MIR-203A-5P SUPPRESSES CERVICAL CANCER CELL TUMORIGENESIS AND EMT VIA TARGETING MAPK1

Haiyan Wang[#], Xia Song[#], Qinghua Zhang, Chun Zhang, Yalan Li, Ying Chen, Weina Du, Zhuoqun Zhang, Liwen Jin[*]
Department of gynecology, The Central Hospital of Wuhan Affiliated to Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, China
[#]*These authors contributed equally to this work*

ABSTRACT

Introduction: *Cervical cancer (CC) is the fourth most prevalent malignancy worldwide. CC is usually asymptomatic in initial stage, if the treatment is not timely, it can be life-threatening. MiR-203 was downregulated in CC tissues, and a potential biomarker for the early stages of CC. In this study, the role and underlying mechanisms of miR-203a-5p in CC cells were investigated.*

Case report: *The expressions of miR-203a-5p and mitogen-activated protein kinase 1 (MAPK1) in CC tissue and cells were measured by real time reverse transcription polymerase chain reaction (RT-qPCR). The protein levels of MAPK1 and epithelial to mesenchymal transition (EMT)-related proteins (E-cadherin, N-cadherin, Vimentin and Snail) in CC cells were detected by western blot assay. The effects of miR-203a-5p and MAPK1 on proliferation, migration, invasion and apoptosis were analyzed by cell counting kit-8 (CCK-8), transwell and flow cytometry assays, respectively. MiR-203a-5p-MAPK1 binding interaction was predicted and demonstrated by Targetscan and dual luciferase reporter assays, respectively. MiR-203a-5p was downregulated, and MAPK1 was upregulated in CC tissues and cells. MiR-203a-5p suppressed proliferation, migration, invasion, and induced apoptosis of CC cells. MiR-203a-5p elevated the protein level of E-cadherin and attenuated the protein levels N-cadherin, Vimentin and Snail in CC cells. MAPK1 was demonstrated as a target of miR-203a-5p. MiR-203a-5p retarded proliferation, migration, invasion, EMT, and boosted apoptosis of CC cells through targeting MAPK1.*

Conclusion: *MiR-203a-5p could suppress CC cell proliferation, migration, invasion, EMT, and boosted apoptosis by regulating MAPK1 expression, hinting that miR-203a-5p is a potential prognostic biomarker and therapeutic target for CC.*

Keywords: *miR-203a-5p, MAPK1, cervical cancer, proliferation, migration, invasion, apoptosis, EMT.*

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Introduction

Cervical cancer (CC) is the fourth most prevalent malignancy worldwide and has the highest mortality rates in women, with an approximated 570,000 new cases and 311,000 deaths in 2018 worldwide[1]. Despite the great progress in early detection and treatment, CC is usually asymptomatic in initial stage[2]. Therefore, it is imperative and urgent to identify new accurate biomarker and molecular mechanisms for predicting the prognosis of CC.

MicroRNAs (miRNAs) are small non-coding RNA molecules of 19-22 nts in length, which modulate gene expression by inhibiting translation of mRNAs[3].

Mounting evidence suggests that miRNAs function as oncogenes or tumor suppressors in various cellular processes, including proliferation, invasion, migration and apoptosis[4,5]. Moreover, the dysregulation of miRNAs has been linked to the tumor formation and tumorigenesis in diverse tumors, such as CC[6,7]). MiR-203 is located in the chromosome 14q32.33 and acts as a potential biomarker for the early stages of CC[8]. A prior report showed that miR-203 expression was downregulated in CC tissues[9]. Furthermore, Zhu et al. had also confirmed that high expression of miR-203 could block tumor growth and angiogenesis via regulating VEGFA in CC[10].

However, the exact roles and underlying mechanisms of miR-203a-5p in CC is still unclear. Mito-

gen-activated protein kinase 1 (MAPK1), an extracellular signal-regulated kinases, has been reported to be involved in multiple cellular processes, such as proliferation, apoptosis and epithelia-mesenchymal transition (EMT)[11-13]. Moreover, MAPK1 was found to be upregulated in CC tissues and knocking-down of MAPK1 hindered cell proliferation, migration and invasion in CC[14]. These results suggested that MAPK1 plays a vital role in the development of CC.

EMT is the biological process that epithelial cells lose polarity and reduced adhesion, which led to the higher abilities of migration, invasion, anti-apoptosis and extracellular matrix degradation in epithelial cells[15, 16]. In other words, EMT is the transformation of epithelial cells into mesenchymal phenotype cells. Previous study verified that E-cadherin is the epithelial marker, while N-cadherin, Vimentin and Snail are the mesenchymal markers[17]. Moreover, some reports have demonstrated miRNAs were closely associated with EMT in CC[18, 19]. But the effect of miR-203a-5p expression on EMT of CC remains unknown. Hence, in this paper, we aimed to explore the function of miR-203a-5p and to elucidate the molecular mechanisms of miR-203a-5p in proliferation, migration, invasion, apoptosis and EMT of CC.

Materials and methods

Tissue samples and cell culture

CC tumor tissues samples (n=48) and adjacent normal tissues were acquired from CC patients undergoing surgery at The Central Hospital of Wuhan Affiliated to Tongji Medical College. This research was carried out with the approval of The Central Hospital of Wuhan Affiliated to Tongji Medical College Ethics Committee and informed consent was signed by every participant.

Human keratinocytes cell line HaCaT was obtained from CLS Cell Lines Service GmbH (EppeIheim, Germany) and human CC cell lines (HeLa, C33A, CaSki, SiHa and HT-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Invitrogen, CarIsbad, CA, USA) at 37°C in a cell incubator with an atmosphere of 5% CO_2.

Cell transfection

The cDNA sequence of MAPK1 was sucloned into pcDNA3.1 empty vector (Invitrogen) to synthesize overexpression plasmid pcDNA3.1-MAPK1. MiR-203a-5p mimic and scrambled mimics control (miR-NC mimic) were purchased from GeneCopoeia (Rockville, MD, USA). All these plasmids or oligomers were transfected into CaSki and SiHa cells by Lipofectamine 2000 reagent (Invitrogen) in line with the instruction of manufacturer.

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from CC tissues and cells by TRIzol reagent (Invitrogen) based on the manufacturer's protocol. Briefly, RNA reverse transcription assay was carried out to synthesize cDNA first strand using M-MLV reverse transcriptase (Themo Fisher Scientific, Rockford, IL, USA). Then, the relative expression level miR-203a-5p was assessed by All-in-One™ miRNA RT-qPCR Detection Kit (GeneCopoeia) and the quantitative analysis of MAPK1 mRNA was carried out with SYBR® Premix Ex Taq™ reagent (TaKaRa, Tokyo, Japan). GAPDH or U6 snRNA were acted as the endogenous control to normalize the expression of MAPK1 or miR-203a-5p. The relative level of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of primers miR-203a-5p, U6, MAPK1 and GAPDH were listed as below: MiR-203a-5p: 5'-GTGAAATGTTTAGGACCACTAG-3' (sense), 5'-AGTGGTCCTAAACATTTCACTT-3' (antisense); U6: 5'-CTCGCTTCGGCAGCACA-3' (sense), 5'-AACGCTTCACGAATTTGCGT-3' (antisense); MAPK1: 5'-TGGATTCCCTGGTTCTCTCTAAAG-3' (sense), 5'-GGGTCTGTTTTCCGAGGATGA-3' (antisense); GAPDH: 5'-AGAAGGCTGGGGCTCATTTG-3' (sense), 5'-AGGGGCCATCCACAGTCTTC-3' (antisense).

Dual luciferase reporter assay

MAPK1 3'UTR fragment harboring wild-type or mutant-type of miR-203a-5p binding sites was amplified by PCR and sub-cloned into the psiCHECK-2 firefly luciferase reporter vector (Promega, Madison, WI, USA) to synthesize wt-MAPK1 and mut-MAPK1 reporter plasmids, respectively. Then, constructed reporters were co-transfected with miR-NC mimic or miR-203a-5p mimic into CaSki and SiHa cells. At 48 h post-

transfection, the luciferase activities were measured using the dual luciferase reporter system (Promega) in accordance with the producer's instructions.

Western blot assay

Total protein was extracted from cultured cells by RIPA buffer (Sigma Aldrich, St Louis, MO, USA) with protease inhibitor, then were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Subsequently, membrane was blocked in 5% skim milk for 1 h, then was probed with primary antibody against rabbit anti-MAPK1 (1:1000, Abcam, Cambridge, MA, USA), E-cadherin (1:400, Abcam), Vimentin (1:400, Abcam), N-cadherin (1:400, Abcam), Snail (1:500, Abcam) and GAPDH (1:1000, Abcam) at 4˚C overnight. After repeated washes, the membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibody (1:5000, CWBIO, Beijing, China) at room temperature for 1 h. Bands were detected with an enhanced chemiluminescence kit (GE Healthcaare UK Ltd, Little Chalfont, UK) and images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Cell proliferation assay

Cell proliferation was detected by cell counting kit-8 (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) based on the manufacturer's instructions. Generally, transfected CaSki and SiHa cells were seeded in 96-well plate at a density of 2 × 10^3 cells/well for 48 h. Afterwards, 10 μL CCK-8 solution was added and incubated for 2 h at 37˚C in a humidified air atmosphere with 5% CO_2. Finally, cell proliferation was detected using a microplate reader (Elx800, BioTek Inc, North Brunswick, NJ, USA) by measuring absorbance at 450 nm.

Cell apoptosis assay

The flow cytometry assay was employed to detect the effect of miR-203a-5p and MAPK1 on apoptosis of CaSki and SiHa cells at 48 h post-transfection. Cells were resuspended and then stained with the Annexin V-fluorescein isothiocyanate (FITC) / Propidium Iodide (PI) at room temperature in the dark for 10 min. Next, apoptosis cells were assessed using the FACSan flow cytometry (BD Bioscience, San Jose, CA, USA) with Apoptosis Detection Kit (Invitrogen) according to the producer's instructions.

Cell migration and invasion assay

Cell migration and invasion assays were appraised by Transwell chamber (8 μm, BD Bioscience) based on the producer's instructions. In brief, 8 x 10^4 cells were seeded in the upper chamber of transwells with a non-coated membrane for migration assay, whereas 2 x 10^5 cells were plated in the upper chamber pre-coated with matrigel (BD Bioscience) for invasion assay. Cells in the upper chamber were resuspended in serum-free medium, and the medium complemented with serum was used as chemoattractant in the lower chamber. After incubation at 37˚C for 24 h, cells in the upper chamber were removed with a cotton swab. Migrated / Invaded cells on the lower surface were fixed and stained, then counted and photographed under a microscope (Nikon, Tokyo, Japan).

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) with at least three independent experiments. The data were analyzed using SPSS version 17.0 software (Chicago, IL, USA). Difference in groups was analyzed using Student's t-test or one-way ANOVA. P values less than 0.05 were considered significant.

Results

MiR-203a-5p expression is upregulated in CC tumor tissues and cells

To investigate the function of miR-203a-5p in CC, its expression pattern was firstly measured by RT-qPCR assay. As exhibited in Fig. 1A, miR-203a-5p expression was obviously decreased in CC tumor tissues in comparison with that in the paired adjacent normal tissues. Besides, lower expression of miR-203a-5p was found in CC cell lines (HeLa, C33A, CaSki, SiHa and HT-3) relative to normal human keratinocyte cell line HaCaT (Fig. 1B). Together, these data hinted that the involvement of miR-203a-5p in CC progression.

MiR-203a-5p suppresses proliferation, migration, invasion and promotes apoptosis of CC cells in vitro

Then, to identify the function of miR-203a-5p in CC cells, the overexpression plasmid of miR-203a-5p was synthesized. As exhibited in Fig. 2A,

the expression level of miR-203a-5p was apparently upregulated in CC cells transfected with miR-203a-5p mimics, when compared with cells transfected with miR-NC mimics. Thus, we used the overexpression system to further explore the role of miR-203a-5p in proliferation, migration, invasion and apoptosis. CCK-8 assay suggested that proliferation of CaSki and SiHa cells was dramatically retarded after introduction with miR-203a-5p mimics (Fig. 2B).

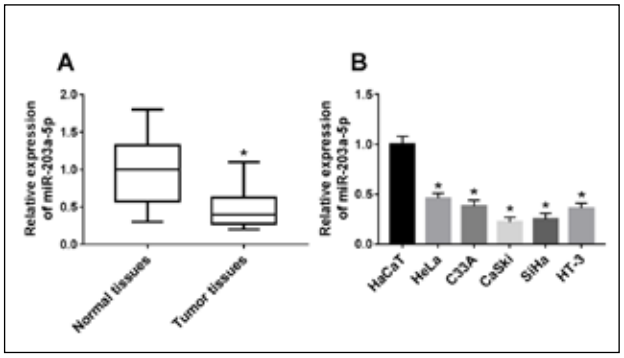

Fig. 1: MiR-203a-5p expression is downregulated in CC tumor tissues and cells. (A) RT-qPCR assay was performed to detect the expression profiles of miR-203a-5p in 48 pairs of CC tissues and adjacent nontumor samples. (B) Expression level of miR-203a-5p in CC cell lines (HeLa, C33A, CaSki, SiHa and HT-3) and normal human keratinocyte cell line (HaCaT). *P <0.05.

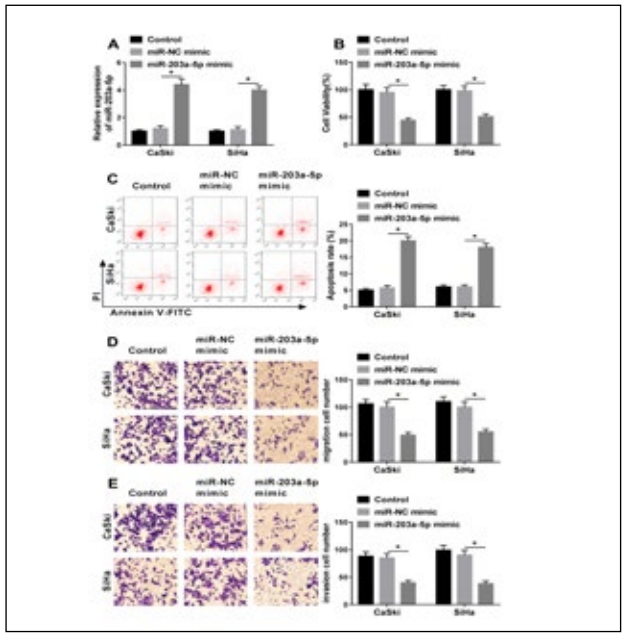

Fig. 2: MiR-203a-5p suppresses proliferation, migration, invasion, and promotes apoptosis of CC cells *in vitro*. (A) Transfection efficiency of miR-203a-5p mimics in CaSki and SiHa cells. (B) Cell viability in miR-203a-5p mimics-transfected CaSki and SiHa cells was detected by CCK-8 assay. (C) Apoptosis rate was assessed by flow cytometry assay in these transfected cells. (D and E) Migration and invasion were measured by transwell assay in these transfected cells. *P <0.05.

Afterwards, we further probed that the effect of miR-203a-5p on migration and invasion of CaSki and SiHa cells. Transwell assay presented that overexpression of miR-203a-5p effectively blocked migration and invasion of CaSki and SiHa cells (Fig. 2D and 2E). In addition, we also observed the role of miR-203a-5p on the apoptosis of CC cells. The flow cytometry assay suggested that miR-203a-5p overexpression led to a prominent enhancement in apoptotic rate in CaSki and SiHa cells (Fig. 2C). In a word, all of these proved that miR-203a-5p could contribute to tumorigenesis of CC cells.

MiR-203a-5p inhibits EMT of CC cells in vitro

Next, to identify the effect of miR-203a-5p in CC metastasis, the expression EMT-related proteins was measured by western blot assay. The results displayed that the EMT maker E-cadherin was remarkably upregulated and N-cadherin, Vimentin and Snail were evidently downregulated in CaSki and SiHa cells transfected with miR-203a-5p mimics (Fig. 3A and 3B). In a word, miR-203a-5p could suppress EMT in CC cells.

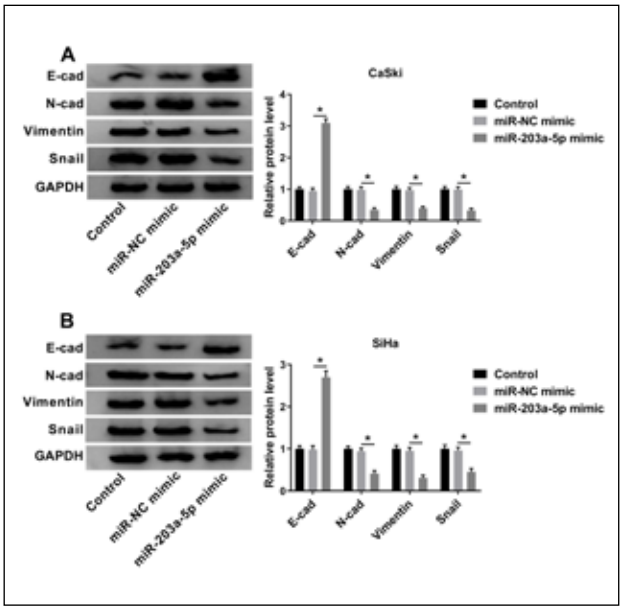

Fig. 3: MiR-203a-5p inhibits EMT of CC cells *in vitro*. (A and B) Levels of EMT-related proteins (E-cadherin, N-cadherin, Vimentin and Snail) were assessed by western blot assay in CaSki and SiHa cells transfected with miR-203a-5p mimics. *P <0.05.

MAPK1 is a direct target of miR-203a-5p in CC cells

To explore the mechanisms of miR-203a-5p suppressing tumor activity in CC, bioinformatic-based target prediction analysis by TargetScan bioinformatics algorithm was used to probe the

potential targets of miR-203a-5p. As displayed in Fig. 4A, MAPK1 contains binding sites of miR-203a-5p. To further confirm the interaction between MAPK1 and miR-203a-5p, the dual luciferase reporter assays were carried out. As showed in Fig. 4B and 4C, upregulation of miR-203a-5p resulted in a significant reduction in luciferase activity of wt-MAPK1 but had no evident inhibitory effect on mut-MAPK1 in CaSki and SiHa cells. In parallel, miR-203a-5p overexpression obviously repressed the protein level of MAPK1 in CaSki and SiHa cells, as proved by western blot (Fig. 4D). Together, all results suggested that miR-203a-5p directly targeted MAPK1 and regulated its expression.

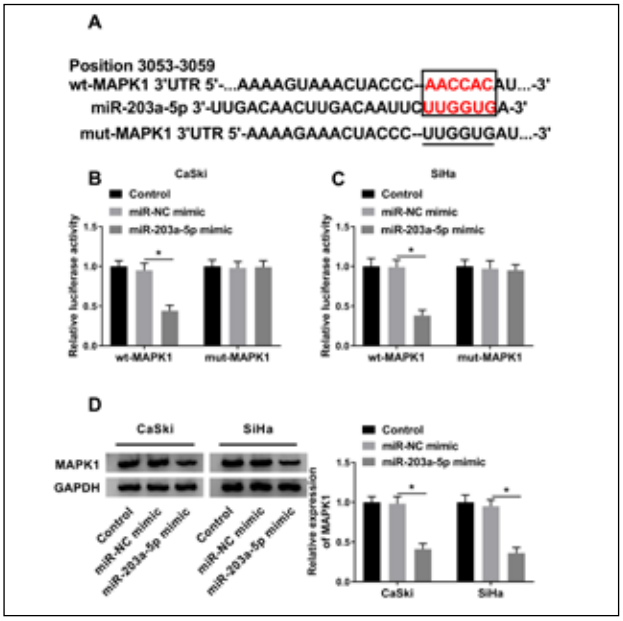

Fig. 4: MAPK1 acts as a target of miR-203a-5p in CC cells. (A) The predicted binding sites of miR-203a-5p on MAPK1, as well as the mutant. (B and C) The relative luciferase activity in CaSki and SiHa cells co-transfected with luciferase reporter plasmids wt-MAPK1 or mut-MAPK1 and control or miR-NC mimic or miR-203a-5p mimic. (D) Western blot analysis of MAPK1 expression in CaSki and SiHa cells transfected with miR-203a-5p mimics. *P <0.05.

MAPK1 expression is upregulated in CC tumor tissues and cells

As mentioned above, MAPK1 as a target of miR-203a-5p in CC, we speculated that the involvement of MAPK1 in CC progression. In order to confirm the hypothesis, we measured the expression of MAPK1 in CC tissues and cells. The data presented that MAPK1 expression was upregulated in CC tissues and cells in comparison with respective controls (Fig. 5A and 5B), suggesting that MAPK1 participates in the progression of CC.

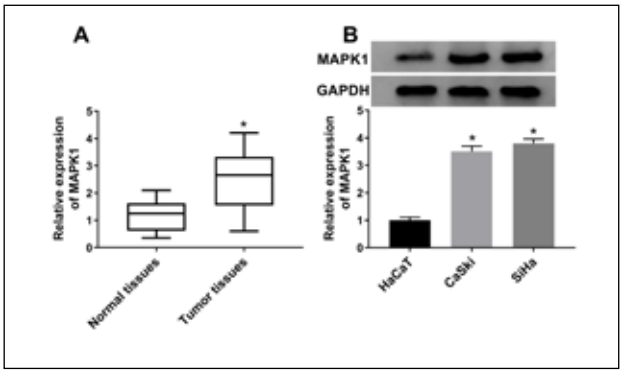

Fig. 5: MAPK1 expression is upregulated in CC tumor tissues and cells. (A) The expression of MAPK1 in 48 pairs of CC tumor tissues and paired adjacent nontumor tissues was measured by RT-qPCR. (B) Expression level of MAPK1 in CC cell lines (CaSki and SiHa) and normal human keratinocyte cell line (HaCaT). *P <0.05.

MiR-203a-5p suppresses CC cells progression by targeting MAPK1

To further explore the mechanisms of miR-203a-5p involved in CC progression, we conducted rescue experiments by introducing miR-203a-5p mimic + pc-NC or miR-203a-5p mimic + pc-MAPK1 into CaSki and SiHa cells. Firstly, western blot results indicated that the upregulation of miR-203a-5p decreased the expression of MAPK1, and reintroduction of MAPK1 obviously overturned the inhibitory action (Fig. 6A).

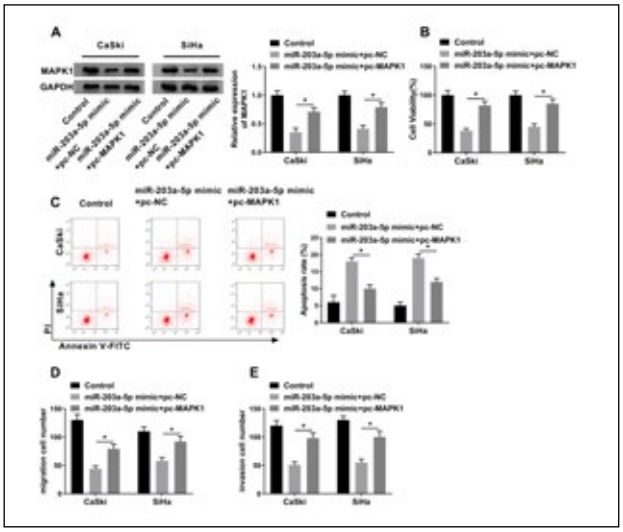

Fig. 6: MiR-203a-5p suppresses CC cells progression by targeting MAPK1. (A) MAPK1 expression level was assessed by western blot in CaSki and SiHa cells transfected with control or miR-203a-5p mimic+ pc-NC or miR-203a-5p mimic + pc-MAPK1. (B) Cell viability was assessed by CCK-8 assay in transfected CaSki and SiHa cells (C) Apoptosis rate was measured by flow cytometry assay in transfected CaSki and SiHa cells. (D and E) Migration and invasion analysis were conducted by transwell assay in transfected CaSki and SiHa cells. *P <0.05.

Subsequently, cell proliferation, migration, invasion and apoptosis were measured. As showed in Fig. 6B, 6D and 6E, overexpression of miR-203a-5p remarkably curbed proliferation, migration and invasion of CaSki and SiHa cells, while recover of MAPK1 expression evidently attenuated these effects. Furthermore, miR-203a-5p overexpression led to a notably enhancement in apoptotic rate in CaSki and SiHa cells, which was dramatically eliminated by reverting of MAPK1 (Fig. 6C). All of these results illuminated that miR-203a-5p inhibited CC development by regulating MAPK1.

MiR-203a-5p inhibits EMT of CC cells by targeting MAPK1

Next, to further investigate whether miR-203a-5p could affect EMT-related proteins expression by regulating MAPK1, western blot assay was carried out. The results suggested that miR-203a-5p overexpression elevated the expression of E-cadherin and weakened the expression of N-cadherin, Vimentin and Snail. However, rescue of MAPK1 expression effectively reversed these effects (Fig. 7A and 7B). Taken together, these data indicated that miR-203a-5p could constrain EMT via regulating MAPK1.

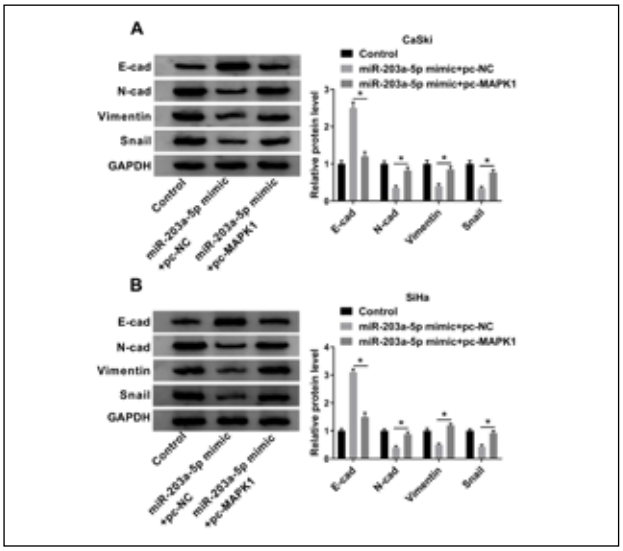

Fig. 7: MiR-203a-5p inhibits EMT of CC cells by targeting MAPK1. (A and B) EMT-related proteins level (E-cadherin, N-cadherin, Vimentin and Snail) were measured by western blot assay in CaSki and SiHa cells transfected with control or miR-203a-5p mimic+ pc-NC or miR-203a-5p mimic + pc-MAPK1. *P <0.05.

Discussion

Amounting evidence presents that miRNAs can act as prognostic biomarkers and the aberrant expression are correlated with the formation and tumorigenesis in CC[20, 21]. MiR-203a-5p is a form of mature miR-203, which has been confirmed as a tumor suppressor in various cancers, including CC. For instance, Li et al. reported that upregulation of miR-203 inhibited cell proliferation, colony formation, migration and invasion by binding to IRF1 in CC[22]. Besides, the targets of miR-203 and miR-203a-5p, such as VEGFA (10) and FOXN2[23] have also been identified in CC. However, the accurate function and molecular mechanism of miR-203a-5p in CC remain largely unknown.

In this study, we firstly investigated that the expression level of miR-203a-5p in CC tumor tissues and CC cells. The data exhibited that miR-203a-5p expression was notably downregulated in CC tumor tissues and CC cell lines relative to their respective controls. Then, we assessed that the role of miR-203a-5p on proliferation, migration, invasion and apoptosis of CC cells. The results suggested that the overexpression of miR-203a-5p impeded proliferation, migration and invasion, as well as boosted the apoptosis of CC cells. In other words, miR-203a-5p performed as a tumor-suppressor in the development of CC.

Next, we further explored the potential molecular mechanisms of miR-203a-5p in inhibiting CC progress. Previous report demonstrated that MAPK1 was upregulated in CC tissues and curbed cell proliferation, migration, invasion and metastasis in CC by regulating miRNA[14, 24]. Hence, MAPK1 as a target gene of miR-203a-5p in CC cells was further confirmed by TargetScan (http: //www.targetscan. org) and dual luciferase reporter assays. Moreover, in this paper, we found that MAPK1 expression was upregulated in CC tumor tissues and CC cell lines, and inversely associated with miR-203a-5p expression in CC cells. Consequently, we further explored whether the effect of miR-203a-5p on CC development was mediated via regulating MAPK1. The following function analysis disclosed that miR-203a-5p overexpression retarded the proliferation, migration and invasion, as well as promoted the apoptosis of CC cells, while recover of MAPK1 partially overturned tumor inhibition effect of miR-203a-5p. We firstly proved that miR-203a-5p suppressed CC progression probably by targeting MAPK1.

It is widely accepted that EMT, acting as the characteristics of tumor invasion ability, is a vital factor in the tumorigenesis containing local infiltration and metastasis[25]. Prior reports showed that the downregulated expression of E-cadherin and the upregulated expression of mesenchymal markers,

such as Vimentin, N-cadherin and fibronectin are the characteristic of EMT[26]. Meanwhile, Li et al. reported that Snail inhibited the expression of E-cadherin, and the overexpression of Snail led to the occurrence of EMT in pancreatic cancer[27]. In addition, some studies have demonstrated dysregulation of miRNAs play critical roles for EMT in various kinds of cancers, including CC[28, 29].

In the present study, we demonstrated that overexpression of miR-203a-5p upregulated E-cadherin protein level and downregulated N-cadherin, Vimentin and Snail protein level in CC cells, suggesting that miR-203a-5p repressed EMT of CC cells. Moreover, MAPK1, as a target gene of miR-203a-5p MAPK1, has also been pointed out to be closely correlated with the generation of EMT (30). Thus, in the paper, we further investigated whether the miR-203a-5p on EMT was mediated by targeting MAPK1. Western blot analysis manifested that overexpression of miR-203a-5p upregulated E-cadherin protein level as well as downregulated N-cadherin, Vimentin and Snail protein level in CC cells, however, the reintroduction of MAPK1 partly reversed these effects, suggesting that miR-203a-5p could repress EMT by targeting MAPK1 in CC cells.

Conclusions

Taken together, our study demonstrated miR-203a-5p overexpression inhibited proliferation, migration, invasion, EMT and induced apoptosis of CC by targeting MAPK1, providing an effective therapy for predicting the prognosis of CC.

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Corresponding Author:
Liwen Jin
Department of gynecology, The Central Hospital of Wuhan Affiliated to Tongji Medical College, Huazhong University of Science and Technology, No. 26 Shengli Street, Jiangan District, Wuhan City, Hubei Province, 430014, Wuhan, China
Email: xianyu134565@126.com
(China)