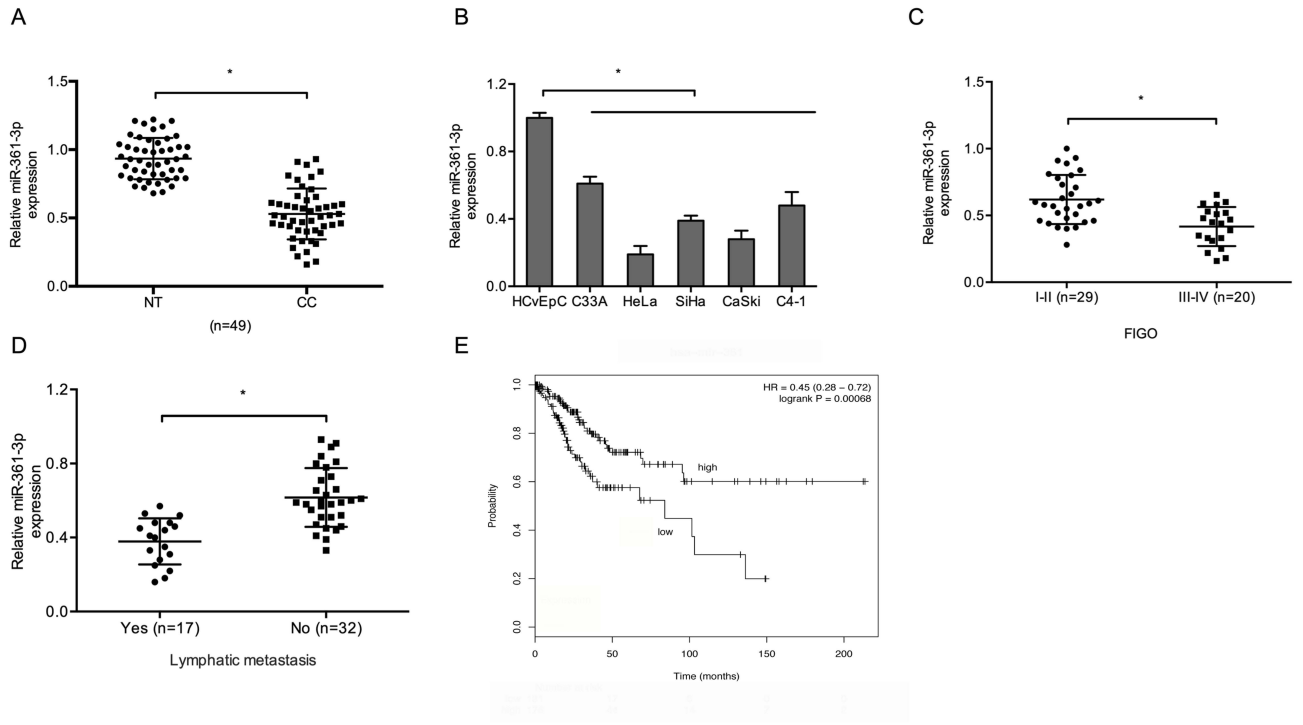

Figure 4 miR-361-3p was down-regulated in CC. (**A, B**) miR-361-3p expression was decreased in CC tissues and cell lines. (**C, D**) Low miR-361-3p expression was associated with advanced FIGO stage, and lymph node metastasis in CC patients. (**E**) Low miR-361-3p expression was associated with poor overall survival rate in CC patients. *P < 0.05.

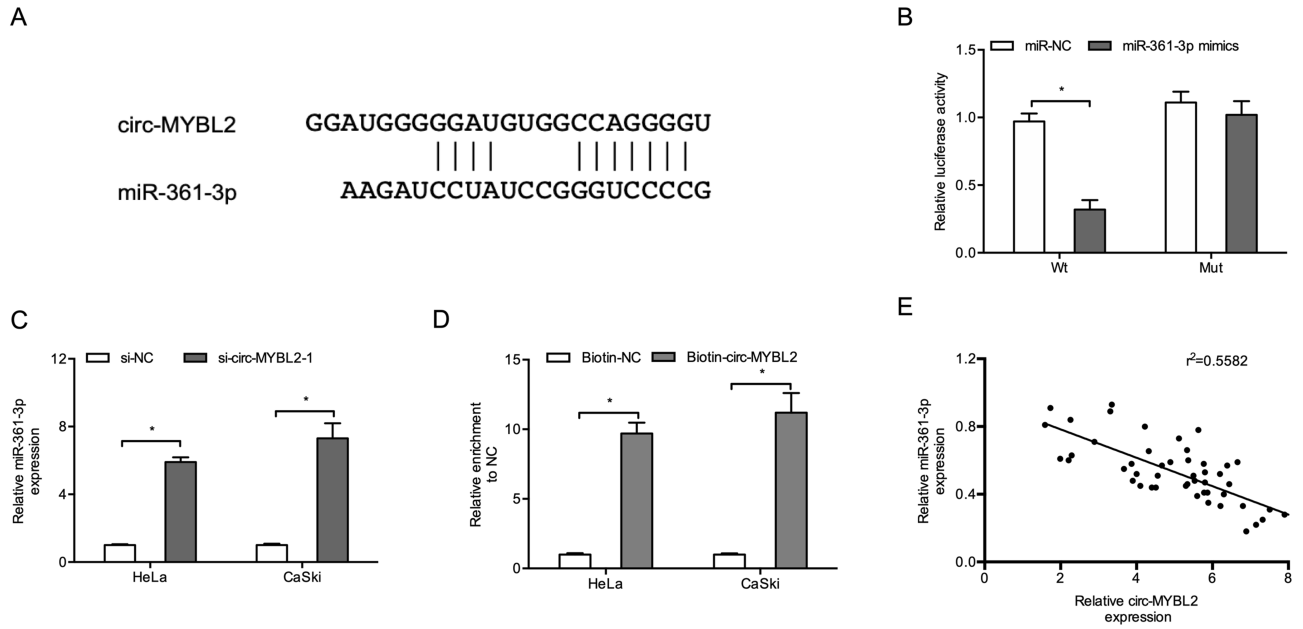

Figure 5 circ-MYBL2 directly interacted with miR-361-3p. (**A**) Binding site of circ-MYBL2 and miR-361-3p. (**B**) miR-361-3p mimics decreased the luciferase activity of circ-MYBL2-Wt. (**C**) circ-MYBL2 inhibition induced miR-361-3p expression in CC cells. (**D**) RNA pull-down assay showed that miR-361-3p was enriched in CC cells by circ-MYBL2 probe. (**E**) miR-361-3p expression was negatively correlated with circ-MYBL2 expression in CC tissues. *P < 0.05.

inhibition induced miR-361-3p expression in CC cells. Furthermore, RNA pull-down assay confirmed the correlation between miR-361-3p and circ-MYBL2 in CC. In addition, rescue assays showed that miR-361-3p inhibitors could reverse the effects of circ-MYBL2 inhibition on CC cells proliferation, invasion and EMT processes. Therefore, we suggested that

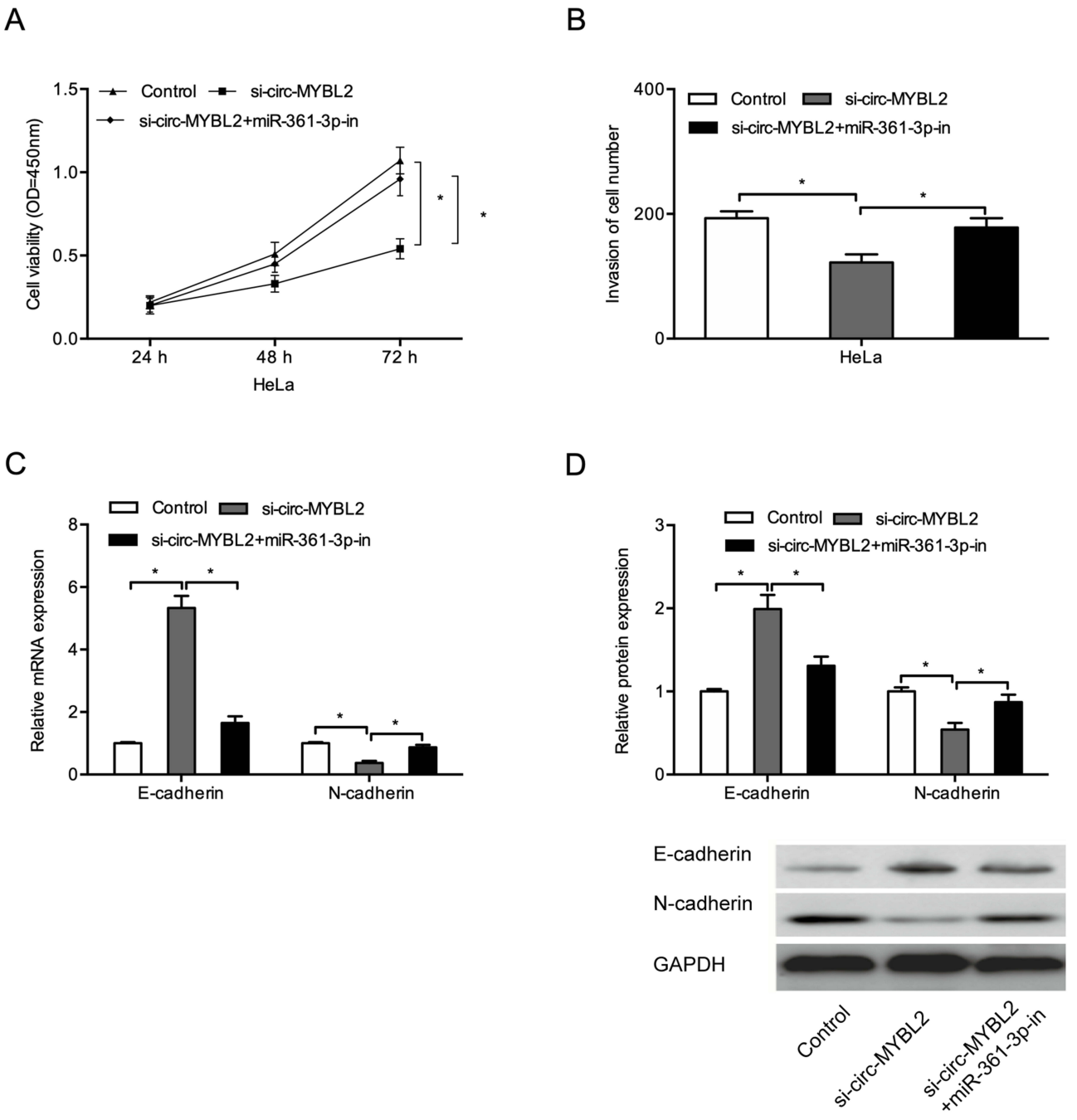

Figure 6 miR-361-3p inhibitors reversed the effect of silencing circ-MYBL2 on CC progression. (**A**) miR-361-3p inhibitors abolished the proliferation capability caused by circ-MYBL2 inhibition. (**B**) miR-361-3p inhibitors abolished the invasion ability caused by circ-MYBL2 suppression. (**C, D**) miR-361-3p inhibitors reversed the anti-EMT effects of circ-MYBL2 inhibition on CC cells. *P<0.05.

circ-MYBL2 might act as a oncogenic circRNA in CC progression through regulation of miR-361-3p.

Conclusion

We demonstrated an oncogenic role of circ-MYBL2 in CC progression by regulating miR-361-3p expression, which might provide a potential therapeutic target for the management of CC.

Availability Of Data And Materials

The dataset supporting the conclusions of this article is included within the article.

Acknowledgments

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Disclosure

All the authors declare that they have no conflicts of interest in this work.

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