Abstract. Of gynecological cancers, cervical cancer has the second highest incidence globally and is a major cause of cancer-associated mortality in women. An increasing number of studies have reported that microRNAs (miRNAs) have important roles in cervical cancer carcinogenesis and progression through regulation of various critical protein-coding genes. The aim of the present study was to investigate the expression and biological roles of miRNA-211 (miR-211) in cervical cancer and its underlying molecular mechanism. The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) demonstrated that the expression levels of miR-211 in cervical cancer tissues and cell lines were significantly lower compared with adjacent normal tissues and the normal human cervix epithelial cell line, respectively. Furthermore, upregulation of miR-211 by transfection with miR-211 mimics inhibited cell proliferation, migration and invasion of cervical cancer, as determined by MTT, Transwell and Matrigel assays, respectively. Bioinformatics analysis and luciferase reporter assay results indicated that zinc finger E-box binding homeobox 1 (ZEB1) may be a direct target gene of miR-211. In addition, RT-qPCR and western blot analysis results demonstrated that miR-211 overexpression markedly reduced ZEB1 expression at mRNA and protein levels in cervical cancer. Furthermore, the effects of ZEB1 downregulation on the proliferation, migration and invasion of cervical cancer cells were similar to those induced by miR-211 overexpression. These results indicate that miR-211 may act as a tumor suppressor in cervical cancer by directly targeting ZEB1. Therefore, miR-211/ZEB1-based targeted therapy may represent a potential novel treatment for patients with cervical cancer.

Introduction

Globally, cervical cancer has the second highest incidence among gynecological cancers and is a major cause of cancer-associated mortality in women (1). It is particularly widespread in developing countries, indicating the importance of screening programs for cervical cancer (2,3). It is estimated that there will be 529,800 new cases and ~275,100 deaths each year worldwide (4). It is considered that early sexual intercourse, promiscuity and human papillomavirus (HPV) infection, particularly HPV16, are closely associated with the development of cervical cancer (5). At present, the first-line treatments for patients with cervical cancer are surgical resection, chemotherapy and radiotherapy (6). Despite advances in the diagnosis, treatment and prevention of the disease, the prognosis of patients remains poor. The median progression-free survival and overall survival range between 2.5 and 13.2 months, and 4.2 and 12.87 months, respectively (7,8). Therefore, a complete understanding of the mechanisms underlying the occurrence and development of cervical cancer are required for the development of novel, effective therapeutic strategies.

MicroRNAs (miRNAs) are endogenous, conserved and non-coding RNAs of 19-25 nucleotides in length that originate from distinct hairpin precursors that are present in animals, plants and fungi (9,10). miRNAs post-transcriptionally downregulate the expression of various target genes via direct interaction with the 3’-untranslated regions (UTRs) of their target genes in a base pairing manner, which leads to mRNA degradation and/or translational inhibition (11). Notably, increasing evidence indicates that miRNAs are abnormally expressed in various human cancer types and have important functions in various areas of oncogenesis, including survival, proliferation, the cell cycle, apoptosis, angiogenesis, migration, invasion and metastasis (12-14). Specifically, numerous studies have reported that miRNAs have important roles in cervical cancer carcinogenesis and progression via the regulation of various protein-coding genes (15-17). For example, the expression levels of miRNA-206 (miR-206) are reduced in cervical cancer tissues and are significantly associated with adverse clinicopathological features, including advanced Federation of Gynecology and Obstetrics (FIGO) stage, positive lymph node metastasis, poor differentiation and HPV infection. Furthermore, miR-206 was demonstrated to exhibit tumor suppressor activity in cervical cancer by suppressing cell
proliferation, migration and invasion, and enhancing apoptosis (18). Therefore, regarding miRNAs may be developed as novel therapeutic targets for patients with cervical cancer.

miR-211 has been investigated in various forms of human cancer (19-21). However, to the best of our knowledge, there is currently no information available concerning the role of miR-211 in cervical cancer. Therefore, the present study aimed to investigate the expression of miR-211 in cervical cancer tissues and cell lines, and the biological roles of miR-211 in cervical cancer cells. In addition, the potential molecular mechanisms underlying its tumor suppressive roles were also investigated. The results of the present study may contribute towards identifying a novel therapeutic target for cervical cancer.

Materials and methods

Tissue specimens. Cervical cancer tissues and corresponding adjacent normal tissues were collected from 34 patients (age range, 39-72 years) who underwent surgical resection without radiotherapy and/or chemotherapy in the Department of Gynaecology, Songgang People's Hospital (Shenzhen, China) between January 2010 and July 2013. All fresh tissues were immediately frozen in liquid nitrogen and stored at -78˚C until further experiments. The present study was approved by the Research Ethics Committee of Songgang People's Hospital. Written informed consent was also obtained from all patients that participated in the study.

Cell lines and culture conditions. Four human cervical cancer cell lines (HeLa, C33A, SiHa and CaSki), a normal human cervix epithelial cell line (Ect1/E6E7) and the 293T cell line were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 100 µg/ml gentamicin (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37˚C in a humidified atmosphere with 5% CO₂.

Oligonucleotide transfection. The human miR-211 mimic and miRNA mimic negative control (miR-NC) were designed and provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-211 mimics sequence was 5'-UUCUCUCUCCGCCUCCUG-3' and the miR-NC sequence was 5'-UUCCUGAAGCGUGACUGTT-3'. Small interfering (si)RNA targeting zinc finger E-box binding homeobox 1 (ZEB1) and NC siRNA were chemically synthesized by Guangzhou Ribobi Co., Ltd. (Guangzhou, China). The ZEB1 siRNA sequence was 5'-GACAGAUCGCGCAAAAGAUtdT-3' and the NC siRNA sequence was 5'-UUCCUGCGAAGCGUGACUGTT-3'. HeLa and C33A cells were seeded into 6-well plates at a density of 8x10⁴ cells per well, and transfected with miR-211 mimics (100 pmol), miR-NC (100 pmol), ZEB1 siRNA (100 pmol) or NC siRNA (100 pmol) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. Transfected cells were used for subsequent experiments. Then, 48 h after transfection, RT-qPCR was performed to detect miR-211 or ZEB1 mRNA expression. MTT and cell migration and invasion assays were conducted at 24 h and 48 h following transfection. Western blot analysis was carried out 72 h following transfection.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cells by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The relative expression of miR-211 was determined using a SYBR PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol, with U6 small nuclear RNA as an internal control. The thermocycling conditions for qPCR were as follows: 42˚C for 5 min, 95˚C for 10 sec, followed by 40 cycles of 95˚C for 5 sec, 55˚C for 30 sec and 72˚C for 30 sec. For ZEB1 mRNA expression, cDNA was synthesized with an M-MLV Reverse Transcription System (Promega Corporation, Madison, WI, USA), which was followed by qPCR using the reagents of the SYBR Green I Mix (Takara Biotechnology Co., Ltd., Dalian, China). The temperature protocol for reverse transcription was as follows: 95˚C for 2 min; 20 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 2 min; and 72˚C for 5 min. The thermocycling conditions for qPCR were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The primers were as follows: miR-211, 5'-GATCTTCCCCCTTTGTCACTCC-3' (forward) and 5'-GTGTCGAGTCTCAGCAAA-3' (reverse); U6, 5'-GCTTCGCGCA GCACATACTAAAT-3' (forward) and 5'-CGCTTCACG AATTTGCAGTCAT-3' (reverse); ZEB1, 5'-AAGTGGCAGG TAGATGTA-3' (forward) and 5'-TGTAGCGACTGGAT TTT-3' (reverse); and GAPDH, 5'-ACCGATTGTGTCG ATT-3' (forward) and 5'-GGAAAGATGTTAGGATT-3' (reverse). GAPDH was used as an internal control for ZEB1 mRNA expression and mRNA levels were quantified using the 2⁻ΔΔCq method (22). Each sample was performed in triplicate.

MTT assay. Cell proliferation was assessed using an MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Transfected HeLa and C33A cells were collected after 24 h incubation and seeded in 96-well plates (3x10⁴ cells/well). Subsequently, cells were incubated at 37˚C with 5% CO₂ for 24, 48, 72 and 96 h. At each time point, an MTT assay was performed by adding 20 µl MTT solution (5 mg/ml) to each well and incubating at 37˚C for 4 h. Dimethyl sulfoxide (200 µl; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added and the absorbance at 490 nm was determined using a microplate reader. All experiments were performed in triplicate.

Cell migration and invasion assays. Transwell chambers (pore size, 8 mm; Corning Incorporated, Corning, NY, USA) were used for cell migration and invasion assays. For the cell migration assay, 1x10⁵ cells were suspended in 100 µl of FBS-free DMEM medium and added into the upper Transwell chamber. For the cell invasion assay, 1x10⁵ cells were suspended in 100 µl of FBS-free DMEM medium and added into the upper part of a Transwell chamber coated with 50 µl Matrigel (2 mg/ml; BD Biosciences, San Jose, CA, USA). For the chemoattractant, 500 µl DMEM medium containing 20% FBS was added to the lower chamber. Following incubation for 48 h, the cells...
removing on the upper surface of the membrane were carefully removed with cotton swabs. The cells that had crossed the membrane onto the lower surface were fixed with 100% methanol at room temperature for 10 min, stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 10 min and counted under an IX51 inverted light microscope (x200 magnification; Olympus Corporation, Tokyo, Japan). The average cell number in five fields was taken as the final result for both migration and invasion assays. All experiments were performed in triplicate.

Bioinformatics analysis. The putative target genes for miR-211 were predicted by bioinformatics analysis using TargetScan (http://www.targetscan.org/) (23).

Luciferase reporter assay. Wild-type (Wt) or mutant (Mut) versions of 3'UTR of ZEB1, containing the putative binding sites for miR-211, were separately cloned into the pMIR-REPORT miRNA Expression firefly Reporter vectors. pRL-CMV was obtained from Promega Corporation (E2261). For the luciferase reporter assay, 293T cells were seeded in 24-well plates at a density of 50-60% confluence. Following incubation overnight at 37°C, cells were transfected with miR-211 mimics (50 pmol) or miR-NC (50 pmol), in addition to pMIR-ZEB1-3'UTR Wt (0.2 µg) or pMIR-ZEB1-3'UTR Mut (0.2 µg), using Lipofectamine 2000. After 48 h at 37°C, cells were harvested and luciferase activities were determined with a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Renilla luciferase activity served as an internal reference. All experiments were performed in triplicate.

Western blot analysis. Total proteins were isolated from cells or tissues using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The concentration of total protein was determined by a BCA protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 µg) were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were subsequently blocked with 5% non-fat milk in TBS at room temperature for 2 h and incubated at 4°C overnight with primary antibodies, followed by incubation with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. The primary antibodies used in the present study were as follows: Mouse anti-human monoclonal ZEB1 (sc-81428; 1:1,000; Santa Cruz Biotechnology, Inc.) and mouse anti-human GAPDH (sc-59540; 1:1,000; Santa Cruz Biotechnology, Inc.). Protein bands were detected by Western Chemiluminescent HRP Substrate (ECL; EMD Millipore, Billerica, MA, USA). The band density of each protein was quantified with ImageJ version 1.49 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Data were analyzed using student’s t-test or one-way analysis of variance with the Student-Newman-Keuls multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference. SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis.

Results

miR-211 is downregulated in cervical cancer. The expression of miR-211 in cervical cancer tissues and corresponding adjacent normal tissues was measured by using RT-qPCR. As demonstrated in Fig. 1A, miR-211 expression was significantly lower in cervical cancer tissues compared with adjacent normal tissues (P<0.05). Furthermore, the expression levels of miR-211 in cervical cancer cell lines were also determined. Compared with the Ect1/E6E7 normal human cervix epithelial cell line, as determined by RT-qPCR, *P<0.05 vs. normal tissues or Ect/E6E7 cell line in A and B, respectively. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

miR-211 inhibits HeLa and C33A cell proliferation. To identify the biological roles of miR-211 in cervical cancer, HeLa and C33A cells were transfected with miR-211 mimics or miR-NC. The transfection efficiency was evaluated using RT-qPCR, which demonstrated that miR-211 was markedly upregulated in miR-211 mimic-transfected HeLa and C33A cells compared with miR-NC-transfected cells (P<0.05; Fig. 2A). Subsequently, the effect of miR-211 on HeLa and C33A cell proliferation was determined by an MTT assay. As demonstrated in Fig. 2B, upregulation of miR-211 significantly suppressed the growth rate of HeLa and C33A cells at 72 and 96 h.
miR-211 inhibits HeLa and C33A cell migration and invasion. The effects of miR-211 on HeLa and C33A cell migration and invasion capacities were assessed by cell migration and invasion assays. Results of cell migration and invasion assays revealed that overexpression of miR-211 using mimics reduced the migration and invasion abilities of HeLa and C33A cells compared with miR-NC groups (P<0.05; Fig. 3). These results indicate that miR-211 may act as a tumor suppressor in cervical cancer by inhibiting cell growth and metastasis.

ZEB1 is a direct target of miR-211 in cervical cancer. To investigate the potential molecular mechanism underlying the inhibition of HeLa and C33A cell growth and metastasis by miR-211, bioinformatics analysis was performed to identify the potential target genes of miR-211. Based on bioinformatics analysis, hundreds of potential targets were identified. Among these putative targets, a number of them had previously been reported as direct targets of miR-211 in different cancer types, including preferentially expressed antigen in melanoma (PRAME) as a target of miR-211 in melanoma (24), chromodomain helicase DNA binding protein 5 (CHD5) in colorectal cancer (21), transforming growth factor β receptor II (TGFβRII) in head and neck carcinomas (25), SATB homeobox 2 (SATB2) in hepatocellular carcinoma (26) and cyclin D1 in ovarian cancer (27). The present study selected ZEB1 for further analysis as it was previously reported to be expressed at abnormally high levels in cervical cancer (28), and has been implicated in the tumorigenesis and progression of cervical cancer (29,30).

To confirm whether ZEB1 was a putative target of miR-211, luciferase reporter assays were performed. 293T cells were cotransfected with miR-211 mimics or miR-NC, and pMIR-ZEB1-3’UTR Wt or pMIR-ZEB1-3’UTR Mut (Fig. 4A). As demonstrated in Fig. 4B, transfection with miR-211 mimics decreased the luciferase activities of the luciferase reporter construct carrying the Wt 3’UTR, which contains the potential miR-211 binding site (P<0.05). However, miR-211 overexpression did not affect the luciferase activities in the luciferase reporter construct carrying the Mt 3’UTR containing the potential miR-211 binding site.

Subsequently, RT-qPCR and western blotting analysis was performed to measure ZEB1 mRNA and protein expression in HeLa and C33A cells transfected with miR-211 mimics or miR-NC. The results indicated that the mRNA (Fig. 4C) and protein (Fig. 4D) levels of ZEB1 were significantly reduced following transfection with miR-211 mimics, compared with the miR-NC-transfected cells (P<0.05). The results of these experiments indicate that ZEB1 may be a direct target gene of miR-211 in cervical cancer.

Downregulation of ZEB1 inhibits HeLa and C33A cell proliferation, migration and invasion. Finally, to investigate whether ZEB1 knockdown exhibits similar tumor suppressive roles to miR-211 overexpression in HeLa and C33A cells, siRNA targeting ZEB1 was employed to downregulate ZEB1 expression. Following transfection, ZEB1 expression in HeLa and C33A cells was detected by western blot analysis. As demonstrated in Fig. 5A, the expression levels of ZEB1 were significantly reduced in HeLa and C33A cells following transfection with ZEB1 siRNA, compared with NC siRNA-transfected cells (P<0.05).

Subsequently, an MTT assay was performed to determine the effect of ZEB1 downregulation on the proliferation of HeLa and C33A cells. The results demonstrated that downregulation of ZEB1 suppressed the proliferation of HeLa and C33A cells compared with NC siRNA-transfected cells at 72 and 96 h (P<0.05; Fig. 5B). Furthermore, the present study...
also investigated the effect of ZEB1 knockdown on HeLa and C33A cell motility by cell migration and invasion assays. The results demonstrated that in ZEB1 downregulated HeLa and C33A cells, the migration and invasion abilities were significantly reduced compared with NC siRNA-transfected groups (P<0.05; Fig. 5C). Collectively, these results indicate that ZEB1 knockdown exhibited similar tumor-suppressive effects to miR-211 overexpression in cervical cancer, which further confirms that ZEB1 may be a direct functional target gene of miR-211.
miR-211 is located at chromosome 15q13, which is a locus that is frequently deleted in cancer (31-33). Numerous studies have reported that miR-211 is abnormally expressed in various cancer types. For example, miR-211 was demonstrated to be upregulated in oral carcinoma samples and high miR-211 expression was associated with advanced nodal metastasis.
vascular invasion and a poor prognosis (19). Expression levels of miR-211 were also reported to be increased in colon cancer tissues and statistically associated with age. Survival analysis revealed that patients with colon cancer that exhibited high miR-211 expression had a shorter survival time compared with patients with lower miR-211 expression. Furthermore, miR-211 was validated as a risk factor for colon cancer prognosis (20). An upregulation of miR-211 has also been reported in colorectal cancer (21) and head and neck carcinomas (25). However, in melanoma, Mazar et al (34) reported that miR-211 was downregulated in tumor cell lines compared with normal melanocytes. In addition, expression levels of miR-211 were reduced in melanoma tissues. In addition, Maftouh et al (35) demonstrated that miR-211 was downregulated in pancreatic cancer and significantly associated with prognosis, and miR-211 was also reported to be downregulated in breast cancer (36), hepatocellular carcinoma (26) and ovarian cancer (27). These studies indicate that the expression levels of miR-211 in human cancers exhibits tissue specificity and may have important roles in these types of human cancer.

Dysregulation of miR-211 expression is reported to be implicated in the initiation and progression of human cancers. In oral carcinoma, a high expression of miR-211 was associated with increases in cell proliferation, migration and the formation of anchorage-independent colonies (19). Cai et al (21) reported that miR-211 overexpression enhanced the cell growth and invasion of colorectal cancer in vitro and in vivo. Additionally, in non-small cell lung cancer, miR-211 promoted cell proliferation, colony formation and invasion (37). These results indicate that miR-211 may exhibit oncogenic roles in human cancer. However, in melanoma, miR-211 functioned as a tumor suppressor with suppressive roles in cell growth and invasion (34). In pancreatic cancer, induction of miR-211 expression reduced the migration and invasion of pancreatic cancer cells. Furthermore, enhanced miR-211 expression enhanced the chemosensitivity of pancreatic cancer cells to gemcitabine (35). In breast cancer, overexpression of miR-211 suppressed the growth, cell cycle, migration and invasion of triple-negative breast cancer cells (36). Additionally, in ovarian cancer, enforced miR-211 expression inhibited the cell proliferation, enhanced apoptosis and arrested cells in the G0/G1-phase (27). Furthermore, miR-211 has been identified as a tumor suppressor in numerous types of human cancer, including hepatocellular carcinoma (26) and gastric cancer (38). These studies reported contradictory results in that miR-211 was reported to be an oncogene in certain cancer types and a tumor suppressor in others. These conflicting results may be explained by the ‘imperfect complementarity’ of the interactions between miRNAs and target genes (39).

To the best of our knowledge, the present study was the first to provide sufficient evidence that the expression levels of miR-211 were reduced in cervical cancer tissues and cell lines compared with those in corresponding adjacent normal tissues and the normal human cervix epithelial cell line, respectively. Therefore, it may be hypothesized that miR-211 acts as a tumor suppressor in cervical cancer carcinogenesis and progression. Notably, overexpression of miR-211, using miR-211 mimics, inhibited the cell proliferation, migration and invasion of cervical cancer cells. These results indicate that miR-211 may provide potential therapeutic targets for cervical cancer treatment.

miRNAs exert functional roles through incomplete pairing with the 3’UTR of their target genes and regulating the expression level of target genes. Therefore, it is important to validate the direct target genes of miR-211. Previously, various target genes of miR-211 were identified, including laZ in oral carcinoma (19), SRC kinase signaling inhibitor I in non-small cell lung cancer (37), PRAME in melanoma (24), CHD5 in colorectal cancer (21), TGFβRII in head and neck carcinomas (25), cell division cycle 25B in breast cancer (36), SATB2 in hepatocellular carcinoma (26), and cyclin D1 and cyclin-dependent kinase 6 in ovarian cancer (27). In the present study, a molecular association between miR-211 and ZEB1 was demonstrated. ZEB1, a member of the zinc finger family, is located on the short arm of human chromosome 10 (40). Previous studies have reported that ZEB1 was upregulated in breast, ovarian, endometrial and prostate cancer. Upregulation of ZEB1 was also reported to be associated with poor differentiation, aggressive disease, the development of metastases and a poor clinical prognosis in abovementioned cancer types (41-44). Additionally, Ma et al (28) demonstrated that ZEB1 expression was increased in cervical cancer tissues, and was significantly associated with differentiation status, the occurrence of vascular invasion and metastasis to lymph nodes in cervical cancer. Furthermore, Chen et al (29) demonstrated that the expression of ZEB1 was associated with FIGO stage and lymph node metastasis. Functionally, downregulation of ZEB1 decreased the proliferation and metastasis capacities of cervical cancer cells (30). These results indicate that ZEB1 may be considered a valuable therapeutic target for cervical cancer treatment.

In conclusion, the present study investigated miR-211 expression and its functional roles in regulating the growth and metastasis of cervical cancer cells at the cellular level. The present study reported that miR-211 may act as a tumor suppressor in cervical cancer, providing a theoretical basis for its application in the treatment of cervical cancer.

References
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