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MicroRNA-590 Promotes Cervical Cancer Cell Growth and Invasion by Targeting CHL1

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ABSTRACT

MicroRNAs (miRNAs) may function as oncogenes or tumor suppressors. Here, we identified that miR-590-5p was up-regulated in human cervical cancer. Over-expression of miR-590-5p promoted cervical cancer cell growth, cell cycle and invasion via Growth curve, Colony formation, FACS and Transwell assays in HeLa and C33A cell lines. Subsequently, CHL1 was identified as a potential miR-590-5p target by bioinformatics analysis. Moreover, we showed that CHL1 was negatively regulated by miR-590-5p at the posttranscriptional level, via a specific target site within the 3′UTR by luciferase reporter assay. Furthermore, the mRNA and protein levels of CHL1 in cervical cancer cells were downregulated by miR-590-5p. And we identified the cell phenotype altered by miR-590-5p can be rescued by over-expression of CHL1. Therefore, our findings suggest that miR-590-5p acts as an oncogene by targeting the CHL1 gene and promotes cervical cancer proliferation. The findings of this study contribute to current understanding of the functions of miR-590-5p in cervical cancer. J. Cell. Biochem. 115: 847–853, 2014.

KEY WORDS: miR-590-5p; CHL1; CERVICAL CANCER; CELL INVASION; CELL CYCLE

Cervical cancer is the second leading cause of cancer morbidity and mortality for women around the world [Gocze et al., 2013]. However, the high mortality rate in most developing countries is largely due to the lack of organized screening and diagnostic programs, as well as effective therapies [Chung et al., 2010; Su et al., 2013]. It is estimated approximately 500,000 new cases of cervical cancer are reported annually and about 230,000 women die of cervical cancer annually [Shanta et al., 2000; Paavonen, 2007]. Therefore development of effective prevention and therapeutic procedures is needed to reduce the high disease burden [Khaliq et al., 2012].

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous small non-coding and double stranded RNA molecules that function as post-transcriptional gene regulators [Gilabert-Estelles et al., 2012]. Accumulating evidence shows that different cell types and cancers at each developmental stage could have a distinct miRNA expression profile, that may involved in many physiological and pathological progress such as inflammation, cell cycle regulation, differentiation, tumorigenesis and apoptosis, including miR-143 and miR-145 in prostate cancer, miR-30b/30d, miR-370 in gastric carcinoma and miR-345 in human colorectal cancer [Tang et al., 2011; Gilabert-Estelles et al., 2012; Lo et al., 2012; Roa et al., 2012; Altmae et al., 2013; Olivieri et al., 2013].

Close Homologue of L1 (CHL1), a type I transmembrane protein which belongs to L1 family of cell adhesion molecules (CAMs), is initially identified during neuronal development and essential for nervous system [Demyanenko et al., 2010]. Recently many studies have shown that CHL1 is expressed in a variety of human cancer cell lines and primary tumor tissues. Moreover, a frequent decrease of an expression of CHL1 was observed in breast, colon, rectum, thyroid, kidney and small intestine cancers [Senchenko et al., 2011], indicating that CHL1 may acts as a putative tumor suppressor. Functional studies showed that ectopic expression of CHL1 in NPC cells significantly inhibited their clonogenicity, cell cycle and induced apoptosis as compared to control cells without CHL1 expression [Chen et al., 2012]. Thus, these reports suggest that CHL1 plays a critical role in cancer development, not only in neuronal activities. It is predicted that many miRNAs can target CHL1, therefore we

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explored the predicted miRNA, miR-590-5p, in the regulation of CHL1 in cervical cancer cells.

In this study, we identified miR-590-5p as an oncogene in cervical cancer cell lines HeLa and C33A. We found over-expression of miR-590-5p increased cell proliferation and invasion while miR-590-5p ASO decreased cell proliferation and invasion via MTT, colony formation, cell invasion assays. CHL1 was validated as a target of miR-590-5p and down-regulated by miR-590-5p at both the transcriptional and translational level. Finally we found miR-590-5p induced cell proliferation and invasion can be rescued by over-expression of CHL1. These results provide a better understanding of the molecular mechanism of miR-590-5p in the initiation and progression of cervical cancer.

MATERIALS AND METHODS

HUMAN CANCER TISSUES CLINICAL SPECIMEN

Twenty-two fresh frozen human cervical cancer tissue samples and matched normal cervical tissue samples were obtained from the Cancer Institute and Hospital and the National Foundation of Cancer Research. The category of specimens was confirmed by pathological analysis. Large and small RNAs were isolated from tissue samples using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions.

CELL CULTURE AND TRANSFECTION

The human cervical cancer cell line HeLa cells and C33A cells were grown in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum separately, incubated at 37°C in a humidified chamber with 5% CO_2. Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Briefly, cells were seeded in plates the day before transfection to ensure a suitable cell confluence on the day of transfection. miR-590-5p mimics and ASO were purchased from GenePharma Biotechnology (Shanghai, China).

VECTOR CONSTRUCTION AND LUCIFERASE REPORTER ASSAY

To construct CHL1-3′UTR plasmid, a wild-type 3′-UTR fragment of human CHL1 mRNA containing the putative miR-590-5p binding sequence was amplified by PCR and cloned into downstream of the firefly luciferase gene in the pMIR-REPORT vector (Ambion) between *Hin*dIII and *Spe*I sites to produce the pMIR-CHL1-3′UTR luciferase vector (CHL1 3′UTR) using the primer: CHL1-3′UTR-Sense, 5′-CGGACTAGTCTATACATCCACAGGGTT-3′ and CHL1-3′UTR-anti-sense, 5′-CCCAAGCTTTCTTTAGCCACTTCAGTT-3′. For the mutant reporter vector, seed sequences of miR-590-5p-binding sites in CHL1-3′UTR fragment were mutated using the QuikChange Mutagenesis Kit (Stratagene, USA). The mutated CHL1-3′-UTR fragment was cloned into pMIR-REPORT vector to develop the pMIR-CHL1-3′UTR-mut vector (CHL1-3′UTR-mut). For the luciferase assay in HeLa cells, cells were cotransfected in 48-well plates with CHL1 3′UTR or CHL1-3′UTR-mut, miR-590-5p mimics or ASO using Lipofectamine 2000 reagent. Twenty-four hours later, luciferase activity was measured by using a dual luciferase reporter assay (Promega, USA) on a Fluorescence Spectrophotometer F4500 (HITACHI). The results were expressed as relative luciferase activity (firefly Luc/Renilla Luc). All experiments were repeated three times in triplicate.

CELL GROWTH CURVE AND COLONY FORMATION ASSAY

For Cell growth curves, treated cells were seeded in 24-well plates (4,000 cells per well) in triplicate. At the different times, the cells were detached using trypsinization, and counted by a hemocytometer. For colony formation assay, the cells were seeded in 12-well plates at a density of 5900 cells per well. Fresh culture medium was replaced every 3 days. After 7 days of culture, the cells were stained with crystal violet, and the numbers of colonies were counted.

TRANSWELL INVASION ASSAY

BD Falcon™ 8.0-μm pore Transwell cell culture inserts (353097; BD Biosciences, Franklin Lakes, NJ) were used to evaluate cell migration. The inserts were placed in a 24-well plate, containing 700 μl of medium with 10% FBS (lower chamber), for 30 min before seeding cells. After 48 h of transfection, 5×10^4 HeLa cells and 7×10^4 C33A cells (in 0.2 ml RPMI 1640 without FBS) were placed on the top chamber of each insert without or with 30 μl of 1 mg/ml Matrigel. The lower chamber was filled with 600 μl of RPMI 1640 medium with 20% FBS to act as the nutritional attractant. At the end of incubation, non-migrated cells on the top surface of membrane were removed using cotton swabs, followed by washing with PBS. Migrated cells on the bottom surface of membrane were fixed, stained, and counted. All the experiments were performed independently in triplicate.

REAL-TIME RT-PCR AND WESTERN BLOT

Real-time RT-PCR was used to detect the relative level of CHL1 transcription, Briefly, cDNA library was generated through reverse transcription using M-MLV reverse transcriptase (Promega) with 5 μg of total RNA. The cDNA was used to amplify the CHL1 gene, and the β-actin gene was used as an endogenous control. The PCR primer sequences were as follows: CHL1-3UTR-sense, 5′-CGGACTAGTCTATACATCCACAGGGTT-3′; and CHL1-3UTR-anti-sense, 5′-CCCAAGCTTTCTTTAGCCACTTCAGTT-3′; β-actin sense, 5′-CGTGACATTAAGGAGAAGCTG-3′; and β-actin antisense, 5′-CTAGAAGCATTTGCGGTGGAC-3′. The PCR conditions were as follows: 94°C for 5 min, then did the procedure which is 94°C for 1 min, 56°C for 1 min and 72°C for 1 min by 35 cycles. For western blot, the cells were lysed with RIPA lysis buffer and proteins were harvested. All proteins were resolved on a 8% SDS denatured polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membranes were incubated overnight at 4°C with polyclonal rabbit anti-human CHL1 and rabbit anti-human GAPDH. The membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody. Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. Lab Works™ Image Acquisition and Analysis Software (UVP, Upland, CA) was used to quantify band intensities. Antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

FLOW CYTOMETRY ANALYSIS

Transfected HeLa cells were seeded into six-well plates for 24 h in complete medium. Next, cells were deprived of serum for 48 h and then returned to complete medium for an additional 24 h. All cells

were collected by centrifugation, fixed in 95% ethanol, incubated at −20°C overnight and washed with phosphate buffered saline (PBS). Then, cells were resuspended in 1 ml of FACS solution (PBS, 0.1% TritonX-100, 60 μg/ml propidium iodide (PI), 0.1 mg/ml DNase free RNase, and 0.1% trisodium citrate). After a final incubation on ice for 30 min, cells were analyzed using a FACS Calibur flow cytometer (Beckman Coulter). A total of 10,000 events were counted for each sample.

STATISTICAL ANALYSIS

Statistical analysis utilized two-tailed Student's *t*-test. Statistical significance was set to $P < 0.05$.

RESULTS

miR-590-5P IS UP-REGULATED IN HUMAN CERVICAL CANCER TISSUES

In order to investigate the role of miR-590-5p in cervical cancer, we performed research to measure the expression levels of miR-590-5p in 22 pairs of human cervical cancer tissues and adjacent normal tissues by real-time PCR assay. As shown in Figure 1, the expression level of miR-590-5p was higher in 22 cervical cancer tissues compared to their matched normal cervical tissues with two exceptions. The result implies that miR-590-5p is increased in cervical cancer tissues and may be serve as oncogene involved in cervical carcinogenesis.

miR-590-5p promotes cervical cancer cell line HeLa and C33A growth and invasion. With the above findings that miR-590-5p may serve as cervical cancer oncogene, we then tested the effect of miR-590-5p on HeLa and C33A cell growth using growth curve and colony formation. Firstly, we transfected either miR-590-5p mimics or miR-590-5p ASO into HeLa cells and detected miR-590-5p level by Real-time PCR. As shown in Figure 2A, the level of miR-590-5p was obviously increased in HeLa cells transfected with miR-590-5p mimics as compared to cells transfected with NC mimics. Transfection with miR-590-5p ASO resulted in a significant reduction in miR-590-5p level comparing to NC ASO. Next, we used growth curve assay to determine the effect on cell viability. As shown in Figure 2B,C, cells transfected with miR-590-5p had increased cell viability, whereas cells transfected with miR-590-5p ASO had reduced cell viability. In consistent with this result, we confirmed the effect of miR-590-5p on the long-term proliferative capacity of HeLa cells with colony formation. As shown in Figure 2D, the colony number of HeLa cells with miR-590-5p mimics increased about twofold than that of control group. Conversely, the ability of colony formation in HeLa cells with miR-590-5p ASO was 60% lower than that of HeLa cells transfected with NC ASO. Furthermore, FACS was used to analyze effect of miR-590-5p on the cell cycle of HeLa cells. Overexpression of miR-590-5p resulted in an increase of cells in S phase (from 19% to 39%) and a decrease of cells in G1 phase (from 76% to 57%) compared to the negative control (Fig. 2E). We observed similar results in another cervical cancer cell line, C33A cells.

To further test the function of miR-590-5p on cervical cancer cell, we performed cell invasion assays to identify whether miR-590-5p was associated with the invasion of cervical cancer cells. Compared with the cell transfected with NC mimics, the number of cells that migrated through the basement membrane of the transwell chamber increased approximately 2.7-fold in HeLa and 2.4-fold in C33A cells transfected with miR-590-5p mimics, whereas it decreased 58% in HeLa cells and 63% in C33A cells transfected with miR-590-5p ASO (Fig. 2F). These results demonstrated that miR-590-5p can promote the proliferation and invasion of cervical cancer cells.

miR-590-5P NEGATIVELY REGULATE CHL1 THROUGH DIRECTLY TARGETING ITS 3′UTR

Bioinformatics searches identified CHL1 might be a putative target gene of miR-590-5p (Fig. 3A). Therefore, we assessed whether miR-590-5p had a functional role in the regulation of CHL1 expression. As

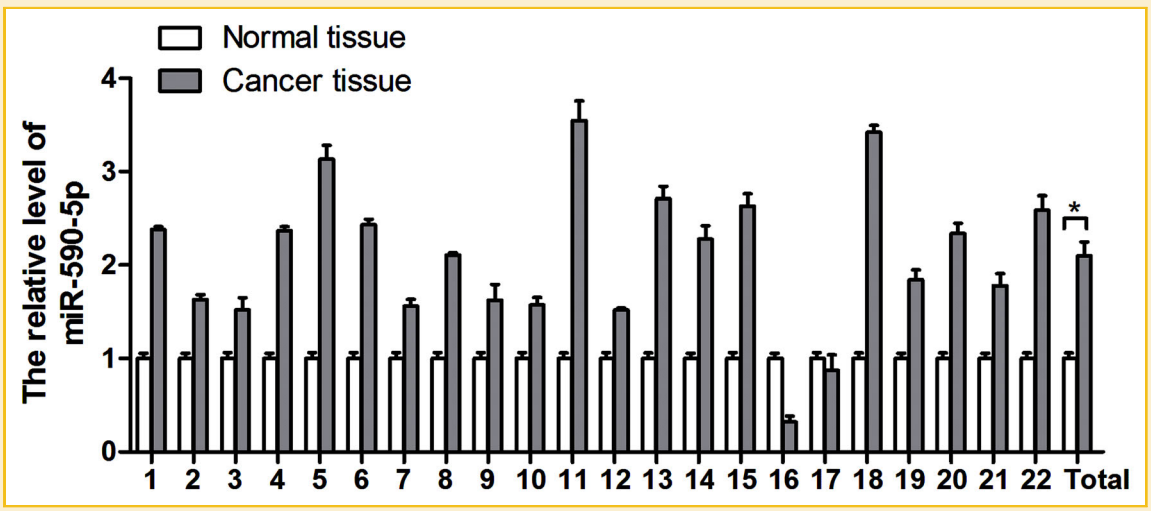

Fig. 1. Quantitative analysis of miR-590-5p expression in human cervical cancer tissues. miR-590-5p expression levels in 22 pairs of cervical cancer tissues and matched adjacent normal tissues were measured by real-time PCR. U6 snRNA was used as an endogenous control, and the relative expression levels of miR-590-5p are shown (*$P < 0.05$).

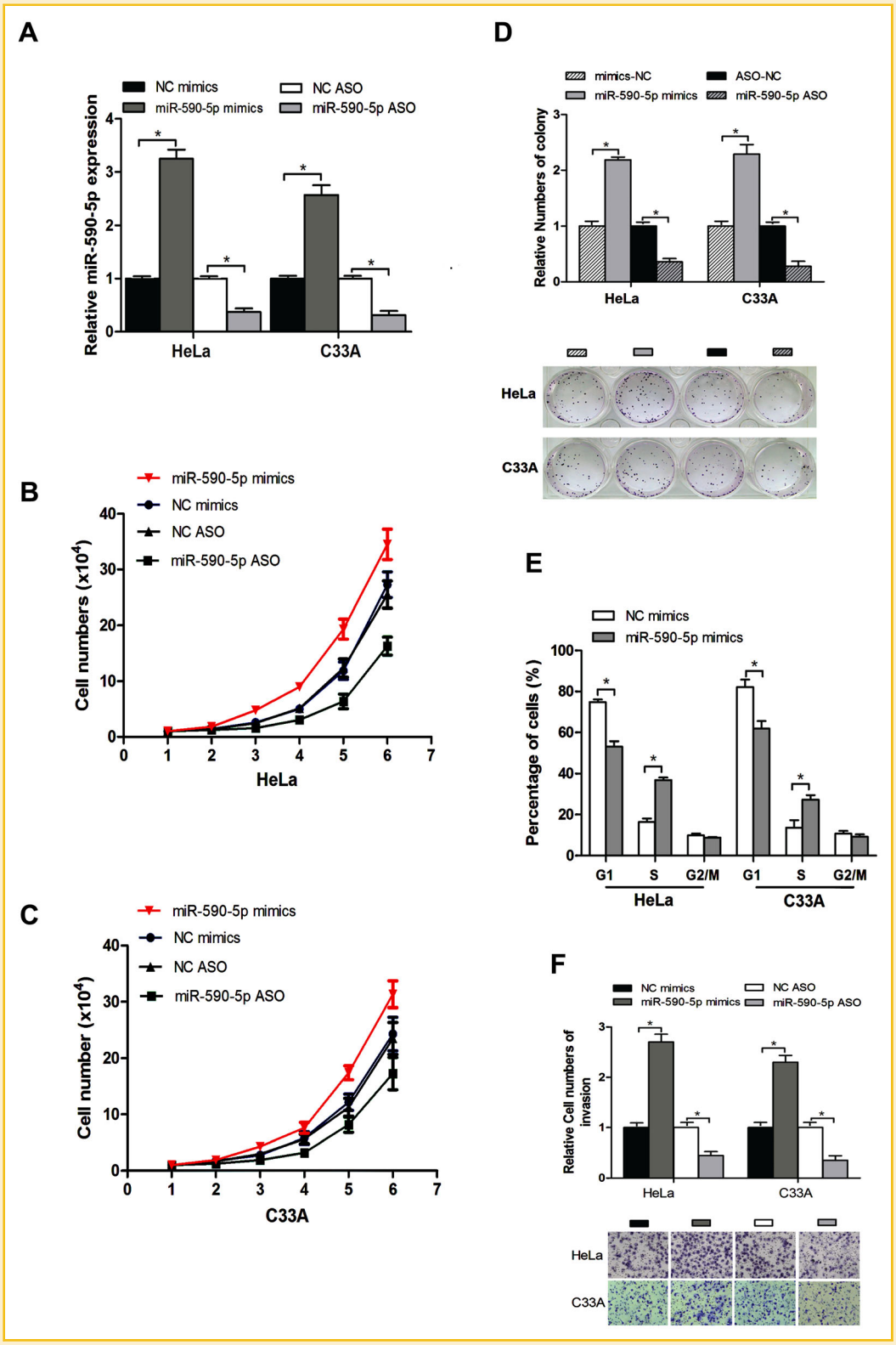

Fig. 2. miR-590-5p promotes growth and invasion of HeLa and C33A cells. A: miR-590-5p mimics or ASO was transfected into the cells and real-time PCR was performed to determine miR-590-5p expression. The effects of overexpression or knockdown of miR-590-5p on cell viability (B,C) was detected by growth curve assay and its long-term proliferative capacity (D) was detected by colony formation. E: FACS analysis was performed to determine the cell cycle distribution of HeLa and C33A cells transiently transfected with miR-590-5p mimics or ASO. F: The changes of cell invasion ability induced by miR-590-5p over-expression were determined by Transwell assay. Representative images are shown at the top ($^{*}P < 0.05$).

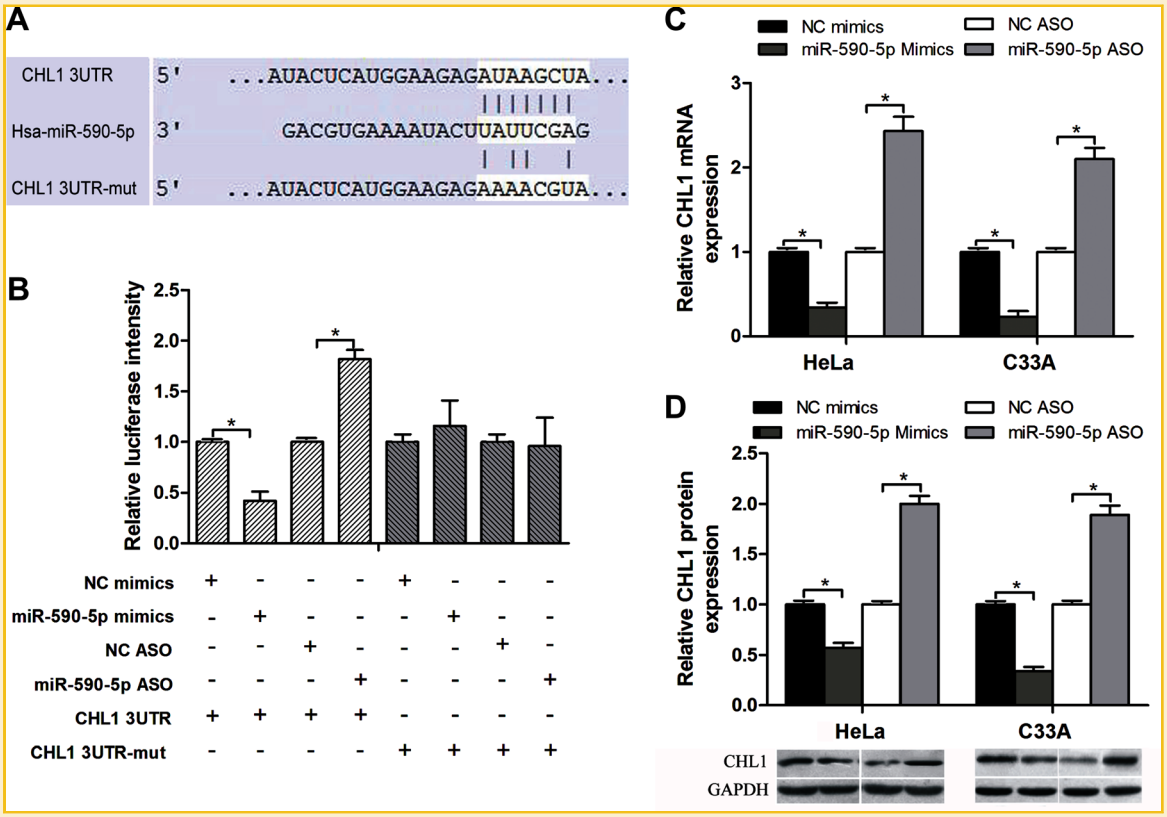

Fig. 3. miR-590-5p directly targets CHL1 and inhibits its expression. A: A schematic of the bioinformatics predicted seed region in the 3′UTR of CHL1, as well as mutated 3′UTR used in this study. B: Effect of miR-590-5p mimics or ASO on the luciferase activity of the plasmid CHL1-3′UTR and CHL1-3′UTR-mut. For the reporter assays, HeLa cells were transfected and then harvested for lysis of cells 48 h after transfection. (C,D) Real-time PCR and Western blot shows suppression of the CHL1 mRNA and protein by miR-590-5p mimics. Blockade of miR-590-5p by ASO causes up-regulation of CHL1.

expected, we confirmed that the ectopic expression of miR-590-5p mimics substantially suppressed CHL1 mRNA and protein expression with real-time PCR (Fig. 3B) and Western blot (Fig. 3C), respectively. To further determine the specificity that whether miR-590-5p directly targets CHL1, we constructed a luciferase reporter carrying CHL1 3′-UTR with a putative miR-590-5p binding site. We detected a reduction of luciferase activity by 56% in the miR-590-5p mimics transfected cells compared with NC mimics and miR-590-5p ASO increased the luciferase activity (Fig. 3D). This suppression effect was specific to miR-590-5p because miR-590-5p mimics and ASO had no effect on the luciferase activity when cells cotransfected with CHL1 3′-UTR-mut in which the seed sequence was mutated (Fig. 3A). Thus, these results demonstrated that miR-590-5p directly targets CHL1 and this specific silencing of CHL1 by miR-590-5p accounts at least in part for the miR-590-5p mediated inhibition of cervical cancer cell growth.

Role of CHL1 in the miR-590-5p mediated cell proliferation and invasion. To further determine the role of CHL1 in miR-590-5p regulated cervical cancer cells, we transfected pcDNA3-CHL1 into HeLa cells to evaluate the effects caused by over-expression of miR-590-5p. First, Western blot was used to detect the CHL1 protein level and we identified that the level of CHL1 can be rescued in miR-590-5p and pcDNA3-CHL1 cotransfected cells compared to the control. The colony formation confirmed that miR-590-5p induced cell proliferation can be rescued by CHL1 over-expression (Fig. 4A,B). Cell invasion assay was performed in HeLa cells co-transfected miR-590-5p and pcDNA3-CHL1. As shown in Figure 4C, the induction effect on cell invasion caused by miR-590-5p can be entirely abrogated by the over-expression of CHL1. These results suggested that miR-590-5p-induced HeLa cell growth was mediated by CHL1.

DISCUSSION

Recently accumulated evidences support a role for miRNAs as diagnostics biomarkers and therapeutic targets for human cancers [Iorio and Croce, 2012]. MicroRNA expression has higher tissue specificity and regulates multiple targets to amplify their biological effects, with biologically and clinically significance [Su et al., 2012].

It is identified that miR-590-5p could promote cardiomyocyte proliferation in both neonatal and adult animals [Eulalio et al., 2012]. Watson et al. [2013] found in high risk cases of Wilms's tumor blastema, miR-590-5p was upregulated. Downregulation of miR-590-5p inhibited proliferation and invasion of hepatocellular carcinoma cells by directly targeting TGF-beta RII [Jiang et al., 2012]. In the present study, we identified miR-590-5p has

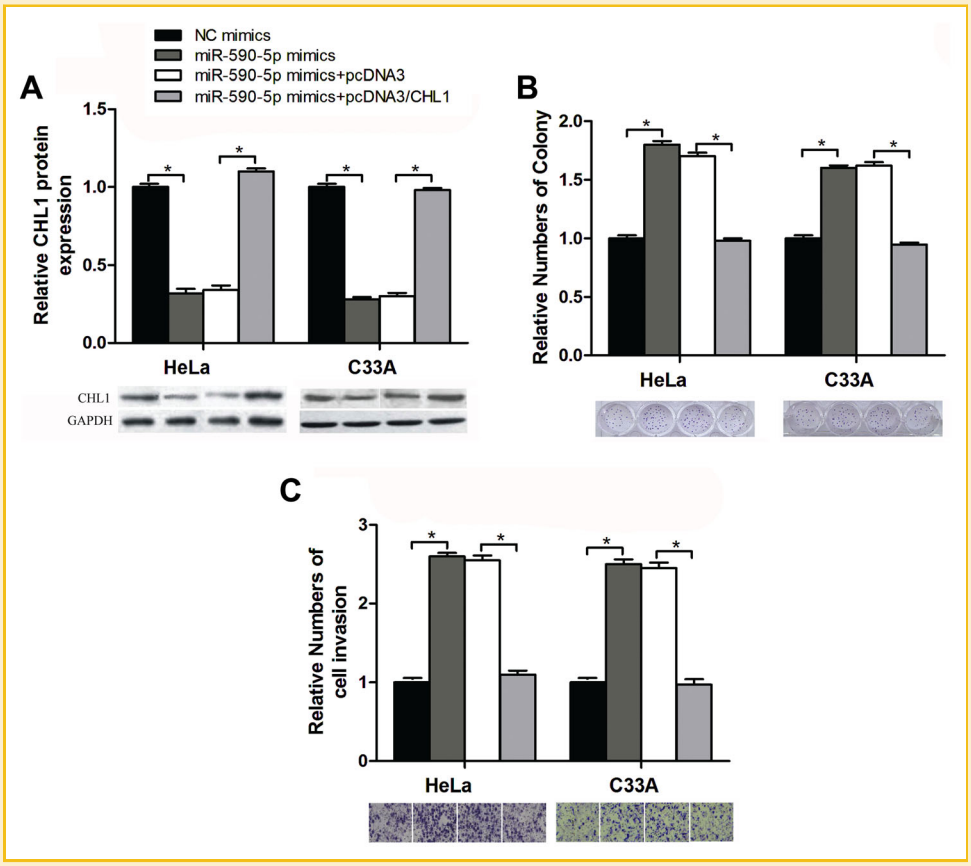

Fig. 4. CHL1 can rescue miR-590-5p induced cell proliferation and invasion. Cells were transfected with miR-590-5p mimics, or cotransfected with pcDNA3/CHL1. Then cells were lysed for protein and Western blot was used to detect CHL1 protein (A), seeded in the six-well for colony formation assay (B), placed in transwell chamber for invasion assay (C).

significant oncogenic activity. We detected the expression of miR-590-5p in 22 pairs of human cervical cancer and normal tissues by real-time PCR. Our data showed that the expression of miR-590-5p in 22 pairs of human cervical cancer was increased with two exceptions, which prompted miR-590-5p may play the role of oncogene. To elucidate its functional role in cervical cancer cells, we performed MTT and colony formation assays to detect miR-590-5p on cell viability and long-term proliferative capacity. The assays showed miR-590-5p mimics increased cell proliferation, while miR-590-5p ASO decreased cell proliferation. Furthermore, cell invasion assay was performed and demonstrated that miR-590-5p can induce cervical cancer cell invasion significantly.

The functional role of miRNA that promote proliferation and invasion is through its target genes incomplete pairing with mRNA 3′UTR to influence the expression level of target gene. Therefore, it is of great importance to find and identify the direct target gene of miR-590-5p and clarify the role of its regulation of biological processes. Since miR-590-5p is highly expressed in cervical cancer and associated with the malignant phenotype, therefore target genes of miR-590-5p may have anti-proliferative activity. Using the biological information technology, we found predicted target genes for the miR-590-5p, in which we were interested in cell adhesion molecule CHL1 which are Close Homologue of L1 family of cell adhesion molecules (CAMs). Studies have shown that CHL1 was down-regulated in many types of tumors, which indicated a possible correlation with miR-590-5p. To detect whether miR-590-5p affected CHL1 expression, RT-PCR and Western blot were used. The results showed that CHL1 was down-regulated in mRNA and protein level when cells were transfected with miR-590-5p mimics, while the level of CHL1 was increased in cells transfected with miR-590-5p ASO. Furthermore, we constructed a CHL1 3′UTR fluorescent reporter vector containing the potential miR-590-5p binding site and showed that miR-590-5p can down-regulate the luciferase activity significantly, indicating miR-590-5p can directly target CHL1 and negatively regulate CHL1 expression. The subsequent rescue assay further confirmed miR-590-5p induced cell proliferation and invasion was partly mediated by CHL1. Recently, CHL1 was also confirmed as a direct target of miR-590-5p in cervical cancer cells [Long et al., 2012], which indicate important role of CHL1 in cervical cancer pathogenesis.

The target gene CHL1 is a member of the L1 family of neural cell adhesion molecules [Ye et al., 2008]. It is originally identified as a neural recognition protein that may be involved in signal transduction pathways. The gene is also involved in general cognitive activities and neurological diseases such as schizophrenia [Sakurai et al., 2002]. Recently, several cell adhesion molecules, including CHL1, were shown to be involved in cancer growth and metastasis

[Senchenko et al., 2011]. Therefore miR-590-5p may function in more types of diseases. Because tumor growth and invasion are responsible for the majority of deaths of cancer patients, the development of therapeutic agents that inhibit tumor development is of paramount importance. Our present study of miR-590-5p/CHL1 may function as a therapeutic target and clinical biomarker. A further study is needed to confirm their clinic application in other cancer.

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