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miR-196a targets netrin 4 and regulates cell proliferation and migration of cervical cancer cells

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ABSTRACT

Recent research has uncovered tumor-suppressive and oncogenic potential of miR-196a in various tumors. However, the expression and mechanism of its function in cervical cancer remains unclear. In this study, we assess relative expression of miR-196a in cervical premalignant lesions, cervical cancer tissues, and four cancer cell lines using quantitative real-time PCR. CaSki and HeLa cells were treated with miR-196a inhibitors, mimics, or pCDNA/miR-196a to investigate the role of miR-196a in cancer cell proliferation and migration. We demonstrated that miR-196a was overexpressed in cervical intraepithelial neoplasia 2–3 and cervical cancer tissue. Moreover, its expression contributes to the proliferation and migration of cervical cancer cells, whereas inhibiting its expression led to a reduction in proliferation and migration. Five candidate targets of miR-196a chosen by computational prediction and Cervical Cancer Gene Database search were measured for their mRNA in both miR-196a-overexpressing and -depleted cancer cells. Only netrin 4 (NTN4) expression displayed an inverse association with miR-196a. Fluorescent reporter assays revealed that miR-196a inhibited NTN4 expression by targeting one binding site in the 3′-untranslated region (3′-UTR) of NTN4 mRNA. Furthermore, qPCR and Western blot assays verified NTN4 expression was downregulated in cervical cancer tissues compared to normal controls, and in vivo mRNA level of NTN4 inversely correlated with miR-196a expression. In summary, our findings provide new insights about the functional role of miR-196a in cervical carcinogenesis and suggested a potential use of miR-196a for clinical diagnosis and as a therapeutic target.

1. Introduction

Cervical cancer, the third most common gynecologic malignancy, brings about approximately 233,000 deaths per year worldwide [1]. Significant research efforts have revealed that high-risk human papillomavirus (HPV) infection is a necessary factor for cervical cancer development [2]. HPV infection contributes to the stepwise progression to cervical cancer, but single HPV infection is not sufficient to induce the malignant transformation. There must be other unidentified molecular events downstream of the HPV infection which results in the transformation of cervical epithelium to cancer. Therefore, the identification of such molecular mechanisms would be of considerable importance for the screening and treatment of cervical cancer [3].

MicroRNAs (miRNA) are a class of endogenous small noncoding RNAs recently identified to regulate gene expression [4]. Mature miRNAs, often 19–25 nucleotide-length noncoding RNA, that bind within the 3′ untranslated region (3′-UTR) of target genes to abolish or inhibit translation [5]. miRNAs play pivotal regulatory roles in cell growth, proliferation, differentiation and cell death as well as tumorigenesis [6]. Past studies have identified a range of aberrantly regulated miRNAs in cervical cancer tissues and cell lines, with miRs-127, 9, 203, 199a, 218, 21, 143, 205, 214, 126, 15b, 16, 146a and 155 amongst the most common [7]. Furthermore, functional experiments have revealed that miRs-203, 205 and 214, play important roles in mediating malignant behavior of human cervical cancer cells [8–10]. In cervical cancer cells, miR-203 targets vascular endothelial growth factor A to suppress tumor cell growth and angiogenesis [10], miR-205 targets genes including cysteine-rich angiogenic inducer 61 and connective tissue growth factor to maintain the proliferative and migratory property of the cervical cancer cells [9], and miR-214 targets polypeptide N-acetylgalactosaminyltransferase 7 to suppress growth and invasiveness of cervical cancer cells [8]. These findings indicate miRNAs are important triggers for imbalance between oncogenes or tumor suppressors underlying carcinogenesis.

miR-196a has been found to express aberrantly in multiple types of cancer and regulate the behavior of malignant cells

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[11,12]. miR-196a is upregulated in gastric cancer and promotes cell proliferation [11], has been shown to drive malignant characteristics in gastrointestinal stromal tumors [12]. More recently, Gocze et al. found that miRNA-196a was differentially expressed in cervical squamous cell carcinoma and adenocarcinoma [13], suggesting miR-196a involvement in pathogenesis of cervical cancer. However, at present there is limited information about the role of miR-196a in cervical carcinogenesis. In this study, we investigated the miR-196a expression in cervical specimens ranging from histologically normal through to invasive cervical cancer, and evaluated its functional influence on proliferation and migration of cervical cancer cells. Moreover, we found that netrin 4 is a target for miR-192a in cervical cancer cells.

2. Materials and methods

2.1. Specimen collection

We collected 70 snap-frozen specimens from the patients who underwent colposcope examination or excision treatment in Liaocheng People's Hospital. The specimens were bisected immediately after removal from patients; one-half was used for conventional histopathologic examination, and the other half was flash-frozen in liquid nitrogen for later analysis. The human HPV genotyping was performed for these specimens using the HPV GenoArray test kit (HybriBio). The samples were grouped based on histopathological examination of hematoxylin and eosin-stained paraffin sections and HPV status. The samples consisted of 15 cervical squamous cancer tissues (all infected with high-risk HPV); 18 precancerous CIN2-3 lesions (2 tissues with low-risk HPV; 16 tissues with high-risk HPV); 13 mildly dysplastic CIN1 lesions (9 tissues with low-risk HPV; 4 tissues with high-risk HPV); and 24 normal cervical tissues (9 HPV negative and 15 HPV positive tissues). All study procedures were approved by the Ethics Committee of Liaocheng People's Hospital, and all patients gave informed written consent.

2.2. Cervical cancer cell lines

Four human cervical cell lines were used, including CaSki, C33A, HeLa and SiHa. These lines were all purchased from the Type Culture Collection of Chinese Academy of Sciences. The cell lines HeLa and C33A were grown in RPMI 1640 (GIBCO), and SiHa and CaSki were cultured in DMEM medium. All cells were supplemented with FBS and 1% penicillin/streptomycin (Invitrogen) and cultured at 37 °C and 5% CO_2 in a humidified incubator.

2.3. miRNA mimic and inhibitor transfection

The functional effects of miR-196a expression on cervical cancer were investigated by manipulating miRNA expression. miR-196a inhibitor (Anti-miR-196a; Ambion) was transfected into CaSki cells to inhibit miR-196a expression with Anti-miR-NC (GenePharma) as a negative control. A mature miR-196a mimic (Sigma–Aldrich) was transfected into HeLa cells to overexpress miR-196a, with miR-NC (GenePharma) as a negative control. In addition, pCDNA/miR-196a was transfected into HeLa cells to stably sustain the expression of miR-196a with pCDNA/miR-NC as a negative control [11]. All cells were transfected using Lipofectamine 2000 (Invitrogen).

2.4. Cell proliferation assay and colony formation

We measured cell proliferation by colorimetric assay using the WST-1 reagent Kit (Roche). After 48 h of transfection, 5000 cells/well were incubated for another 48 h. Then, 10 μl of WST-1 reagent was added and incubated for 3 h. The absorbance at 450 nm (for measurement) and 650 nm (as reference) was determined using a microplate reader and analyzed with SoftMax Pro 5 software. The relative cell proliferation was normalized by the respective control. For colony formation, the cells were counted and seeded in 12-well plates at 200 cells/well after transfection. Culture medium was replaced every 3 days. On the 14th day after seeding, the cells were stained using crystal violet, and the colonies were counted manually. Colony formation rate = (number of colonies/number of seeded cells) × 100%.

2.5. Transwell cell migration assay

Assays were performed using BD Falcon™ 8.0-mm pore Transwell cell culture inserts (BD Biosciences). 48 h after transfection, 3×10^4 cells were placed in each well of upper chamber with 100 μl medium and were incubated for 24 h at 37 °C in 5% CO_2. At the end of incubation, non migrated cells were scraped from the upper surface. Migrated cells on the bottom surface were fixed with 4% paraformaldehyde (Sigma–Aldrich) solution for 10 min, washed with PBS and stained with 0.5% crystal violet (Sigma–Aldrich) for 10 min. Cell counting was done by using a microplate reader.

2.6. miR-196a targets prediction

The putative miRNA targets were predicted by the TargetScan, miRanda, PITA, and RNAhybrid algorithms. The putative target genes were further screened out by inquiring a Cervical Cancer Gene Database (http://crdd.osdd.net/raghava/ccdb/index.php). We selected genes that met two standards: (1) they were identified by all four algorithms; (2) they were revealed to be downregulated along with cervical cancer development in previous studies.

2.7. RNA isolation and quantitative RT-PCR

Expression of miR-196a and putative target genes was quantified by quantitative RT-PCR using an Applied Biosystems 7500 Fast Real-time PCR system. Briefly, total RNA was isolated from tissue samples using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's protocol. For miR-196 quantification, RNA was reverse transcribed to cDNA from 1 μg small RNA-enriched RNA from tissues sample or cell lines with the stem-loop reverse transcriptase primer using M-MLV reverse transcriptase (Promega). For mRNA quantification, cDNA was synthesized from 2 μg large RNA fraction using the M-MLV reverse transcriptase. Real-time PCR analyses were conducted with SYBR *Premix Ex Taq*™ kit (TaKaRa). Expression levels were calculated relative to the endogenous snRNA-U6 control and the values expressed as $2^{-\Delta\Delta Ct}$. All primers were purchased from AuGCT, Inc., and the sequences are shown in Supplementary Table 1.

2.8. Fluorescent reporter assay

A fragment of 3′-UTR of the NTN4 mRNA containing the predicted miR-196a binding site was amplified by PCR using the primers listed in Supplementary Table 1 and inserted downstream of the enhanced green fluorescent protein (EGFP) gene in pCDNA/EGFP (pEGFP-NTN4 3′-UTR). This predicted target site (5′-AAGCACTTTACTACCTA-3) was mutated (5′-AAGCACTTTAGTTCGTT-3′) using QuickChange mutagenesis kit (Stratagene) and was cloned into the pCDNA/EGFP plasmid to construct pEGFP-NTN4 3′-UTR-Mut. All insertions were confirmed by sequencing. HeLa cells were transfected with pEGFP-NTN4 3′-UTR or -Mut together with miR-196a mimics or the control miR-NC vector. CaSki cells were

transfected with pEGFP-NTN4 3′-UTR or -Mut together with Anti-miR-196a or the control Anti-miR-NC. All transfections were performed using Lipofectamine 2000 (Invitrogen). 48 h after transfection, fluorescence intensity was measured with a Fluorescence Spectrophotometer F-4500 (HITACHI, Tokyo, Japan).

2.9. Western blot analysis

Western blotting was performed to determine NTN4 protein expression. The total protein was extracted from the cervical tissues and was quantitated by Bradford's method (Bio-Rad). Samples (50 μg protein) were resolved on a 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane. The membranes were probed with antibodies for NTN4 and GAPDH (R&D Systems) with a dilution of 1:500 and 1:1000, respectively. The LabWorks image acquisition and analysis software (UVP, LLC) was used to quantify band intensities.

2.10. Statistical analysis

All the experiments were performed independently in triplicate. Student's *t*-test (two-tailed), one-way ANOVA and Mann–Whitney test were performed to analyze the data using SPSS 16.0 software. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. miR-196a expression is upregulated in human cervical cancer tissues

The expression of miR-196a was measured in cervical cancer tissues, CIN1 tissues, CIN2-3 tissues, and four cervical cancer cell lines. We found that expression of miR-196a was significantly elevated in cervical cancer tissues and CIN2-3 tissues compared to normal controls ($P < 0.001$, Fig. 1A). Cervical cancer tissue had higher miR-196a expression than CIN2-3 tissue ($P < 0.001$). The increased expression of miR-196a was also seen in four cervical cancer cell lines, C33A, HeLa, SiHa, and CaSki ($P < 0.001$, Fig. 1B).

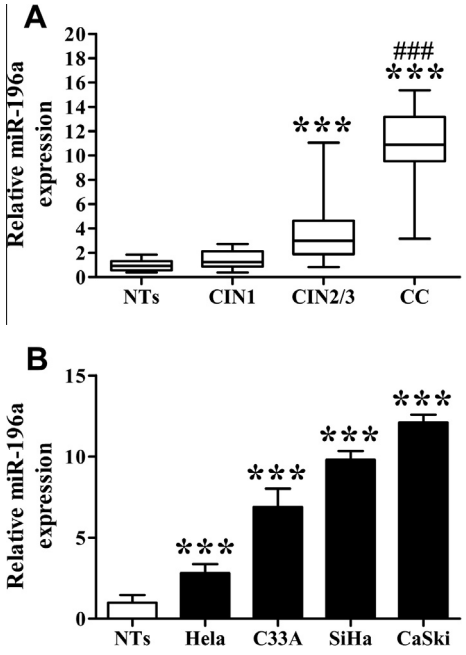

Fig. 1. The level of miR-196a expression in clinical samples stratified by histological diagnosis and cervical cancer cell lines. Data are presented as fold change in lesion tissues or cancer cell lines relative to control specimen. (A) Analysis of miR-196a expression levels in 15 cervical cancer (CC) tissues, 13 CIN1 tissues, 18 CIN2-3 tissues, and 24 adjacent noncancerous tissues (NTs) by qRT-PCR. $^{***}P < 0.001$ vs. NTs; $^{\#\#\#}P < 0.001$ vs. CIN2-3. (B) Analysis of miR-196a expression levels in cervical cancer cell lines by qRT-PCR. $^{***}P < 0.001$ vs. NTs.

3.2. miR-196a expression contributes to proliferation and migration of human cervical cancer cells

We manipulated miR-196a expression to investigate its functional effect of in cancer cells. HeLa cells with relatively low miR-196a level and CaSki cells with relative high miR-196a level were upregulated and downregulated, respectively. miR-196a expression in HeLa cells increased 10.0-fold after transfection with a miR-196a mimic and was induced 6.0-fold in HeLa cells stably transfected with pCDNA/miR-196a, as compared to control (Fig. 2A). As CaSki cells expressed relative high miR-196a level, we downregulated their miR-196a by transfecting them with Anti-miR-196a or the control, Anti-miR-NC. miR-196a expression in CaSki cells was reduced 0.61-fold following transfection with miR-196a inhibitors (Fig. 2B).

The effects of altered miR-196a expression on cancer cells proliferation and migration were investigated in cervical cancer. We found that inhibition of miR-196a expression in CaSki cells caused a significant decrease in cell growth (approximate 19%; $P < 0.01$), whilst overexpression of miR-196a in HeLa cells brought about significant increases of cell proliferation (approximate 31–51%; $P < 0.01$), compared to their respective controls (Fig. 2C). Similarly, the results of colony-formation assays revealed clonogenic survival was decreased following inhibition of miR-196a in CaSki cells, and enhanced in pCDNA/miR-196a transfected HeLa cells (Fig. 2D). Using a Transwell assay, we found cell migration was significantly enhanced by miR-196a overexpression induced by its mimic or pCDNA/miR-196a transfection in HeLa cells (approximate 35% and 29% respectively; $P < 0.05$). Moreover, miR-196a suppression resulted in a significant decrease of cell migration in CaSki cell line (approximate 21%, $P < 0.05$; Fig. 2E). Taken together, both gain- and loss-of-function experiments indicate a role for miR-196a in cell proliferation and migration.

3.3. Validation of NTN4 as miR-196a target gene

A combination of bioinformatic databases and the Cervical Cancer Gene Database identified five genes that met our selection criteria, including MAL, MBNL2, MRPS25, NRAS, and NTN4 gene. Among these genes, only NTN4 expression was downregulated by miR-196a over-expression in HeLa cells and was increased by inhibition of endogenous miR-196a expression in CaSki cells (Fig. 3A).

In order to determine whether miR-196a manipulation by miR-196a mimics and inhibitors could regulate the EGFP reporter construct, we constructed plasmids containing EGFP and either a miR-196a-binding site (pEGFP-NTN4 3′-UTR) or the mutant site (pEGFP-NTN4 3′-UTR-Mut) (Fig. 3B). In the HeLa cell line, coexpression of miR-196a mimic and pEGFP-NTN4-3′-UTR resulted in a significant reduction in the intensity of EGFP fluorescence compared to the mutated 3′-UTR GFP reporter. In contrast, in CaSki cells, coexpression of Anti-miR-196a and the pEGFP-NTN4 3′UTR reporter resulted in an increase in EGFP fluorescence compared to the mutated 3′-UTR (Fig. 3C and D). Together, these results demonstrate that miR-196a binds directly to the 3′-UTR of NTN4 to repress gene expression.

Next, we evaluated the NTN4 expression in 15 cancer tissues compared to 24 normal tissue controls by Western blot, then correlated NTN4 mRNA level with miR-196a expression. We found significantly lower expression of NTN4 in human cervical cancer

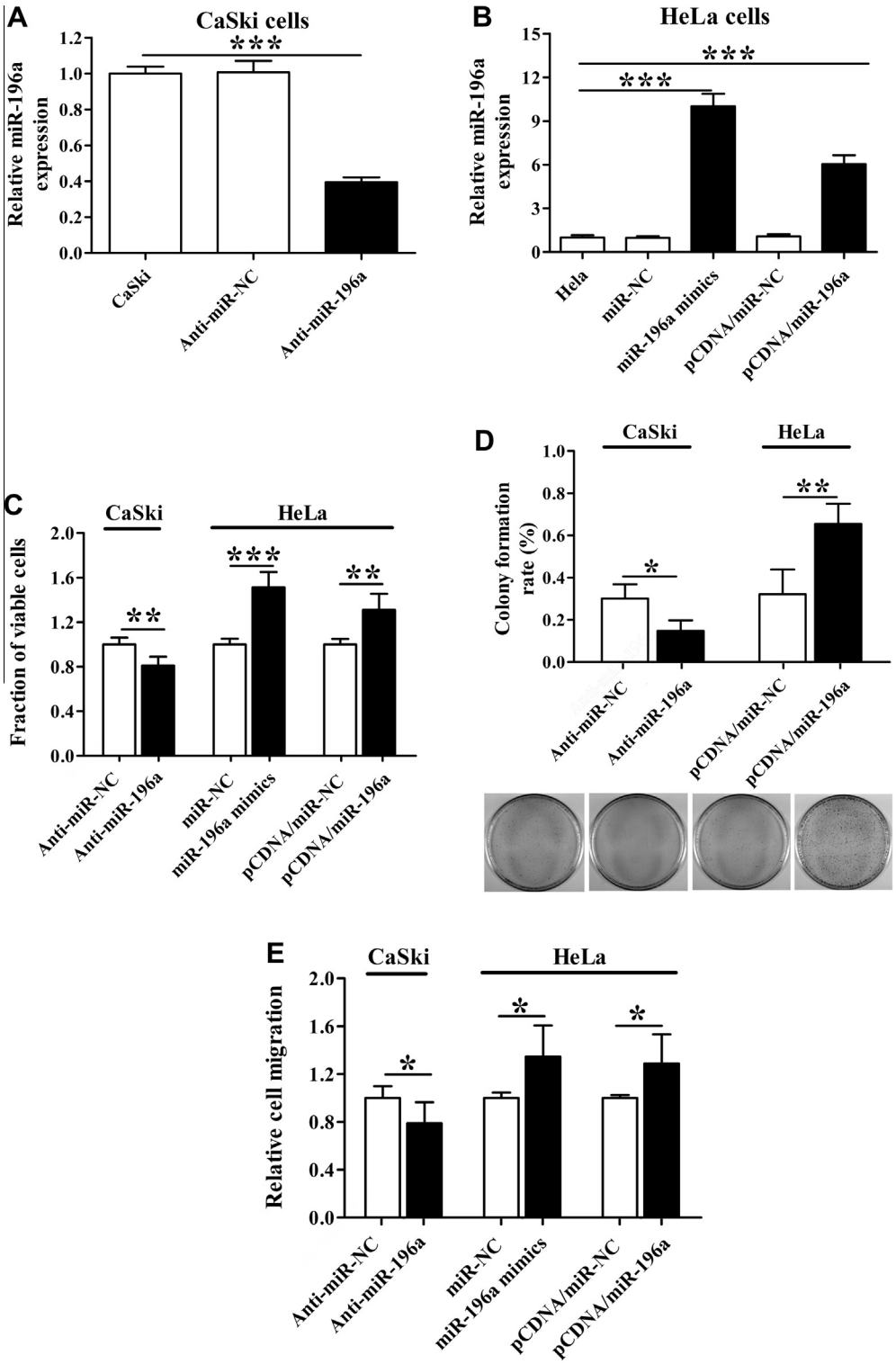

Fig. 2. Functional analyses of miR-196a regulation in cervical cancer cell lines. (A) Analysis of miR-196a expression in CaSki cells with or without transfection of Anti-miR-196a or Anti-miR-NC by qRT-PCR. (B) Analysis of miR-196a expression in HeLa cells with or without transfection of miR-NC, miR-196a mimics, pCDNA-NC or pCDNA-miR-196a by qRT-PCR. (C) Cell proliferation was assessed in cervical cancer cell lines transfected with miR-196a mimics, pCDNA/miR-196a, Anti-miR-196a or corresponding negative control (miR-NC, pCDNA/miR-NC or Anti-miR-NC) using WST-1 assay. Relative cell growth was normalized to its respective control-treated cells. (D) Colony-forming growth assays were performed to determine the proliferation of CaSki and HeLa cells with ectopic expression of miR-196a. (E) Graphs showing relative cell migration in both miR-196a inhibition and overexpression experiments as evaluated by Transwell migration assay. Relative cell migration was normalized to its respective control-treated cells. Data presented represent mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{**}P < 0.001$.

samples as compared with their normal counterparts ($P = 0.0003$; Fig. 3E). Furthermore, the expression patterns of NTN4 were inversely correlated with miR-196a expression (Corr = −0.443, $P = 0.005$; Fig. 3F).

4. Discussion

In the present study, we found that miR-196a levels were dramatically upregulated following the transition from normal cervi-

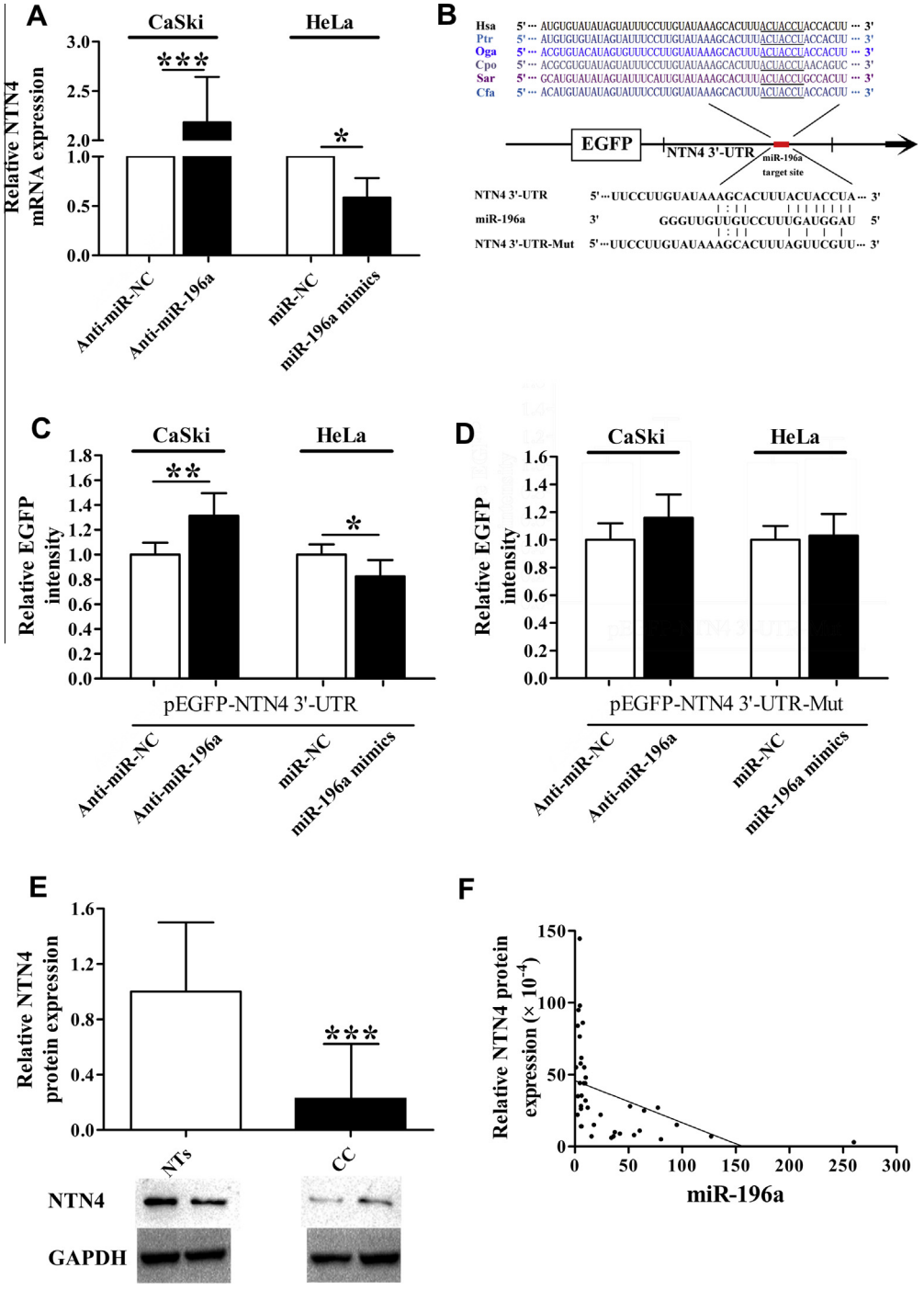

Fig. 3. miR-196a directly targets the NTN4 gene. (A) qRT-PCR analyses of NTN4 expression levels following treatment of CaSki cells with miR-196a inhibitors or HeLa cells with miR-196a mimics. (B) Human NTN4 3′-UTR fragment containing wild-type or mutant (Mut) miR-196a-binding sequence was cloned downstream of the fluorescent reporter gene. (C) The intensity of EGFP fluorescence in cancer cells transfected with miR-196a mimics was decreased and increased following transfection with Anti-miR-196a. (D) miR-196a mimic and Anti-miR-196a had no effect on the intensity of EGFP fluorescence in cells transfected with the 3′-UTR mutant vector. (E) Relatively lower expression of NTN4 was found in cancer samples as compared to their normal counterparts using Western blot assay. Data are presented as fold change in cervical cancer relative to control specimen. (F) Inverse correlation between the expression level of miR-196a and NTN4 protein levels. The expression relationship was evaluated by Pearson's correlation analysis. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{**}P < 0.001$. Hsa, *Homo sapiens*; Ptr, *Pan troglodytes*; Oga, *Otolemur garnettii*; Cpo, *Cavia porcellus*; Sar, shrew; Cfa, Canine family. NTs, noncancerous tissues, CC, cervical cancer.

cal tissues, to precancerous CIN2–3 lesions, and to cervical cancer tissues. These findings suggested miR-196a may be a useful indicator for malignant transformation of cervical epithelial cells. Several studies have revealed an aberrant overexpression of miR-196a in multiple tumors and demonstrated oncogenic functions of miR-196a in the context of tumorigenesis [11,12]. In contrast, miR-196a also exhibited tumor-suppressing effects on other tumors. In melanoma and breast cancer, miR-196a expression significantly inhibited invasive and metastatic behaviors of cancer cells [14,15]. Therefore, the functional role of miR-196a may be dependent on the cancer and tissue type, and the exact action of miR-196 in cervical cancer is worthy of further study. We explored the biological role of miR-196a in malignant behaviors of cervical cancer cells using gain-or-loss function experiments and demonstrated that

the high expression of miR-196a contributes to proliferation and migration of cervical cancer cells.

miRNAs regulate gene expression by binding to complementary sites in the 3′-UTR of target genes to prevent translation; therefore identifying target genes of miR-196a is a key method for understanding its oncogenic function. Indeed, several targets of miR-196a have been identified that mediate cancer-promoting effects in different settings of tumorigenesis. In esophageal cancer, miR-196a directly targets and suppresses annexin A1 expression to promote cancer cell proliferation [16]. In colorectal cancer, high levels of miR-196a activate the Akt signaling pathway, promoting cancer cell detachment, migration, invasion and chemosensitivity [17,18]. miR-196a also contributes to cancer cell proliferation of non-small cell lung cancer and gastric cancer by downregulating homeobox A5 and $p27^{kip1}$, respectively [11,19]. Generally, with each miRNA potentially regulating hundreds of genes [5], sequence-based available target prediction algorithms can often predict hundreds to few thousands of target genes for each miRNA [20]. Identification of real functional targets of individual miRNA from the numerous predicted targets therefore presents a considerable challenge. Increasing evidence indicates that the functional targets of a miRNA can change depending on the specific pathophysiological context [20], which also explains why miR-196a regulates different target genes in various tumors.

Inspired by the conception of context-specific targets of miRNA, we selected candidate targets of miR-196a that were predicted by multiple computational algorithms and displayed a trend of downregulation in cervical cancer genesis or progression. We firstly assessed the gain-or-loss of function effects of miR-196a on the expression levels of these candidate targets in cervical cancer cells. We found that the manipulation of miR-196a expression levels affected expression of NTN4. Targeted knock-down of miR-196a expression in CaSki cells led to a significant NTN4 elevation in mRNA. Conversely, its overexpression in HeLa cells brought about a significant downregulation of NTN4 expression. Using fluorescent reporter assay and qRT-PCR, we further confirmed that miR-196a could directly act on 3′-UTR of NTN4 to suppress gene expression. The negative regulation of NTN4 by miR196a was also supported by the in vivo findings. In human cervical tissues, NTN4 mRNA inversely correlated with the expression levels of miR-196a. Along with the overexpression of miR-196a, both qRT-PCR and Western blot data revealed that NTN4 displayed very low expression in cervical cancer tissue. These findings suggested that NTN4 was a functional target of miR-196a in cervical carcinogenesis and may participate in the oncogenic functions of miR-196a in cervix uteri.

NTN4 encoding netrin-4 is identified as a member of the netrin family, which promotes neurite outgrowth and regulating cell migration and branching [21]. Recently, increasing evidence has suggested that NTN4 acts as a suppressor for multiple tumors. NTN4 expression was down-regulated in prostate tumors and breast cancer [22,23]. Notably, the expression of NTN4 has been associated with a positive prognosis in breast cancer [23]. Similarly, we also found a reduction of NTN4 expression in cervical cancer, suggesting that NTN4 has a protective function in cervical cancer pathogenesis. Functional assays have also demonstrated that netrin-4 inhibits proliferation of a variety of human tumor cells in vitro, including a cervical cancer cell line, HeLa [24]. In addition, netrin-4 delays colorectal cancer carcinomatosis by inhibiting tumor angiogenesis [25]. Currently, the role of NTN4 in cervical cancer has not been well established. Considering the direct target of miR-196a on NTN4 and inhibitory effects of NTN4 on tumorigenesis, we proposed that the NTN4 may at least partially mediate the suppressing effects of miR-196a on proliferation and migration of cervical cancer cells.

In summary, our study indicated that miR-196a is dramatically upregulated in cervical cancer tissues, precancerous CIN2-3 lesions, and cell lines. Moreover, miR-196a directly regulates NTN4 and contributes to cellular proliferation and migration. The miRNA expression characteristic and identification of target genes may provide an understanding of the potential carcinogenic mechanisms in cervical cancer. These findings have therapeutic implications and may be exploited for future treatment of cervical cancer. We note that there are limitations to this study, specifically, our findings are mainly based on in vitro experiment, further in vivo studies using xenograft models are required to further understand the biological role of miR-196a in cervical cancer genesis and progression. Our study provides new insights into cervical cancer pathogenesis and suggests the potential cancer-suppressing role of NTN4 on cervical cancer should be investigated further.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.142.

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