

MiRNA-338-3p regulates cervical cancer cells proliferation by targeting MACC1 through MAPK signaling pathway

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Abstract. – OBJECTIVE: Aberrant expression of miR-338-3p has recently involved in the progression and development of various types of malignant tumors, but its role in the progression of cervical cancer remains unknown. This study aims to investigate the role of miR-338-3p/MACC1 axis in the progression of cervical cancer.

PATIENTS AND METHODS: MiR-338-3p and metastasis-associated in colon cancer 1 (MACC1) expression was determined in cervical cancer by quantitative real-time PCR (qRT-PCR). We explored the association of miR-338-3p expression with pathology and prognosis in cervical cancer patients. We explored the function of miR-338-3p and MACC1 on cell proliferation. A luciferase reporter assay was conducted to confirm the target gene of miR-338-3p in cervical cancer cells.

RESULTS: In the present work, our data showed that the expression of miR-338-3p was substantially decreased in cervical cancer tissues and associated with advanced FIGO stage, lymph node metastasis, depth of cervical invasion and poor overall survival. However, the MACC1 had an opposite expression. Mechanistically, we identified that MACC1 which acted as a functional downstream target for miR-338-3p. Furthermore, overexpression of miR-338-3p decreased expression of MACC1 in cervical cancer cells could significantly inhibit cervical cancer cell proliferation and induce cells apoptosis. Interestingly, miR-338-3p and MACC1 had proven to be involved in the progression of cervical cancer cells by regulating mitogen-activated protein kinase (MAPK) signaling pathway.

CONCLUSIONS: Our results suggested miR-338-3p/MACC1/MAPK regulatory pathway play an important role in the progression of cervical cancer.

Key Words:

miR-338-3p, MACC1, MAPK, Cervical cancer.

Introduction

Cervical cancer is the third most common cancer and the fourth leading cause of malignancy-related mortality in women worldwide¹. Although the incidence and mortality rates of cervical cancer have declined over the past 30 years, the 5-year survival rate of advanced-stage patients has remained below 40%^{2,3}. Therefore, an effective therapeutic strategy is urgently needed and further exploring the mechanism underlying is urgently required.

MicroRNA (miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses and functions in RNA silencing and post-transcriptional regulation of gene expression to play important roles in a wide scope of physiological and pathological processes^{4,5}. Recent investigations showed that miRNA could negatively modulate their matched target genes by binding to the 3'-untranslated regions (3'-UTRs) of mRNA, causing mRNA degradation or translational inhibition⁶. Therefore, miRNA could regulate multiple target genes expression, such as proliferation, invasion, apoptosis, migration, and differentiation^{7,8}. Recent studies showed that miR-338-3p which were considered a putative tumor suppressor miRNA in types of cancers. Although the potential benefits of miR-338-3p are proposed, the mechanism by which miR-338-3p suppressed cervical cancer is still unclear.

The mitogen-activated protein kinase (MAPK) signaling pathway is one of the most important cell signaling networks in tumorigenesis⁹. Aberrant activation of MAPK pathway induced by miRNAs has been observed in types of cancers. For example, Nakanishi et al¹⁰ found that loss of miR-125b-1 contributed to head and neck cancer development by dysregulating TACSTD2 and MAPK pathway. Xu et al¹¹ showed that miR-1 restrained epithelial-mesenchymal transition and metastasis of colorectal carcinoma via the MAPK and PI3K/AKT pathway. Van Jaarsveld et al¹² showed that miR-634 restored drug sensitivity in resistant ovarian cancer cells by targeting the Ras-MAPK pathway. Consistent with these findings, our results implied that the effect of miR-338-3p on cervical cancer might rely on the MAPK signaling pathway.

Metastasis-associated in colon cancer-1 (MACC1) is a transcription factor first described by Stein et al¹³ in 2009 to promote colorectal cancer metastasis via HGF/c-MET/MAPK pathway. Increasing evidence showed that MACC1 played important roles in tumor process. For example, Sun et al¹⁴ found that silence of MACC1 expression could inhibit proliferation, invasion, and metastasis in U251 human malignant glioma cells. Meng et al¹⁵ suggested that MACC1 inhibition reduced proliferation and tumorigenicity of nasopharyngeal carcinoma cells through Akt/ β -Catenin pathway. Li et al¹⁶ found that miR-433 reduced cell viability and promoted cell apoptosis by regulating MACC1 in colorectal cancer. However, until now, the association between miR-338-3p and MACC1 in cervical cancer progression remains unclear.

Patients and Methods

Clinical Samples

Paired cervical cancer and adjacent normal tissues were obtained from 67 patients were obtained from the First Affiliated Hospital of Xinxiang Medical University between 2010 and 2011. The categorization of the clinical samples was confirmed by pathological analysis. No patients had received any form of tumor-specific therapy before diagnosis. Informed consent was obtained from each patient recruited, and the study protocol was approved by the institutional Ethics Committee of First Affiliated Hospital of Xinxiang Medical University. All specimens were immediately frozen in tubes containing RNAlater preservation li-

quid after removal and stored at -80°C until RNA extraction. The results for the clinical information are presented in Table I.

Cell Culture and Transfection

Human cervical cancer cell lines (HeLa, CaSki) were purchased from the Cell Bank of the Chinese Academy of Sciences (CAS, Shanghai, China). HeLa was grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA). CaSki was grown in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Invitrogen, Carlsbad, CA, USA). The medium was supplemented with 10 % fetal bovine serum (FBS, Hyclone, Logan, UT, USA). All cells were grown in at 37°C in a humidified 5 % CO₂. Stable cell lines were derived in HeLa cells by transfecting with miR-338-3p mimics, MACC1 small interfering (si-MACC1), and negative controls were obtained from GenePharma (Shanghai, China), and transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Counting Kit-8 Assay

Cell proliferation was determined by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, transfected cells (5×10^3 cells/well) were seeded into 24-well plates. At indicated time points (24 h, 48 h, 72 h), the cells were incubated in 10% CCK-8 solution in normal culture medium at 37°C until visual color conversion occurred. The absorbance at 450 nm was measured with a microplate reader (Thermo Scientific, Waltham, MA, USA).

Cell Apoptosis Analysis

After 48 h transfection, cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS) twice. Then the cells were resuspended in binding buffer and stained with Annexin V and propidium iodide (PI) for 15 min in the dark at room temperature, according to the manufacturer's recommendations. The stained cells were examined by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were categorized into early apoptotic cells, late apoptotic cells, dead cells, and viable cells. The relative ratio of early apoptotic cells was counted for further comparisons.

Dual-Luciferase Reporter Assay

Cells were seeded into 24-well plates and co-transfected with 200 ng of pMIR-MACC1-Wt or pMIR-MACC1-Mut vector and 100 ng of miR-

338-3p mimics or mimic control, and the pRL-TK plasmid (Promega, Madison, WI, USA) which was used for internal normalization. After 48 h, HEK-293 cells were lysed using the lysis buffer (Promega, Madison, WI, USA). Luciferase reporter gene assay was implemented using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. All experiments were performed at least three times.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from tissues or cultured cells using a Trizol kit (Invitrogen Co, Carlsbad, CA, USA) according to the manufacturer's instructions and then reverse transcribed using Takara RT reagent (TaKaRa, Dalian, Liaoning, China). Expression of candidate genes was normalized to that of GAPDH. Quantitative real-time PCR was performed using ABI 7500 system (Biosystems, Foster City, CA, USA). The primer sequences were listed: miR-338-3p (F: GCCGATCCAGCATCAGTG, R: CAGTGCAGGGTC-CGAGGT); MACC1 (F: ATCCGCCACACATGCTTAA, R: CTTTCAGCCCCAATTTTCATC); GAPDH (F: ACCCAGAAGACTGTGGATGG, R: TCTAGACGGCAGGTCAGGTC).

Western Blot

Proteins were isolated from tissues by lysing frozen tissues in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis,

MO, USA). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes (Millipore, Billerica, MA, USA) at 80 V for 2 h at 4°C. After blocking in 5% nonfat dry milk in Tris-buffered saline (TBS), the membranes were incubated with primary antibodies overnight at 4°C, washed three times with TBS, and then incubated with secondary antibodies conjugated with horseradish peroxidase in TBS for 2 h at room temperature. Membranes were washed again in TBS for three times at room temperature. The proteins were visualized by an ECL (enhanced chemiluminescence) detection kit (Amersham Biosciences, Buckinghamshire, UK).

Statistical Analysis

All statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The data are expressed as the mean±standard deviation from at least three separate experiments. Differences were tested for significance using ANOVA and Dunnett's post-hoc tests. $p < 0.05$ was depicted as statistical significance.

Results

MiR-338-3p Expression Was Decreased in Cervical Cancer

To explore the function of miR-338-3p in cervical cancer progression, we detected miR-338-

Table I. Correlation between miR-338-3p expression and clinicopathological features of cervical cancer patients.

Clinicopathological features	Total	miR-338-3p expression		p-value
		Low	High	
<45	29	16	13	0.397
≥45	38	17	21	
Tumor size (cm)				0.067
<4.0	31	19	12	
≥4.0	36	14	22	0.100
Histologic grade				
G1 + G2	40	23	17	0.002
G3	27	10	17	
FIGO stage				0.003
Ib-IIa	28	20	8	
I Ib-IIIa	39	13	26	0.016
Lymph node metastasis				
No	50	30	20	0.016
Yes	17	3	14	
Depth of cervical invasion				0.016
<2/3	41	25	16	
≥2/3	26	8	18	

3p expression in human cervical cancer tissues and adjacent non-tumor tissues by qRT-PCR. Results showed that miR-338-3p was significantly downregulated in cervical cancer tissues (Figure 1A, $p<0.05$). Furthermore, we explored the associations between miR-338-3p expression and patients' clinicopathological features. According to the median value of miR-338-3p expression level, patients were divided into low miR-338-3p expression group ($n=33$) and high miR-338-3p expression group ($n=34$). As shown in Table I, the miR-338-3p expression was significantly correlated with FIGO stage, lymph node metastasis and depth of cervical invasion. Kaplan-Meier analysis and log-rank test showed that patients with low miR-338-3p expression had shorter overall survival than those with high miR-338-3p expression (Figure 1B, $p<0.05$). These data indicated that miR-338-3p play critical roles in cervical cancer development.

MACC1 Expression Was Inversely Correlated with miR-338-3p Expression in Cervical Cancer

To determine the role of MACC1 in cervical cancer, we detected MACC1 mRNA expression in cervical cancer tissues and adjacent non-tumor tissues by qRT-PCR. Results showed that MACC1 mRNA expression was significantly increased in cervical cancer tissues (Figure 2A, $p<0.05$). Also, we explored MACC1 protein expression in 3 pairs of cervical cancer. The results showed the expression of MACC1 was significantly upregulated in

cervical cancer tissues (Figure 2B, $p<0.05$). Moreover, correlation analysis showed that MACC1 mRNA expression was negatively correlated with miR-338-3p expression in cervical cancer tissues (Figure 2C, $p<0.05$). Thus, the elevation of MACC1 expression and the reducing of miR-338-3p expression might play important roles in the formation of cervical cancer.

MiR-338-3p Inhibited Cervical Cancer Cell Proliferation and Induced Cells Apoptosis

To investigate the function of miR-338-3p in cervical cancer progression. HeLa cells were transfected with miR-338-3p mimics, and miR-NC and the efficiency were determined by qRT-PCR (Figure 3A, $p<0.05$). The CCK-8 assay showed that miR-338-3p mimics significantly decreased HeLa cells proliferation (Figure 3B, $p<0.05$). Then, we explored whether the effects of miR-338-3p on cell proliferation were regulated by cell apoptosis. Flow cytometric analysis showed that miR-338-3p mimics induced HeLa cells apoptosis (Figure 3C, $p<0.05$). Previous studies revealed that cleaved caspase3, Bax, and Bcl-2 were involved in apoptosis process²⁰. Thus, in the present work, we detected the effect of miR-338-3p on caspase 3, Bax, and Bcl-2. Western blot showed that miR-338-3p mimics significantly increased expression of Bax, cleaved caspase3 and decreased expression of Bcl-2 in HeLa cells (Figure 3D, $p<0.05$). These results suggested that miR-338-3p inhibited cell proliferation by induced the apoptosis of cervical cancer cells.

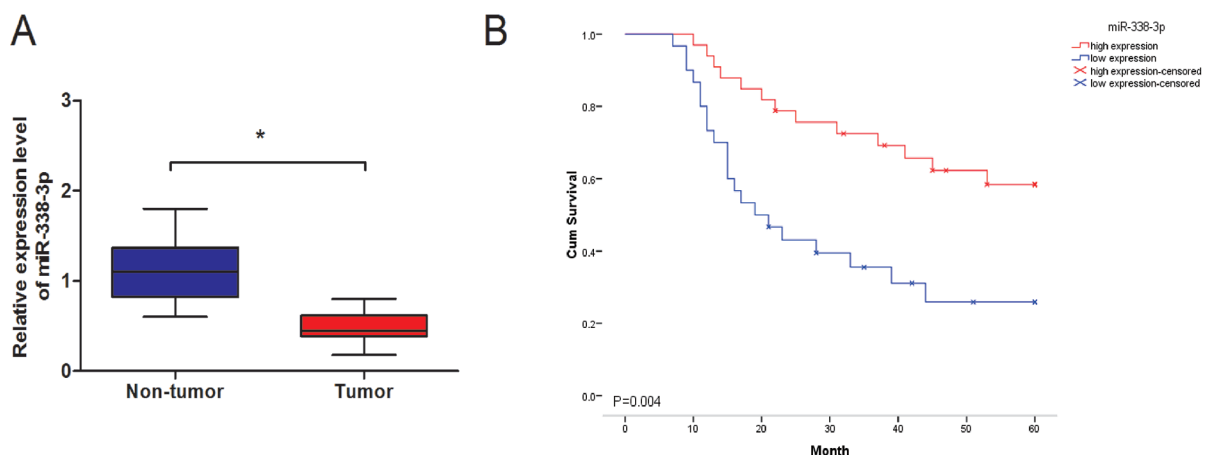


Figure 1. MiR-338-3p expression was downregulated and associated with poor overall survival in cervical cancer patients. (A) MiR-338-3p expression was significantly decreased in cervical cancer tissues compared to adjacent non-tumor tissues. (B) Low miR-338-3p expression was associated with poor overall survival of cervical cancer patients. * $p<0.05$.

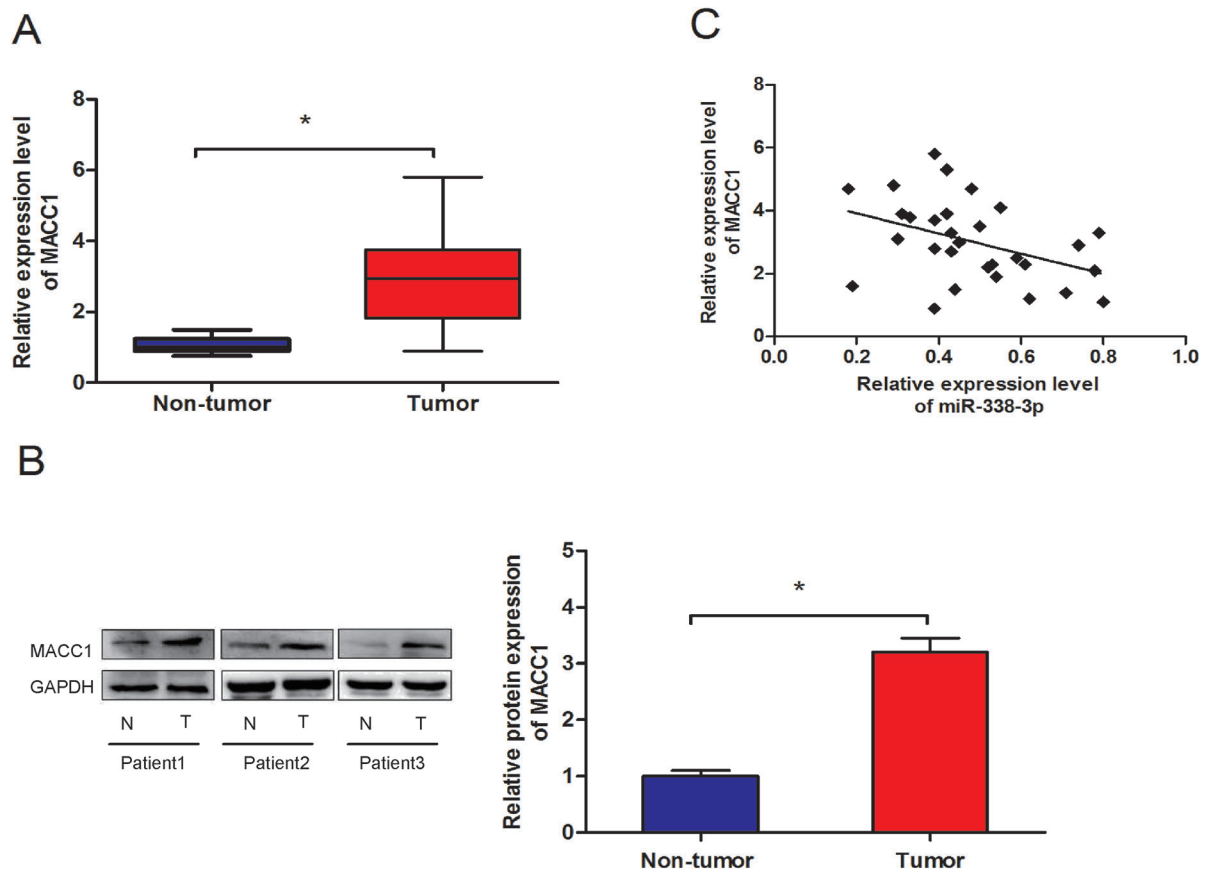


Figure 2. MACC1 expression in cervical cancer tissue. (A) qRT-PCR showed that MACC1 expression was significantly increased in cervical cancer tissues compared to adjacent non-tumor tissues. (B) Western blot showed that MACC1 expression was upregulated in cervical cancer tissues. (C) Inverse correlation between miR-338-3p expression and MACC1 expression in cervical cancer tissues. $*p < 0.05$.

MACC1 was Directly Targeted by miR-338-3p

MiRNAs regulated the target gene expression through the post-transcriptional mechanisms. Thus, TargetScan was utilized together to dig the possible target of miR-338-3p in cervical cancer. As shown in Figure 4A, bioinformatics predicted an interaction between miR-338-3p and the target sites in the MACC1 3'-UTR. Luciferase activity assay showed that cells transfected with miR-338-3p mimics significantly inhibited wild-type MACC1 3'-UTR reporter activity, while had no inhibitory effect on the mutant-type MACC1 3'-UTR reporter activity (Figure 4B, $p < 0.05$). In addition, the mRNA and protein expression of MACC1 were detected in HeLa and CaSki cells transfected miR-338-3p mimics or miR-NC. Results showed that miR-338-3p overexpression significantly attenuated the mRNA and protein levels of MACC1 (Figure 4C and 4D, $p < 0.05$). Ta-

ken together, our results suggested that MACC1 was a target of miR-338-3p.

Downregulation of MACC1 Suppressed Cervical Cancer Cell Proliferation and Induced Cells Apoptosis

To explore the role of MACC1 in cervical cancer cells, we transfected MACC1 small interfering RNA (si-MACC1) into CaSki cells (Figure 5A, $p < 0.05$). The CCK-8 assay showed that MACC1 inhibition significantly suppressed CaSki cells proliferation (Figure 5B, $p < 0.05$). Cell apoptosis assay revealed that knocked down of MACC1 increased CaSki cells apoptosis (Figure 5C, $p < 0.05$). Furthermore, Western blot showed that MACC1 suppression significantly increased expression of Bax, cleaved caspase3 and decreased expression of Bcl-2 in CaSki cells (Figure 5D, $p < 0.05$). These results indicated that MACC1 inhibition could suppress cervical cancer progression.

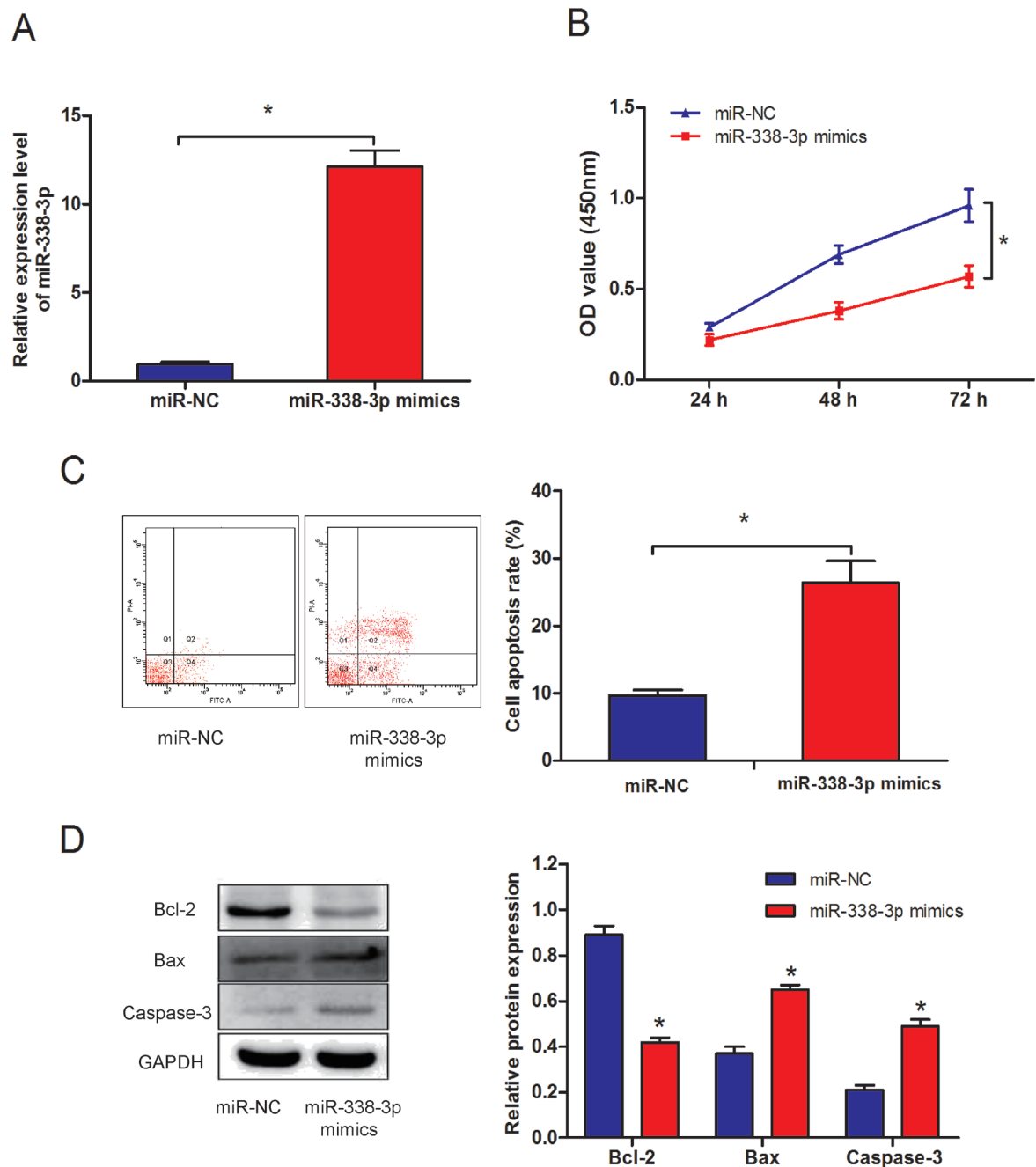


Figure 3. MiR-338-3p mimics suppressed cervical cancer cells proliferation and induced cell apoptosis. **(A)** Cells were transfected with miR-338-3p mimics or miR-NC, and overexpression efficiency was determined by qRT-PCR. **(B)** CCK-8 assay showed that miR-338-3p mimics decreased HeLa cells proliferation. **(C)** Cell apoptosis assay showed that miR-338-3p mimics induced HeLa cells apoptosis. **(D)** Western blot showed that miR-338-3p mimics significantly increased expression of Bax, cleaved caspase3 and decreased expression of Bcl-2 in HeLa cells. * $p < 0.05$

MiR-338-3p Regulated MAPK Pathway by Targeting MACC1

A previous study¹⁷ showed that MACC1 was involved in the regulation of MAPK pathway. However, whether miR-338-3p could regulate MAPK pathway by regulating MACC1 is still unclear. In the

present work, we showed that the protein expression of p38 and Erk1/2 were significantly downregulated in HeLa cells transfected with miR-338-3p mimics (Figure 6A). To further explore whether the effect of miR-338-3p on MAPK pathway was related to MACC1, we transfected CaSki cells with

si-MACC1 and detected the protein expression of p38 and Erk1/2. As expected, our data showed that the protein expression of p38 and Erk1/2 were decreased in cells transfected with si-MACC1 (Figure 6B). Taken together, these data suggested that the negative regulation of miR-338-3p on MAPK signaling pathway partly through regulating MACC1 expression.

Discussion

Aberrant expression of miRNAs is closely related to tumor genesis and has recently been thought to contribute or attenuate the pathophysiology of cervical cancer¹⁸, and several miRNAs have been shown to regulate the activation of cervical cancer cells. Recent studies showed that miR-338-

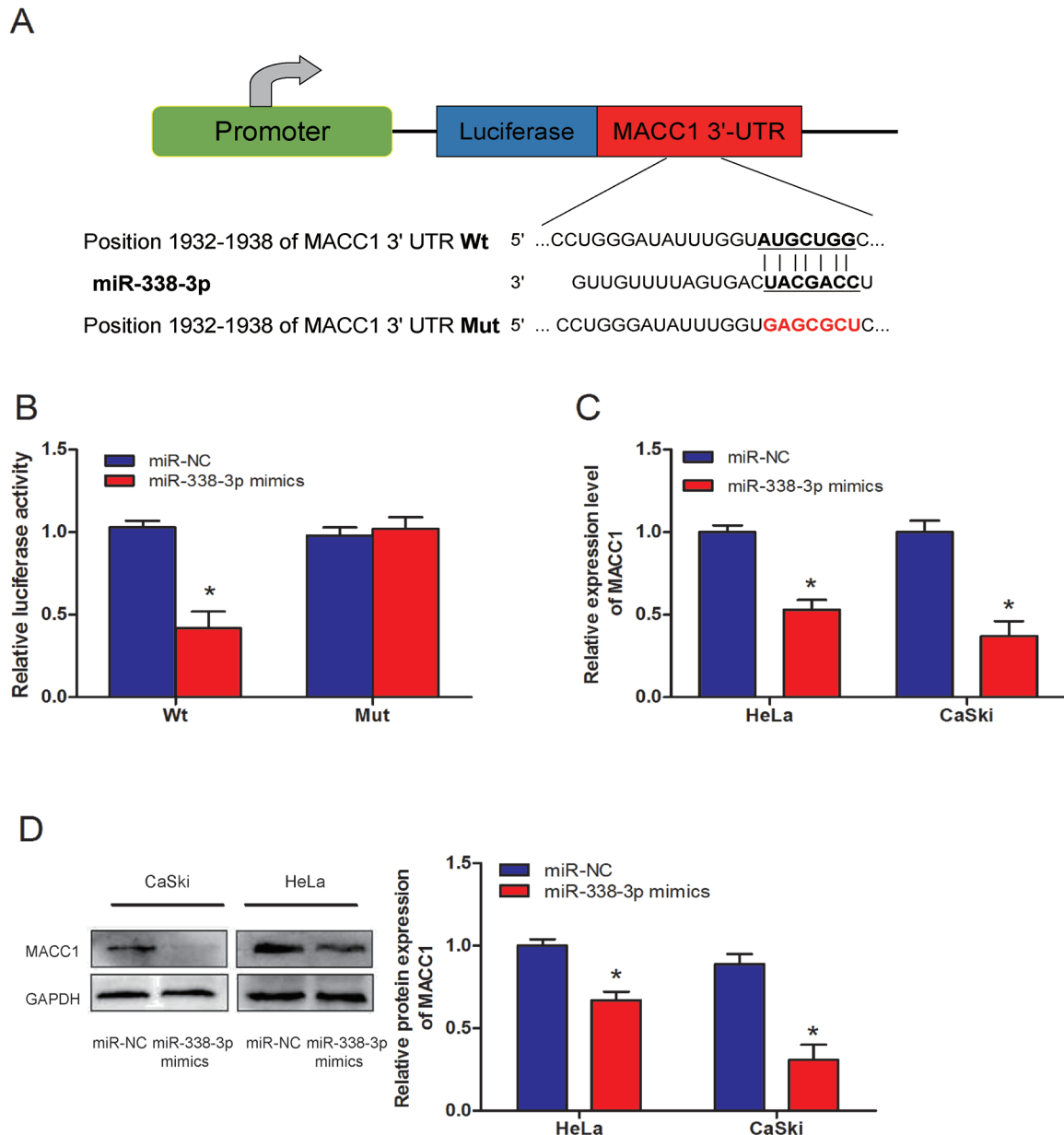


Figure 4. MACC1 is a direct target of miR-338-3p. **(A)** The potential miR-338-3p seed region in the 3'-UTR of MACC1 mRNA was computationally predicted using TargetScan. **(B)** Luciferase activity assay showed that miR-338-3p suppressed Wt MACC1 3'-UTR luciferase activity in HEK-293 cells. **(C)** QRT-PCR showed that MACC1 mRNA expression was decreased in cervical cancer cells transfected with miR-338-3p mimics. **(D)** Western blot revealed that MACC1 protein expression was decreased in cervical cancer cells transfected with miR-338-3p mimics. * $p < 0.05$

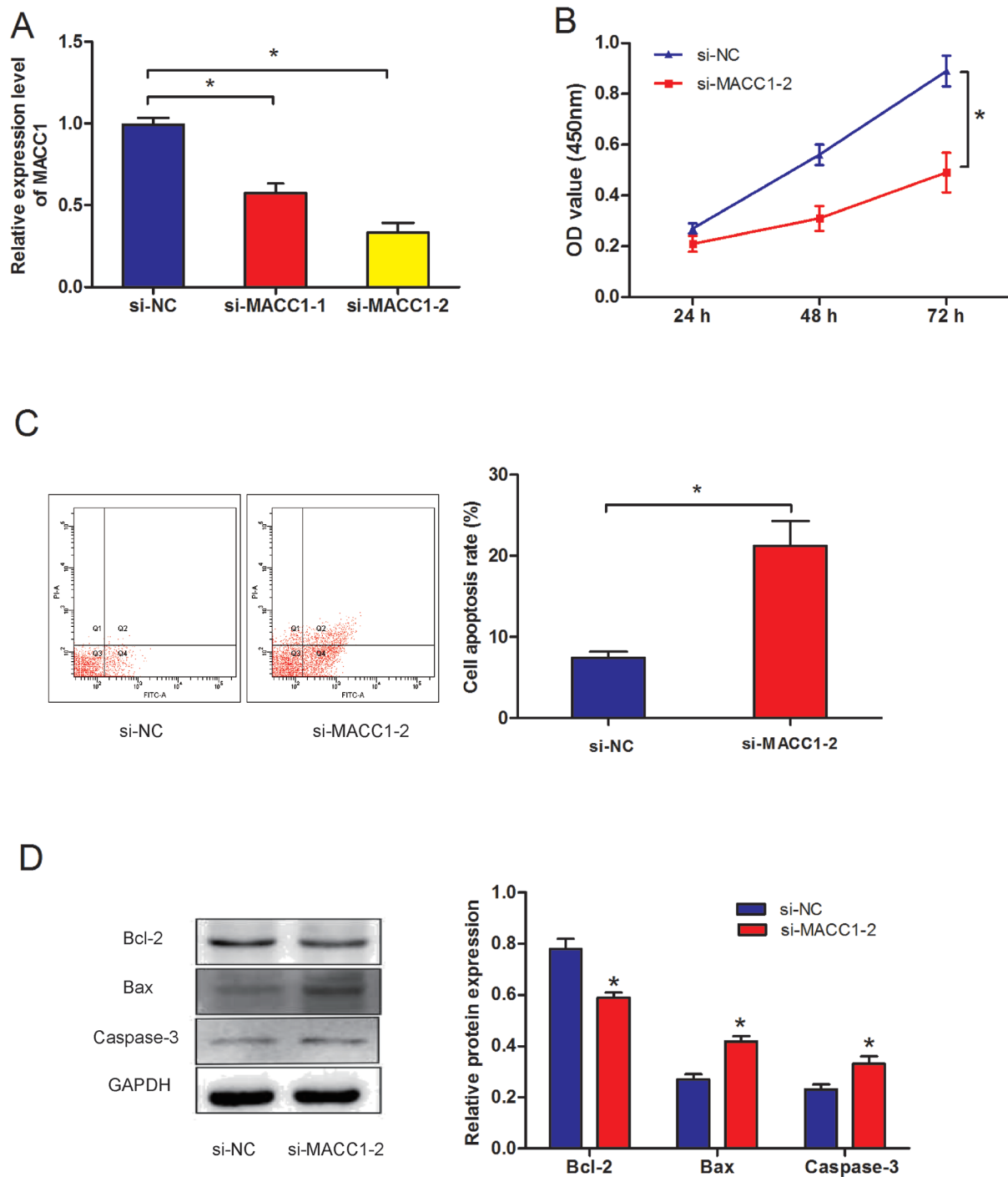


Figure 5. MACC1 inhibition reduced cervical cancer cell proliferation and induced cell apoptosis. (A) CaSki cells were transfected with si-MACC1 or si-NC, and downregulation efficiency was determined by qRT-PCR. (B) CCK-8 assay showed that MACC1 inhibition suppressed CaSki cells proliferation. (C) Cell apoptosis assay indicated that MACC1 suppression induced CaSki cells apoptosis. (D) Western blot found that silencing MACC1 significantly increased expression of Bax, cleaved caspase3 and decreased expression of Bcl-2 in CaSki cells. * $p < 0.05$

3p played important roles in tumor progression. For example, Zhang et al¹⁹ showed that miR-338-3p inhibited the growth and invasion of non-small

cell lung cancer cells by targeting IRS2. Zhang et al²⁰ suggested that miR-338-3p targeted pyruvate kinase M2 and affected cell proliferation and me-

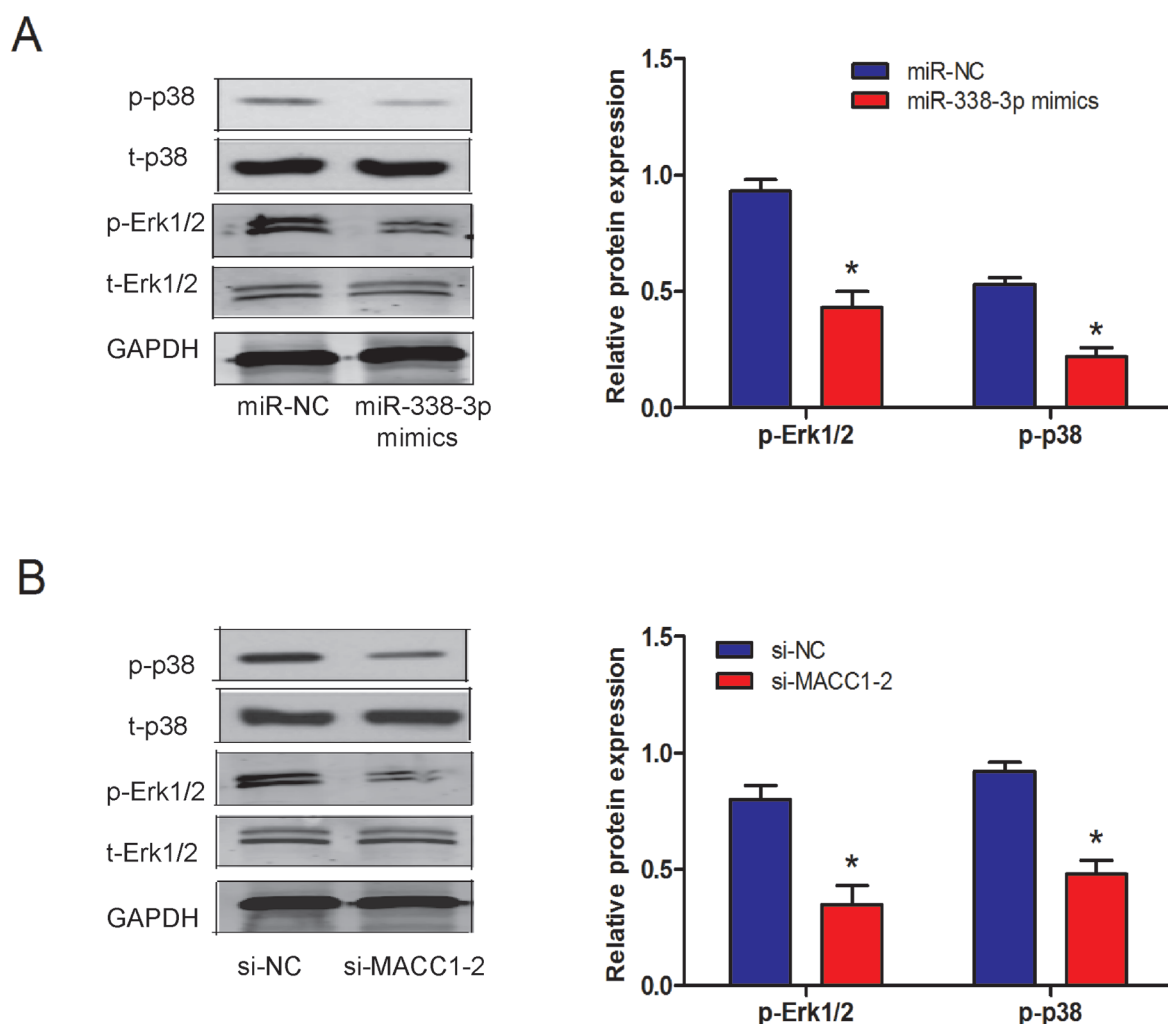


Figure 6. MiR-338-3p regulated MAPK signaling pathway by targeting MACC1. **(A)** The protein expression of p38 and Erk1/2 in cervical cancer cells was determined by Western blot. **(B)** Western blot analyzed MAPK signaling pathway related proteins in cervical cancer cells transfected with si-MACC1 or si-NC. * $p < 0.05$

tabolism of ovarian cancer. Chen et al²¹ showed that miR-338-3p inhibited the proliferation and migration of gastric cancer cells by targeting ADAM17. However, the expression and function of miR-338-3p in cervical cancer are still unclear. In the present research, we showed that miR-338-3p expression was significantly decreased and correlated with FIGO stage, lymph node metastasis, depth of cervical invasion, and poor overall survival of cervical cancer patients. *In vitro* assay results showed that miR-338-3p mimics could inhibit cervical cancer cell proliferation by inducing cell apoptosis. Those findings suggested that miR-338-3p could act a tumor suppressor miRNA in cervical cancer progression.

Increasing evidence showed that MACC1 played important roles in cervical cancer progression.

For example, Zhou et al²² showed that MACC1 was associated with poor prognosis and promoted cell invasion and angiogenesis in human cervical cancer. Our previous investigation²³ showed that MACC1 downregulation suppressed cell proliferation and invasion ability of cervical cancer cells. However, the underlying mechanism of MACC1 on cervical cancer is still unclear. Here, we presented strong evidence that miR-338-3p could inhibit the expression of MACC1 by directly targeting to the 3'-UTR of MACC1, and there was an inverse correlation between miR-338-3p expression and MACC1 expression in cervical cancer tissues. In addition, we showed that MACC1 was increased in cervical cancer tissues and negatively regulated by alteration of miR-338-3p on the mRNA and protein expression level. Silencing of MACC1 suppressed

cervical cancer cell proliferation and induced cell apoptosis. These data indicated that MACC1 was involved in miR-338-3p mediated cervical cancer progression.

Recently, several studies focused on the effect of the MAPK signaling pathway in cervical cancer cells, which may have plasticity and respond to signals from their microenvironment²⁴. Thus, in the present investigation, we explored whether miR-338-3p regulated MAPK pathway by targeting MACC1 in cervical cancer. Western blot showed that the protein expression of p38 and Erk1/2 were significantly downregulated in cells transfected with miR-338-3p mimics, indicating miR-338-3p could suppress the MAPK pathway in cervical cancer. Furthermore, Western blot revealed that inhibition of MACC1 showed a similar effect with miR-338-5p overexpression on MAPK pathway. Taken together, our study suggested that the negative regulation of miR-338-3p on MAPK signaling pathway partly by regulating MACC1 expression in cervical cancer.

Conclusions

Results showed that miR-338-3p inhibited cervical cancer cell proliferation and induced cell apoptosis through downregulating MACC1 expression. Our findings suggested that miR-338-3p-MACC1-MAPK axis might represent a novel therapeutic application in the treatment of cervical cancer.

Conflict of interest

The authors declare no conflicts of interest.

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