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miR-92a is upregulated in cervical cancer and promotes cell proliferation and invasion by targeting FBXW7

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ABSTRACT

MicroRNAs (miRNAs) are involved in the cervical carcinogenesis and progression. In this study, we investigated the role of miR-92a in progression and invasion of cervical cancer. MiR-92a was significantly upregulated in cervical cancer tissues and cell lines. Overexpression of miR-92a led to remarkably enhanced proliferation by promoting cell cycle transition from G1 to S phase and significantly enhanced invasion of cervical cancer cells, while its knockdown significantly reversed these cellular events. Bioinformatics analysis suggested F-box and WD repeat domain-containing 7 (FBXW7) as a novel target of miR-92a, and miR-92a suppressed the expression level of FBXW7 mRNA by direct binding to its 3′-untranslated region (3′UTR). Expression of miR-92a was negatively correlated with FBXW7 in cervical cancer tissues. Furthermore, Silencing of FBXW7 counteracted the effects of miR-92a suppression, while its overexpression reversed oncogenic effects of miR-92a. Together, these findings indicate that miR-92a acts as an onco-miRNA and may contribute to the progression and invasion of cervical cancer, suggesting miR-92a as a potential novel diagnostic and therapeutic target of cervical cancer.

1. Introduction

Cervical cancer is the third most prevalent gynecologic malignancy and the fourth leading cause of cancer-related deaths in women worldwide [1]. It is well known that persistent infection with high-risk human papillomaviruses (HPVs) plays an important role in the initiation and progression of cervical cancer. However, a number of evidences show that HPVs infection alone is insufficient for tumor development and that other factors must be involved in cervical carcinogenesis [2]. Therefore, the identification of such factors would be important for the prevention and treatment of cervical cancer.

MicroRNAs are a group of endogenously expressed, non-coding small RNAs (21–24 nucleotides in length) known to regulate gene expression post-transcriptionally by base pairing with the 3′-untranslated region of target messenger RNAs (mRNAs) to suppress translation or decrease the stability of mRNAs [3]. MiRNAs play vital regulatory roles in cell proliferation, apoptosis, differentiation and migration [4]. Accumulating evidence has indicated that dysregulation of miRNAs occurs in various human cancers and they function as tumor promoter or suppressor depending on the nature of their targets [5]. MiRNAs involved in carcinogenesis and progression of cervical cancer have been widely investigated [6–8]. A recent microRNA microarray analysis showed that miR-92a expression was significantly higher in oncogenic HPVs infected HFK cells as compared to that of HFK without oncogenic papillomavirus infection [9], and a large body of studies has found that miR-92a regulates tumor progression in a variety of cancers as an oncogenic or a tumor-suppressive miRNA [10–15]. Given the complexity of its functionality, it would be of interest to explore the functional roles of miR-92a in cervical cancer development.

In the present study, we investigated the miR-92a expression in cervical cancer tissues and cell lines, and explored its effects on proliferation and invasion of cervical cancer cells. Moreover, we demonstrated that F-box and WD repeat domain-containing 7(FBXW7) is a target for miR-92a in cervical cancer cells and involved in the functional influence of miR-92a on cervical cancer cells proliferation and invasion.

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2. Materials and methods

2.1. Clinical samples, cell lines and transfection

31 paired cervical cancer and matched normal adjacent tissues were provided by Wuhan Tumor Hospital, Wuhan, China. Specimens were obtained with informed consent and the study was approved by local institutional review boards on human subject research and in accordance with the Declaration of Helsinki. All the histological diagnoses for cervical cancer and normal tissues were reviewed and recognized by two pathologists independently. Sample characteristics are described in Supplementary Table S1.

HaCaT cells (an immortalized HPV-negative skin keratinocyte line) and four human cervical cancer cell lines HeLa, SiHa, CaSki and C33A were obtained from Chinese Center for Type Culture Collection (Wuhan, China) and cultured in the recommended conditions. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2. Transfection was performed when cells were grown to 70% confluence, using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.2. MicroRNAs and plasmids construction

miR-92a mimics/inhibitor and corresponding controls were purchased from RiboBio (Guangzhou, China). FBXW7-specific siRNA (Silencer™ Predesigned siRNA) was purchased from Ambion (Shanghai, China). The FBXW7 cDNA was cloned into pcDNA3.1 to construct the FBXW7 expression plasmid. For luciferase reporter, the 3′UTR of FBXW7 containing the putative binding sites for miR-92a was amplified by PCR and cloned into the pGL3-luciferase reporter plasmid (Promega, Madison, WI). Mutations in the miR-92a-binding site of FBXW7 3′UTR were introduced by the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Constructs were verified by sequencing.

2.3. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA and miRNA were extracted from tissues and cells using RNeasy Mini and miRNeasy Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For detection of miR-92a expression, stem–loop RT-PCR was performed as described [16]. Expression of U6 was used as an endogenous control. Real-time PCR was performed using FastStart Universal SYBR Green Master kit (Roche Diagnostics) and analyzed with an Applied Biosystems 7900 Real-Time PCR System. Primer sequences were as follows: FBXW7, 5′-CACTCAAAGTGTGGAATG-CAGAGAC-3′ and 5′-GCATCTCGAGAACCGCTAACAA-3′; GAPDH, 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CAGTGAGCTTCCCGTT-CAG-3′. The qRT-PCR data was analyzed using the method of $2^{-\Delta\Delta Ct}$ relative expression quantity as previously described [17]. All the qRT-PCR reactions were run in triplicate.

2.4. Cell proliferation assay and cell cycle analysis

Transfected cells were seeded into 96-well plates at a density of 2000 cells/well and cultured for 1, 2, 3, 4 and 5 days. MTT (5 mg/mL) was added to each well for 4 h at 37 °C. The reaction was stopped by 150 μl DMSO and absorbance readings at 490 nm were obtained in triplicate using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA). For the cell cycle analysis, cells were harvested by trypsinization, washed twice using cold PBS and fixed in 70% ethanol overnight at 4 °C. Then cells were subsequently incubated with 20 μg/ml propidium iodide (Sigma) for 20 min at room temperature, and cell cycle analysis was performed with FACS flow cytometry (BD Biosciences, Franklin Lakes, NJ).

2.5. Transwell invasion assay

The Matrigel invasion chamber was used to assess cell invasion ability. Aliquots of cells (3×10^4) were placed into upper chambers coated with 150 mg Matrigel (BD Biosciences, Bedford, MD). The lower chambers were filled with DMEM containing 10% FBS. After incubation at 37 °C for 24 h, cells remaining on the upper surface of the membrane were removed. Cells on the lower surface of the membrane were fixed, stained with crystal violet. Stained cells were visualized and counted under a light microscope. The assays were performed in triplicate and were repeated three times.

2.6. Dual luciferase assay

For the dual luciferase assay, HEK293 cells in a 96-well plate were transfected with 50 nM miR-92a or miR-NC. The cells were then co-transfected with 0.2 mg/ml of vector with the wild-type or mutant 3′UTR of FBXW7 gene. After 48 h, luciferase activity was measured with the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was then normalized to the corresponding Renilla luciferase activity. Luciferase assays were performed in quadruplicate and repeated in three independent experiments.

2.7. Western blot

Proteins were extracted by RIPA lysis buffer (Beyongtime, China). Protein concentrations were quantified by the BCA protein assay kit (Beyotime, Haimen, China). Equal amounts of protein were separated by SDS–PAGE, transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA) and blocked for 0.5 h at room temperature. Membranes were probed with primary antibodies against FBXW7 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight followed by incubation with HRP-conjugated secondary antibodies. Blots were detected using an ECL detection system.

2.8. Statistical analysis

SPSS16.0 statistical software package (SPSS, Chicago, USA) was used for statistical analysis. Experiments were repeated independently at least three times, and the results are expressed as mean ± SD. The correlation between miR-92a and FBXW7 was analyzed using Spearman's correlation test. Statistical differences between groups were evaluated using Student's paired two tailed t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-92a is significantly upregulated in cervical cancer tissues and cell lines

To further confirm the altered expression of miR-92a in cervical cancer, we tested the expression of miR-92a in 31 pairs of cervical cancer tissues and adjacent normal cervical tissues using qRT-PCR. In agreement with the microarray-based results, miR-92a expression was significantly higher in human cervical cancers than their normal counterparts (Fig. 1A and B). Furthermore, miR-92a was also significantly increased in four cervical cancer cell lines compared with that of HaCaT cells (Fig. 1C).

3.2. Effect of miR-92a on cervical cancer cell proliferation and invasion

To explore the role of miR-92a in cervical cancer cell growth, HeLa cells expressing relatively high level of miR-92a and C33A

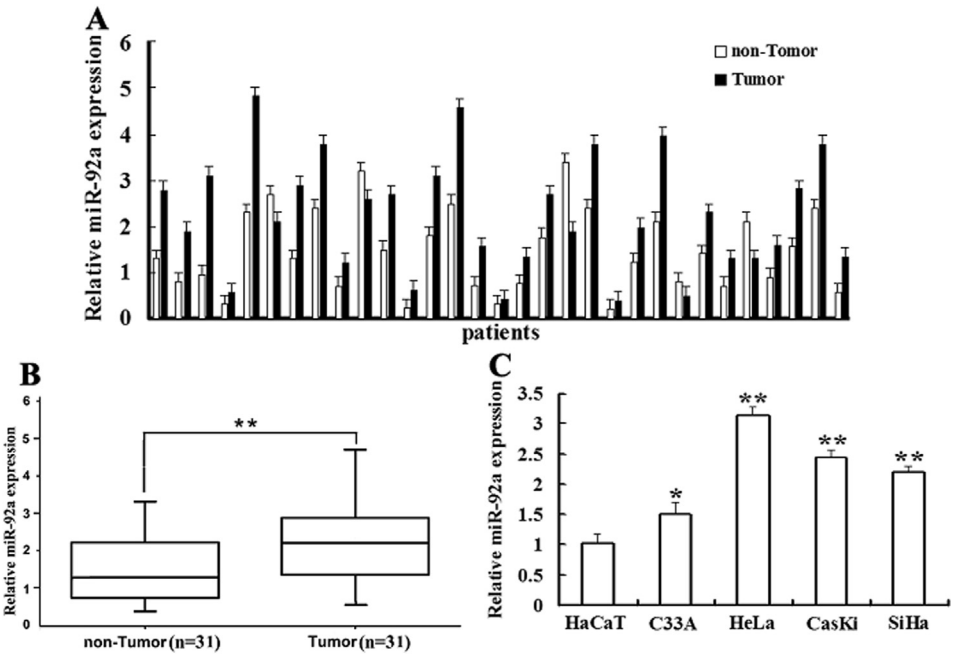

Fig. 1. miR-92a was increased in cervical cancer tissues and cell lines. (A) A paired bar chart shows the expression level of miR-92a in 31 cervical cancer tissues and their pair-matched adjacent noncancerous cervical tissues. (B) The mean expression level of miR-31 in cervical cancer tissues was significantly higher than that in pair-matched adjacent noncancerous cervical tissues ($P < 0.01$). (C) miR-92a was significantly increased in five cervical cancer cell lines compared with that in HaCaT cell. *$P < 0.05$, **$P < 0.01$ compared with control group.

cells expressing relatively low level of miR-92a were transfected with miR-92a inhibitor and miR-92a mimics, respectively, which decreased the level of miR-92a in HeLa by 3.85 folds and increased that in C33A by 14.3 folds, as compared to corresponding negative control (Fig. 2A). MTT assay indicated that downregulation of miR-92a significantly reduced the proliferation of HeLa cells, whereas upregulation of miR-92a significantly promoted the proliferation of C33A cells (Fig. 2B). To further determine the mechanism of how miR-92a promoted cervical cancer cell proliferation, we subsequently explored whether miR-92a has an effect on cell cycle progression of cervical cancer cells using flow cytometry. The results showed that upregulation of miR-92a in C33A cells led to a significant decrease in the cellular population in G0/G1 phase but a sharp increase in S phase, while downregulation of miR-92a in HeLa cells noticeably induced G1 phase arrest (Fig. 2C). Thus, the growth-promoting function of miR-92a may attribute to enhancement of cell cycle progression at G1/S transition in cervical cancer cells. To investigate the effect of miR-92a on the invasion of cervical cancer cells, in vitro invasion assays were performed. Matrigel Transwell assays showed that miR-92a markedly increased the invasive ability of C33A cells while anti-miR-92 inhibited this activity of HeLa cells (Fig. 2D).

3.3. FBXW7 is negatively regulated by miR-92a in cervical cancer cells and inversely correlated with miR-92a in cervical cancer tissues

Four bioinformatics algorithms:TargetScan, pictar, E1MMo2 and miRanda, were used to search for the potential targets of miR-92a. All of these four approaches predicted FBXW7 as a target of miR-92a, and the 3′-UTR of FBXW7 mRNA contains a highly conserved binding site from position 286 to 292 for miR-92a seed sequence (the core sequence that encompasses the first 2–8 bases of the mature miRNA, Fig. 3A). To examine whether FBXW7 was the target of miR-92a , we performed Western blot analysis. The level of FBXW7 protein was markedly decreased by miR-92a overexpression in C33A cells but significantly increased by silencing of miR-92a in HeLa cells (Fig. 3B). We further investigated the expression of FBXW7 mRNA in 31 pairs of cervical cancer and adjacent normal tissues. Results showed that FBXW7 mRNA was significantly downregulated in cervical cancer tissues compared with the paired-adjacent normal tissues and inversely correlated with miR-92a level in cervical cancer tissues (Fig. 3C and D).

3.4. FBXW7 is a direct target of miR-92a

To further validate that FBXW7 is a direct target of miR-92a, we constructed luciferase reporter vector containing wild-type FBXW7 3′UTR with miR-92a binding site (WT) or containing the mutant 3′UTR (mutation of the putative miR-92a target site, MUT, Fig. 3A). Luciferase activity assay indicated that miR-92a significantly suppressed the luciferase activity of FBXW7-3′UTR-WT reporter, compared with control, but did not affect the mutant reporter in HEK293 cells (Fig. 3E).

3.5. FBXW7 is involved in miR-92a-induced effects on cervical cancer proliferation and invasion

We further investigated whether FBXW7 is involved in the oncogenic effects of miR-92a on cervical cancer cells. MTT and in vitro invasion assays showed that overexpression of FBXW7 significantly suppressed miR-92a-induced proliferation and invasion of C33A cells, while silencing of FBXW7 significantly reversed tumor suppressive effects of miR-92a inhibitor on the proliferation and invasion of HeLa cells (Fig. 4A–C). Furthermore, upregulation of FBXW7 markedly abrogated the enhancement of cell cycle progression at G1/S transition induced by miR-92a in C33A cells, whereas knockdown of FBXW7 clearly rescued the G1 phase arrest induced by miR-92a inhibitor in HeLa cells (Fig. 4D). Taken together,

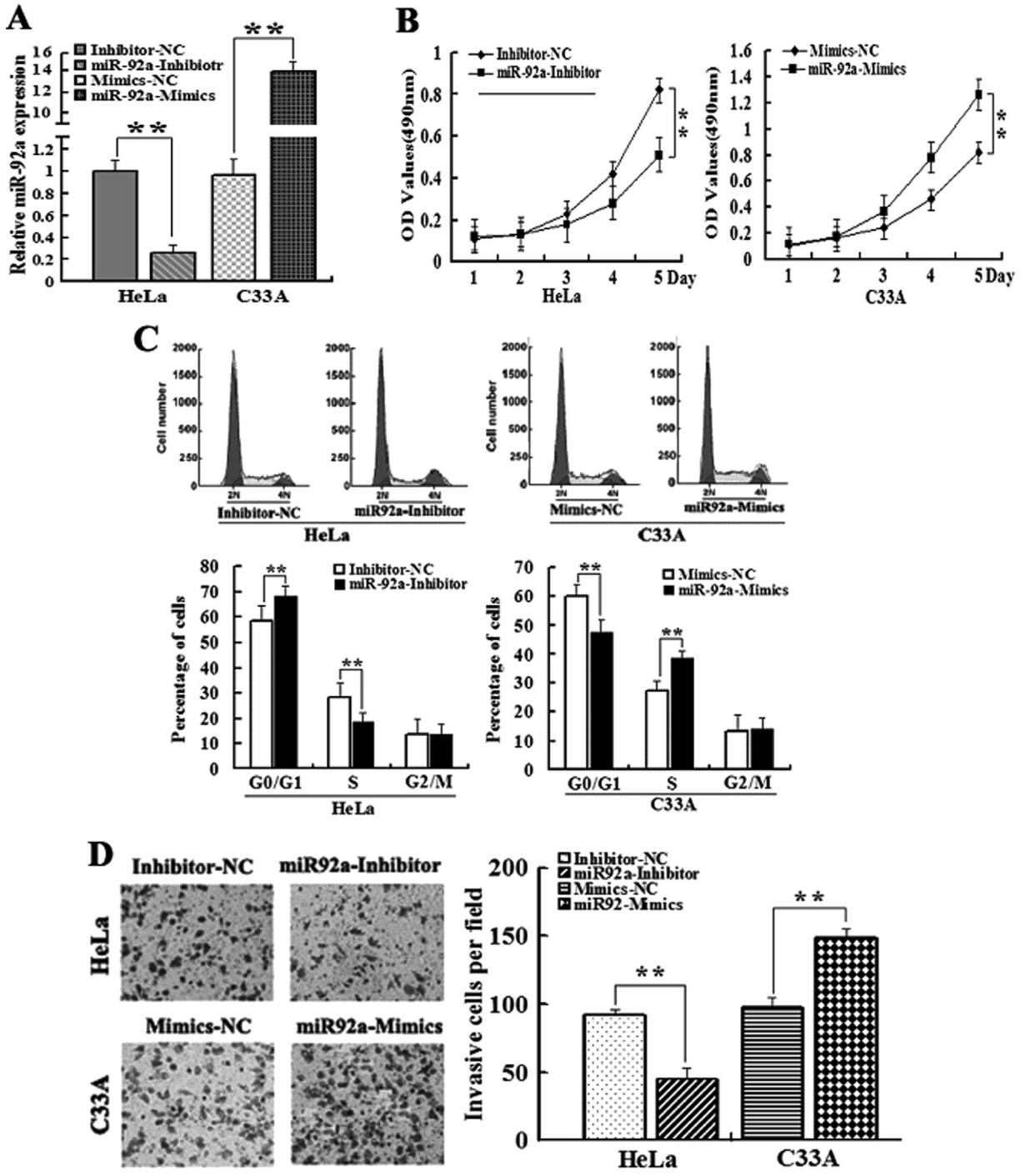

Fig. 2. miR-92a promotes cervical cancer cell proliferation and invasion. (A) qRT-PCR analysis of miR-92a levels in HeLa and C33A cells, transfected with miR-92a inhibitor and miR-92a mimics, respectively. (B) Determination of HeLa and C33A cells proliferation with MTT assay. (C) Determination of HeLa and C33A cell cycle with Flow cytometry. (D) Determination of HeLa and C33A cells invasion with Transwell invasion assay. *P < 0.05, **P < 0.01 compared with control group.

all these data indicate that FBXW7 is a functional mediator of miR-92a in cervical cancer progression.

4. Discussion

Emerging data have shown that the aberrant expression of miRNAs contributes to tumorigenesis by inhibiting the expression of their target genes and potentially serve as biomarkers for prediction and prognosis in various cancers including cervical cancer [18,19]. Hence, identification of specific miRNAs and their targets involved in tumorigenesis would provide vital clue for the diagnosis and therapy of patients with malignancies. In this study, we found that the expression of miR-92a was significantly increased in cervical cancer tissue samples and cell lines, consistent with data from previous microarray analyses. We also showed that miR-92a could promote the proliferation and invasion of cervical cancer cells, accompanied by promoting cell cycle transition from G1 phase to S phase, and miR-92a could inhibit FBXW7 expression by

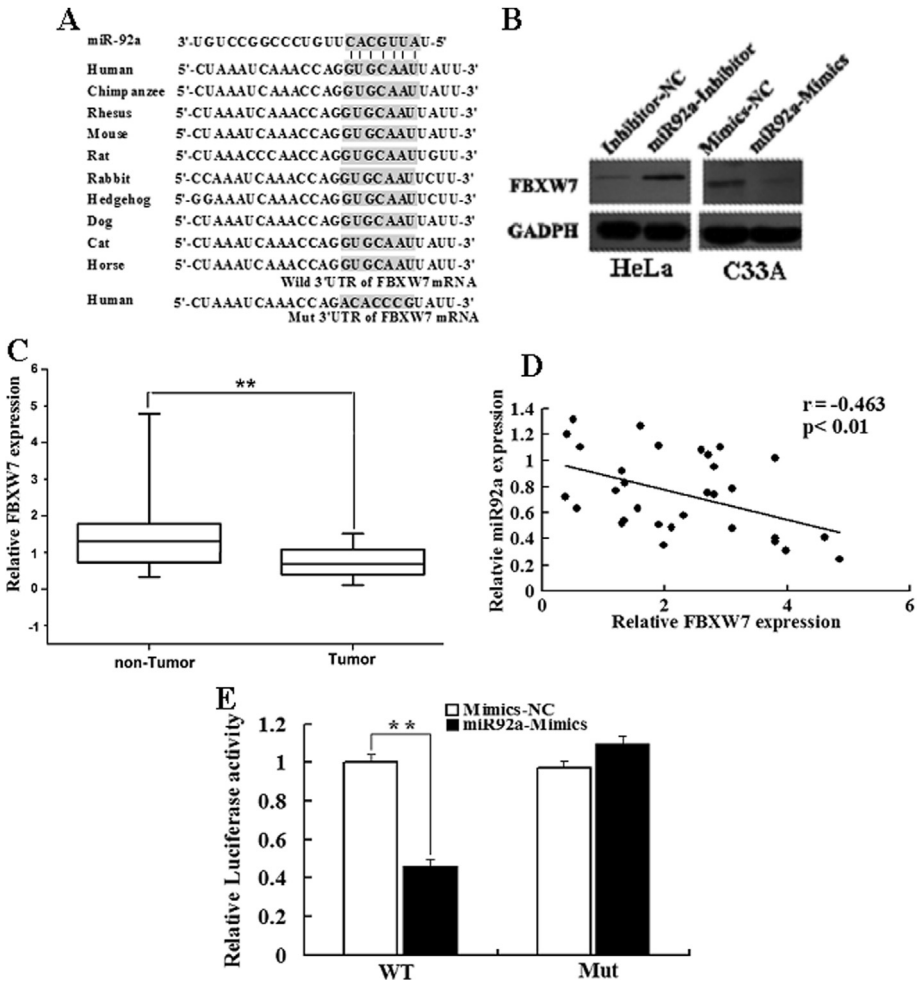

Fig. 3. FBXW7 is a direct target of miR-92a. (A) putative miR-92a binding site in 3′UTR region of FBXW7 and interspecies conservation of seed matching sequences (gray box). Mutation was generated in the FBXW7 3′UTR by mutating seed matching sequence. (B) Protein level of FBXW7 was detected by Western blot in HeLa and C33A cells transfected with miR-92a inhibitor and miR-92a mimics along with corresponding controls, respectively. (C) FBXW7 mRNA level was examined by qRT-PCR and, it was remarkably decreased in cervical cancer tissues. (D) FBXW7 mRNA level was inversely correlated with miR-92a level in cervical cancer tissues (Spearman's correlation analysis). (E) HEK293 cells were co-transfected with miR-92a and WT or Mut FBXW7 3′UTR luciferase reporter construct. *P < 0.05, **P < 0.01 compared with control group.

directly targeting its 3′UTR region. Moreover, FBXW7 could counteract the functional influences of miR-92a on cervical cancer cells. These results suggest that miR-92a functions as an oncogene and plays a critical role in the proliferation and invasion of cervical cancer. However, its roles in vivo and the relationship between deregulation of its expression and clinical outcomes await further studies.

MiR-92a, a member of the miR-17-92 cluster, has been reported to be highly expressed in several cancers and plays a critical role in cancer development. For instance, Zhou et al. reported that miR-92a was upregulated in colorectal cancer and correlates with tumor metastasis and poor prognosis in patients [10]. Chen et al. found that in esophageal squamous cell carcinoma, upregulation of miR-92a was significantly associated with the lymph node metastasis and TNM stage in ESCC patients and modulated the migration and invasion of ESCC cells via targeting CDH1 [11]. Tian et al. suggested that miR-92a promote prostate cell proliferation by suppressing PTEN [12]. However, several studies showed that downregulation of miR-92a was correlated with aggressive breast cancer features and promoted breast cancer migration [13,14]. Ohyashiki et al. found that the expression level of miR-92a was down-regulated in non-Hodgkin's lymphoma, and low levels of miR-92a were related to a significantly high relapse rate [15]. These dual roles of miR-92a could attribute to organ-specific actions and different cellular contexts. The present study expanded the function of miR-92a in cervical cancer and suggested that miR-92a mainly acts as an oncogenic miRNA in cervical cancer.

Since miRNAs usually exert their biologic functions by inhibiting the expression of target mRNAs, we used four bioinformatics algorithms to predict gene targets for miR-92a. FBXW7 was one of cancer-associated genes predicted by all of these algorithms, whose mRNA contains a highly conserved miR-92a binding site on the 3′UTR. Indeed, a previous study has reveal that FBXW7 is a direct target of miR-92a in acute T-cell lymphoblastic leukemia [20]. Although this previous study indicated that miR-92a can inhibit FBXW7 expression by directly targeting its 3′UTR, it did not further validated this potential regulative relation in clinical specimens and did not investigate whether miR-92a-mediated FBXW7 inhibition confers any biological function in T-ALL cells. In addition, this report revealed that miR-92a upregulation significantly accelerated leukemia development in a mouse model, while knockdown of FBXW7 in this mouse T-ALL model did not significantly accelerated leukemia development. Thus this study did not establish a functional link between miR-92a and FBXW7 in tumor. In the present study, we further extended these studies in cervical cancer and showed that the expression level of FBXW7 was significantly lower in cervical

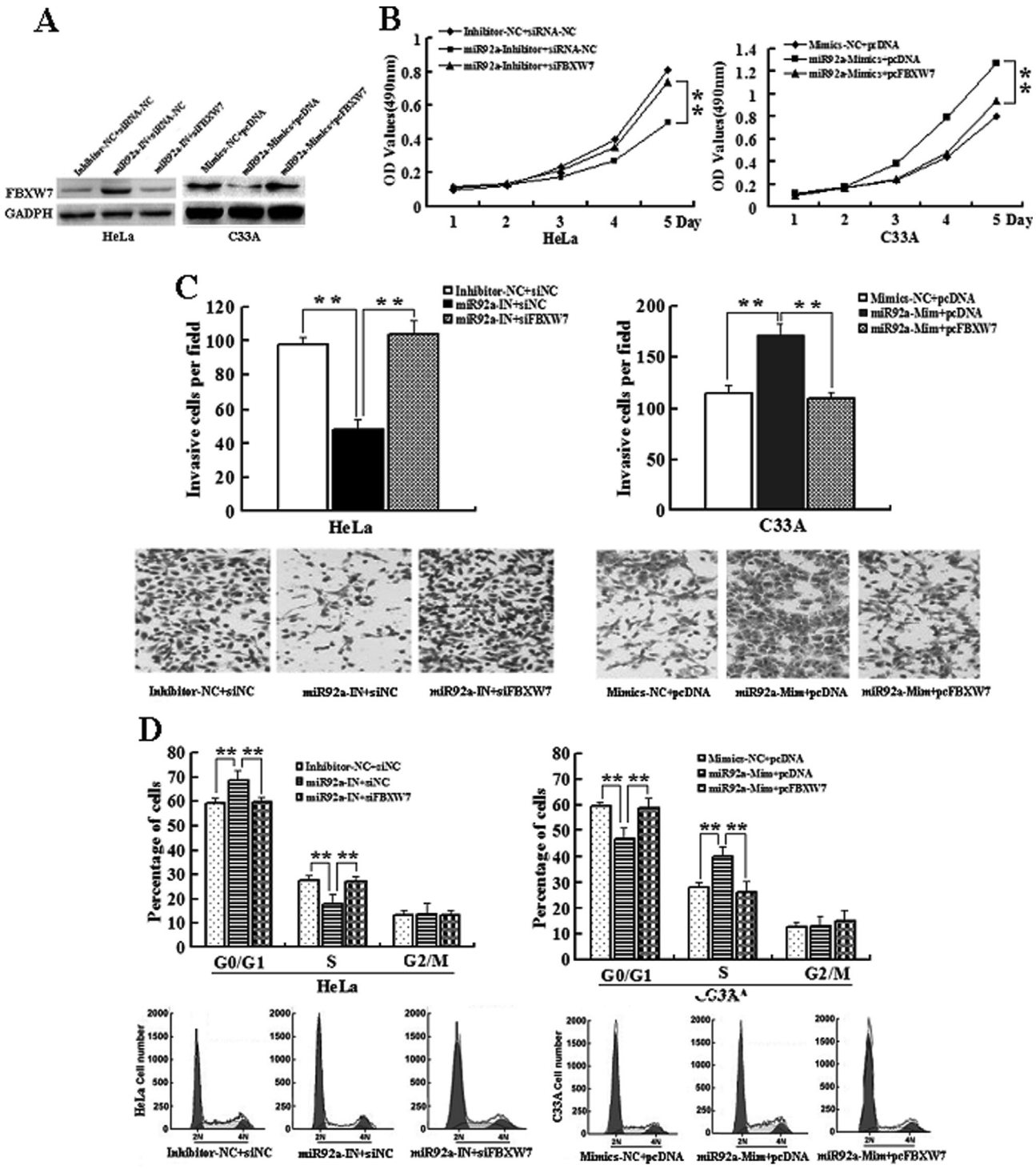

Fig. 4. FBXW7 is involved in miR-92a regulation of cervical cancer cell proliferation and invasion. HeLa cells were transfected with miR-92a inhibitor or cotransfected with miR-92a inhibitor and FBXW7 specific siRNAs (siFBXW7), while C33A cells were transfected with miR-92a mimics or cotransfected with miR-92a mimics and FBXW7 expression vector (pcFBXW7). (A) Western blotting analysis of FBXW7 expression in HeLa and C33A cells. (B) Determination of cell proliferation with the MTT assay. (C) Determination of cell invasion ability with the Transwell assay. (D) Flow cytometry analysis of the cell cycle.*$P < 0.05$, **$P < 0.01$ compared with control group.

cancer tissues as compared with that of normal cervical tissues, which were inversely associated with miR-92a expression patterns. In addition, downregulation of miR-92a increased, while its overexpression inhibited FBXW7 expression in cervical cancer cells. Moreover, our luciferase reporter assay showed that miR-92a caused a significantly decrease in the luciferase activity of a wild-type FBXW7 3′UTR reporter but did not influence that of a 'seed region' mutant FBXW7 3′UTR reporter. These data confirm that miR-92a directly regulates FBXW7 gene expression in cervical cancer via binding to the 3′UTR of its mRNA.

FBXW7 is an ubiquitin ligase substrate receptor. Recent studies have shown that FBXW7 is a potential tumor suppressor that is frequently mutated and deleted in a wide variety of human tumors [21]. Oiesina et al. showed that FBXW7 mutation rate in cervical

cancer is approximately 15%, suggesting that it plays a vital role in cervical cancer development [22]. FBXW7 has been found to regulate diverse cellular processes including proliferation, cell cycle, differentiation, migration and invasion by targeting numerous substrates for degradation, including multiple well-known oncoproteins [23–25]. For example, downregulation of FBXW7 promotes cell proliferation, migration and invasion along with EMT in breast cancer by elevating protein level of mTOR [26]. FBXW7 could also function as a negative regulator of cell proliferation by controlling cell cycle transition via the degradation of crucial cell-cycle regulators such as c-myc [27], Jun [28], Cyclin-E [29], and Notch [30]. Consistent with these reports, our studies showed that overexpression of FBXW7 abrogated oncogenic effects of miR-92a on cervical cancer cell proliferation and invasion. Hence, our results for the first time establish a functional link between miR-92a and FBXW7, and confirm that miR-92a-promoted cervical cancer cell proliferation and invasion is mediated, in part, by suppressing FBXW7.

Collectively, this study provides novel evidence that miR-92a promotes the proliferation and invasion of cervical cancer by suppressing FBXW7. Therefore, our findings implicate miR-92a as a novel therapeutic strategy for the treatment of cervical cancer.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.066.

Transparency document

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