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Up-regulation of miR-203 inhibits the growth of cervical cancer cells by inducing cell cycle arrest and apoptosis

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Summary

Purpose: MicroRNA-203 (miR-203) had previously been proved to alter in various cancers. In the present research, the authors attempted to assess the roles of miR-203 in cervical cancer. *Materials and Methods:* Quantitative reverse-transcriptional polymerase chain reaction (qRT-PCR) was employed to testify the expression level of miR-203 in cervical cancer tissues and normal cervical control. Cell growth assay and colony formation were performed to explore the impact of overexpressed miR-203 on the proliferation of human SiHa cells. Cell cycle and apoptosis rates were also evaluated by flow cytometer, and we also confirmed that Bmi-1 was the direct target of miR-203 via luciferase assay and Western blot. *Results:* MiR-203 was significantly down-regulated in cervical cancer specimens compared with normal cervical tissues ($p < 0.01$). Functionally miR-203 could inhibit SiHa cell growth, increase G1-phase population and induce cell apoptosis. Mature miR-203 might decrease the expression of Bmi-1 assessed by luciferase assay and Western blot analysis. *Conclusions:* The present results indicated that miR-203 participate malignant process of cervical cancer as an anti-oncogenic-miRNA by targeting Bmi-1, inducing cell cycle arrest, and apoptosis. Mature miR-203 was supposed to be a perspective strategy for therapeutic intervention of human cervical cancer.

Key words: Cervical cancer; MicroRNA-203; Bmi-1.

Introduction

Nowadays cervical cancer could be potentially prevented by HPV vaccine, but it is still the fourth most leading cause of cancer-associated mortality among women worldwide [1], although the new technology of cervical cytology screening contributes to early detection and early treatment, but the five-year survival rates have not remarkably improved in the recently decades, How to find more effective therapeutic intervention is a major challenge, but it is most important to understand molecular mechanisms of cervical cancer in details first.

Recently miRNAs gained much attention because of the important roles in carcinogenesis. These non-coding RNAs could negatively regulate gene expression at the post-transcription lever through translational inhibition or degradation of its targets by pairing at 3' untranslated regions [2, 3]. Altered expression of miRNAs have been indicated to participated in the malignant process of multiple cancers including tumor initiation, advanced progression, invasion, and metastasis by acting as oncogenes or tumor suppressor. miRNA microarray datas in the present authors' previous work demonstrated that microRNA-203 (miR-203) was a significantly down-regulated miRNA in cervical cancer. So we assumed that miR-203 could be a tumor suppressor in cervical cancer. miR-203 was identified to locate in a region at chromosome 14, which contains a high density of microRNAs (including about 12% of the known human microRNA gene), exhibited significantly decreased expression in various tumors, especially in squamous cell carcinoma in the recent study [4-6]. So the most important role of miR-203 is to reduce the proliferative capacity of epithelial cells upon differentiation [7]. Based on the present authors previously study, we designed a gain-of-function assays to discover the association of miR-203 expression with cervical cancer onset and progression, which has not been reported before.

Materials and Methods

Cervical squamous cell carcinoma and normal specimens were collected from patients during surgery in the Department of Gynecologic Oncology, Guangxi Tumor Hospital. No chemotherapy or radiotherapy performed prior to surgery. The tissues were removed by surgery and then frozen in liquid nitrogen immediately, stored at -80°C until use. The study was conducted in accordance with the Declaration of Helsinki and under a protocol approved by the Ethics Committee of the Guangxi Medical University. The human cervical cancer cell line: SiHa cell line was kept by the present laboratory. All cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) with penicillin and streptomycin at 37°C with 5% CO_2 in humidified incubator.

MiRNAs was isolated from frozen fresh cervical cancer tissues, normal cervical tissues and SiHa cells using the miRcut miRNA

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isolation kit following the protocol. RNA quantification was performed using a spectrophotometer. miRNA expression was assessed by the reverse-transcription reactions using a standard SYBR Green PCR kit. Reverse transcription of primers to the sequence of miR-203 (5'-TAC GAG TGA AAT GTT TAG GAC CAC TAG-3');U6 (5'- ATT GGA ACG ATA CAG AGA AGA TT-3'); universal reverse primer (5'-GTC CTT GGT GCC CGA GTG-3') were synthetized. PCR with a final volume of 10 µl was performed at 95˚C for ten minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for one minute. All reactions were performed in triplicate a 7900HT fast real-time PCR system. Comparative threshold cycle (CT) method-fold change (2-ΔΔCT) calculated relative changes.

MiR-203 mimic were transfected into SiHa cells using lipofectamine 2000 at a final concentration of 100 nM according to the manufacture protocol. Cells transfected with negative control were used as a control for the transfection. The efficiency of overexpression of miR-203 was evaluated at 24 hours after transfection by quantitative reverse-transcriptional polymerase chain reaction (qRT-PCR).

MiR-203 mimic: 5'-GUGAAAUGUUUAGGACCACUAG-3'
3'-AGUGGUCCUAAACAUUUCACUU-5'
Negative control: 5'-UUCUCCGAACGUGUCACGUTT-3'
3'-ACGUGACACGUUCGGAGAATT-5'

SiHa cell proliferation was assessed using MTT assay. In brief, Twenty-four hours after miR-203 mimic transfection, SiHa cells were harvested and seeded (1×10^4 cells/well) in 96-well plates for 24, 48, 72, and 96 hours, respectively. Then, the cells were incubated with 20 µl of MTT (5 mg/ml, PH=7.4) for four hours at 37°C and 150 µl of dimethyl sulfide was added to solubilize the crystals for 20 minutes at room temperature. The optical density was measured at 540 nm using micro plate reader.

The percentages of cells in G1/G0, S-phase, and G2/M phases as well as the percentage of apoptotic cells were assessed for SiHa cell lines using flow cytometry. Cells were trypsinized (0.05% trypsin/0.53 mM EDTA), washed in phosphate-buffered saline (PBS), and 1×10^6 cells from three independent isolations of each cell line were resuspended in 300 µL of cold PBS. Cells were fixed in 70% ethanol for two hours at 4°C. After washing with PBS, cells were treated with RnaseA (50 µg/ml) and stained with propidium iodide (25 µg/ml) for 30 minutes at 37°C. The cell cycle was performed by flow cytometry and analyzed by ModFit LT software. Cell doublets were identified using fluorescence pulse height *vs*. area measurements and excluded from cell cycle analysis. All experiments were performed three times and were calculated using average results.

Apoptosis analyzed by flow cytometry following double staining with annexin V-FITC and propidium iodide using a specific kit according to the manufacturer's instruction. All experiments were performed three times and were calculated using average results.

Approximately 200 cells in each groups were placed in a fresh six-well plate and incubated in RMPI 1640 containing 10% FBS for two weeks. Colonies were washed by PBS and fixed with methanol and stained with 0.1% crystal violet for 15 minutes. Colonies were counted and experimentation was performed independently three times in duplicates.

Protein was extracted from SiHa cells using M-PER mammalian protein extraction reagent.The lysates were centrifuged at 11,000 x g for 15 minutes at 4˚C and the supernatant was stored at -80˚C. The soluble proteins were quantitated using Bradford reagent according to the manufacturer's protocol. Total protein (20 µg) was separated by SDS-PAGE (10% gel) and transferred onto a nitrocellulose membrane. Following one-hour blocking in 5% non-fat milk, membranes were incubated with mouse monoclonal Bim-1 (1:1000) or mouse monoclonal β-actin (1:10000) overnight at 4°C. Following washing by TBST, the membranes were incubated with rabbit anti-mouse IgG–HRP (1:10000) for one hour. Protein was visualized using enhanced chemiluminescence reagent.

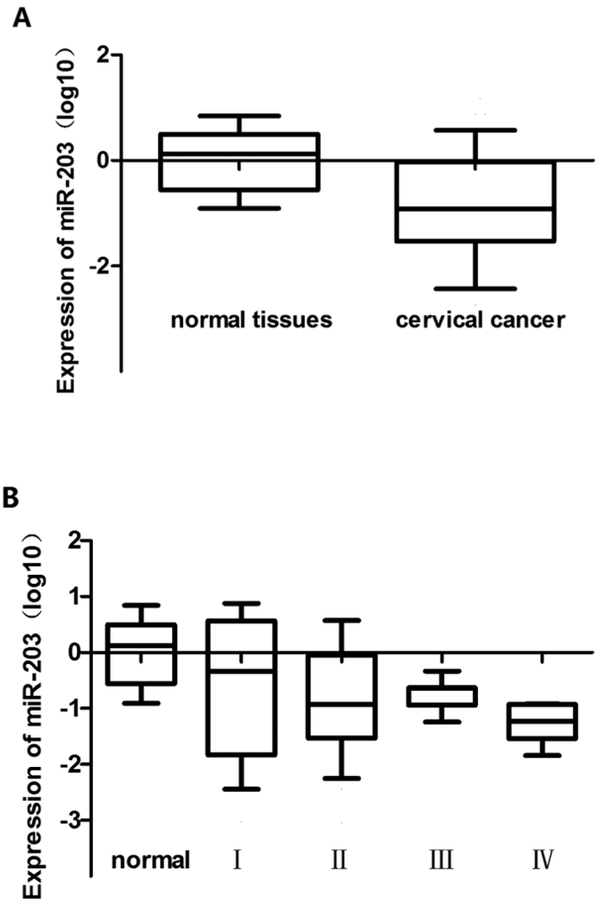

Figure 1. — Expression of miR-203 by reverse transcription-quantitative polymerase chain reaction in cervical cancer tissues. A) The Mann–Whitney test was used to examine the difference between normal tissues and cervical cancer tissues, miR-203 was decreased in cervical cancer tissues compared with control group. B) :Kruskal–Wallis H test was performed to compare the expression among Stages I–IV of cervical cancer patients. Patients with low expression of miR-203 in cervical cancer had a close relationship with LNM and p-value < 0.05 was considered significant.

MiRNAs targets were predicted using the algorithms TargetSan. The miR-203 binding sites from 3'-UTR Bmi-1 or mutant 3'-UTR were cloned into the pGL3 reporter luciferase vector. For reporter assay, co-transfected of SiHa cells with 100 nM miR-203 mimic or control miRNA and 0.1 µg of the pGL3-3'UTR wild type or mutant plasmid DNAs in 96-well plates using lipofectamine 2000 were utilized. Luciferase activity was measured 48 hours post-transfection according to the manufacturer's instructions.

\All data were processed using Graphpad Prism 6 software. Normally distributed groups' data was presented as mean ± SD, and compared by two-tailed Student *t*-test. While the results did not display normal distribution, the authors chose to analyze the

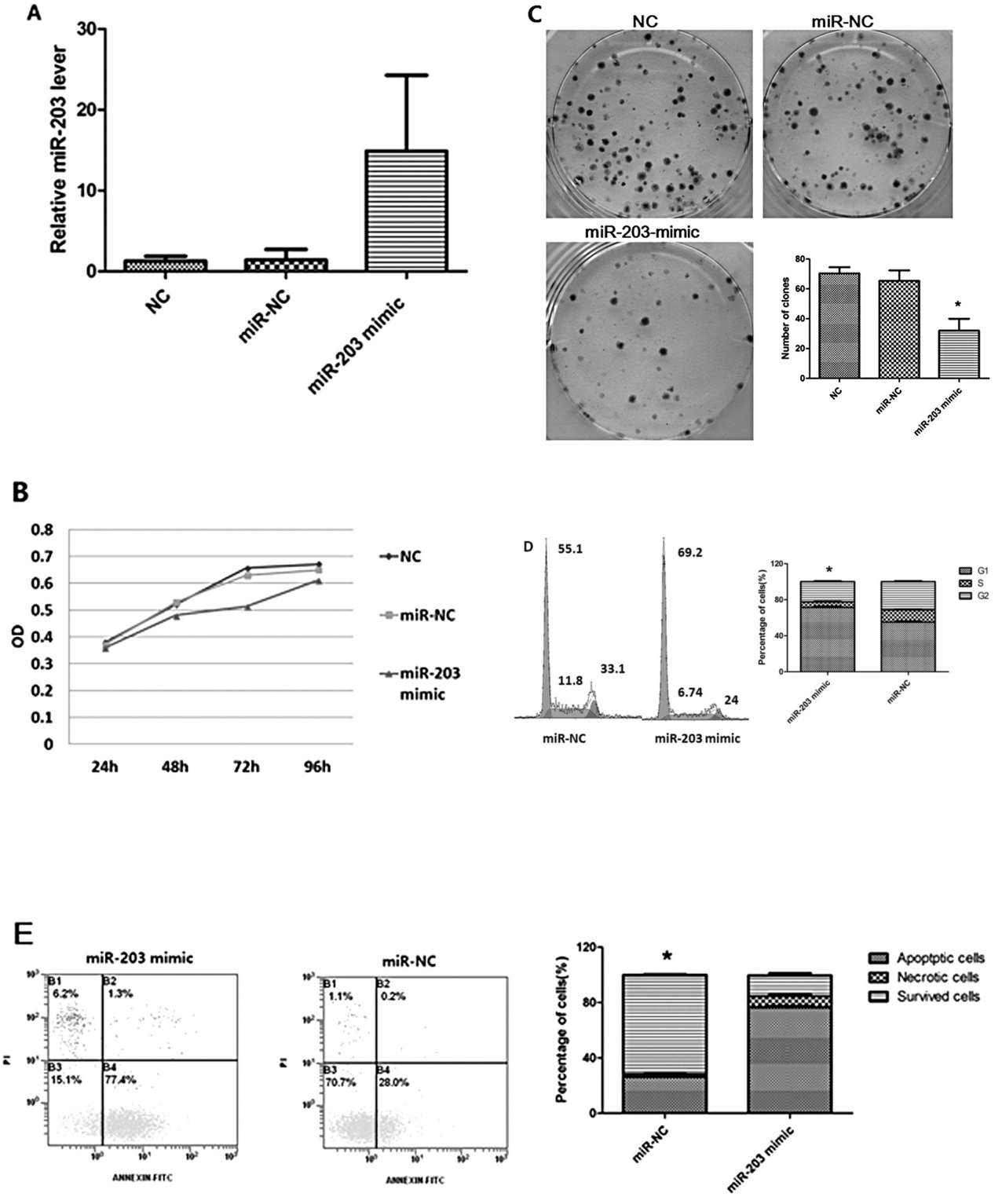

Figure 2. — Cell functional assays following miR-203 overexpression. A) MiR-203 mimic definitely up-regulates the expression level of miR-203 compared to negative control group at 48 hours pos-ttransfection. B) Mature miR-203 significantly inhibits cell growth in SiHa cells by proliferation assay. C) The colony formation capability also is remarkablely reduced. D) The up-regulated miR-203 induces G0/G1 arrest as indicated by flow-cytometry cell cycle analysis. E) Apoptotic rate is increased dramatically in overexpressed miR-203 group.

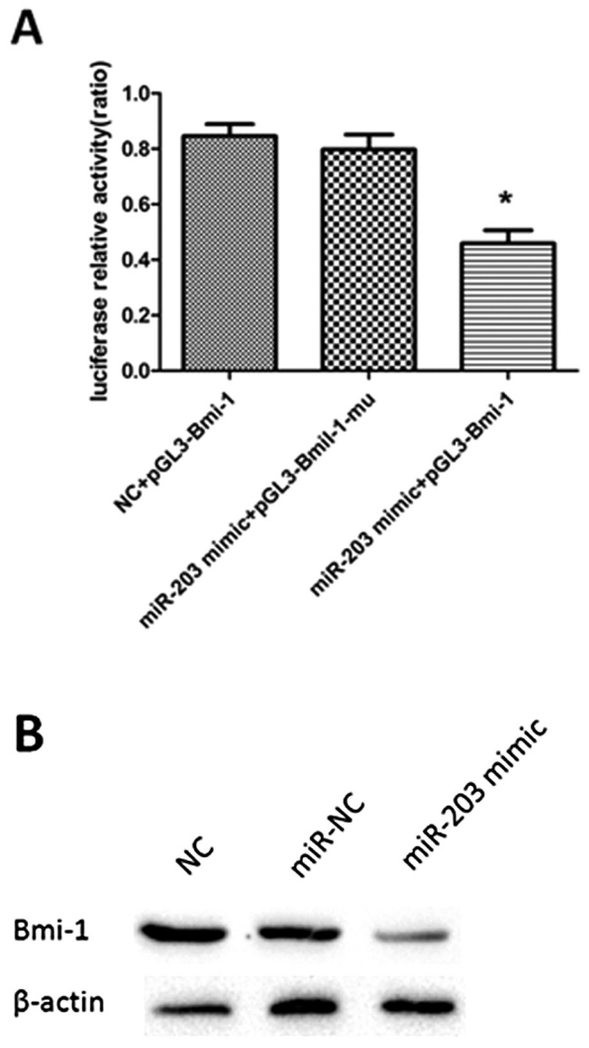

Figure 3. — Bmi-1 is a direct target of miR-203. A) miR-203 remarkably suppressed the luciferase activity of Bmi-3′ UTR-wt at 24 hours compared to the control group. B) Western blot analyses demonstrate up-regulated miR-203 in SiHa cells reducing Bmi-1 protein levels.

data with non-parametric methods. (Mann-Whitney U test between two groups and Kruskal-Wallis H test for three or more groups). A p value less than 0.05 was considered statistically significant.

Results

In order to assess the role of miRNAs in cervical cancer development, qRT-PCR was performed to detect the expression of miR-203 in cervical cancer tissues and normal cervical tissues. In this study, the data reported in Figure 1A indicated that miR-203 was decreased in cervical cancer tissues compared to the control group, by a median of 0.12. Figure 1B shows the clinical data of two subject groups. Patients with low expression of miR-203 in cervical cancer had a close relationship with LNM ($p < 0.05$), There was no significant difference between in the age, histologic grade, and tumor diameter distribution ($p > 0.05$).

Because the expression of miR-203 was lower in cervical cancer tissues, we wanted to discover the inhibition effect of miR-203 on cell proliferation in gain-of- function assays, so we transfected miR-203 mimic into SiHa cells to up-regulated the expression of miR-203, then we performed MTT assay, cell cycle, cell apoptosis, and clone formation experiments. Figure 2A indicated that mature miR-203 was significantly overexpressed in cells transfected with miR-203 mimic compared to negative control.

MTT and colony-forming assays were carried out to evaluate the inhibition potential of miR-203 on SiHa cell line. The overexpression of miR-203 significantly inhibited the proliferation. Growth curves and colony formation rate showed that miR-203 mimic could slow down the growth of SiHa cell line (Figures 2B-C) ($p < 0.05$). At the time points of 24, 48, 72, and 96 hours after transfected, the inhibition rates were 10.5%, 15.3%, 21.9%, and 11.9%, respectively. The up-regulated miR-203 induced G0/G1 arrest as indicated by flow-cytometry cell cycle analysis, there was a large G1 peak (65.6%) in the miR-203 mimic group, in contrast, the control group was 54.3%. The greatly increased G1-phase population suggest that miR-203 could lead to the observed deceleration in the rate of proliferation (Figure 2D). Cell cycle analysis indicated that miR-203 could induce the increase of sub-G1 peak, a characteristic of apoptosis, thus, we hypothesized that miR-203 might induce apoptosis. So flow cytometry was performed, and the results showed that apoptotic rate was increased dramatically in overexpressed miR-203 group ($p < 0.001$) (Figure 2E).

As we all known, miRNAs could bind target genes to fully display its function. To identify targets which have relationship with tumorigenesis, three algorithms (Targetscan, Pictar, and Miranda) were used and finally we had interested Bmi-1. SiHa cells were co-transfected with miR-203 and a reporter plasmid or control vector. As a result, miR-203 remarkably suppressed the luciferase activity of Bmi-3′ UTR-WT at 24 hours compared to the control group. We performed western blot analysis to directly assess the effect of miR-203 on Bmi-1 expression. As shown in Figure 3B, overexpression of miR-203 in SiHa cells down-regulated Bmi-1 protein levels. Taken together, these results indicate that Bmi-1 is a direct downstream targets for miR-203 in SiHa cells, which might be suppressed during tumorigenesis.

Discussion

Dysregulation of miRNA is associated with various diseases, so miRNA might be used not only in early detection of the disease but also possibility in cancer therapy. If

miRNA acts as tumor suppressor, it is necessary to increase the level of its expression by either increasing expression or escape from suppression of the encoded gene or adding mature miRNA into the tumor cell.

Evidence from a previous study of the present group by microarray showed that miR-203 was down-regulated significantly. So we thought that miR-203 might be a tumor suppressor in cervical cancer. To testify this hypothesis, we used q-PCR to discover whether miR-203 was associate with cervical cancer advanced progression. The present results showed that miR-203 was down-regulated in cervical cancer group compared to control group ($p < 0.01$), and low expression of miR-203 prone LNM ($p < 0.01$), this is exactly what Hu *et al.* demonstrated in their study [8]. To confirm this effect of tumorigenesis, we performed cell function assay in vitro. Results of MTT showed up-regulated miR-203 can significantly inhibit cell growth in SiHa cells; it also could reduce the formation of colonies. Then we performed the cell cycle and cell apoptosis assay to explore the mechanisms of tumor growth inhibition by flow cytometry, and the results revealed that miR-203 could block the G1/S transition and induce cell apoptosis, which proved that miR-203 might be involved in the regulation of cell development, cell proliferation, and apoptosis.

To determine the molecular mechanism by which miR-203 suppressed the proliferation of cervical cancer cells, we employed miRNA target prediction databases (TargetScan, miRanda, and PicTar) to predict miR-203 targets. Then B-lymphoma Moloney murine leukemia virus insertion region-1 (Bmi-1) came to mind, which is a member of the polycomb transcription repressors, that plays an important role in various biological processes, including embryonic development, organ formation, stem cell self- renewal, cell differentiation, and tumorigenesis [9]. Bmi-1 is known to be overexpressed in various human cancer tissues and participates in the regulation of malignant transformation, cell growth, cell cycle, apoptosis, invasion, and metastasis [10]. Many studies have indicated that Bmi-1 may accelerate tumor cell growth. Up-regulated Bmi-1 in gastric and breast cancer has been testified to make cancer cell grow faster, suppress cell apoptosis, and improve colony formation capability [11, 12]. We further proved that Bmi-1 is definitely a target of miR-203 by luciferase reporter gene assay and western blot analysis, respectively. We cloned the Bmi-1 3′-UTR into a luciferase reporter vector. Luciferase assay confirmed that miR-203 directly bound to Bmi-1 3′-UTR, and by which it significantly reduced luciferase activities. However, mutation of the admitted miR-203 sites in the 3′-UTR of Bmi-1 abrogated luciferase reactivity to miR-203. To directly verify the effect of miR-203 on Bmi-1 expression, we performed western blot analysis. Figure 3B demonstrates that up-regulated miR-203 in cervical cancer cells could reduced Bmi-1 protein levels.

Above all, the present results indicated that miR-203 might be related with malignant process of cervical cancer by blocking the G1/S transition and inducing cell apoptosis by targeting Bmi-1, and then cell growth suppression accompanied, which suggests that miR-203 might have new perspective for future on therapeutic intervention.

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