Original Article
MiR-630 acts as a tumor suppressor in cervical cancer and inhibits epithelial-mesenchymal transition in cervical cancer

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Abstract: Metastasis and recurrence are the main causes of death in advanced cervical cancer patients. E6/E7, the early genes of the high-risk mucosal human papillomavirus type, plays vital roles in the carcinogenic process of cervical cancer. Beyond this, multifactor can contribute to the formation and evolution of cervical cancer, among which miRNAs play an important role in cancer development and progression. In this study, we found that E6/E7 could remarkably regulate several miRNAs via miRNA microarray analysis. Among these miRNAs negatively regulated by E6/E7, we highlighted the function and molecular mechanism of miR-630 in cervical cancer. We first identified miR-630 expression status in a screen of 10 cervical cancer tissues and matched normal tissues, and found that miR-630 was significantly decreased in tumor tissues compared to normal tissues. Further experiments showed that alteration of miR-630 expression had profound impact on cellular migration and invasion efficiency, down-regulation of miR-630 increased invasion and migration capacity of cancer cells, while overexpression of miR-630 obtained the opposite result. RT-PCR and western blotting analysis demonstrated that miR-630 inhibit EMT in cervical cancer. Further we proved p53 can positively regulate miR-630 expression in cervical cancer cells through CHIP assay. Taken together, miR-630 might act as a tumor suppressor in cervical cancer and inhibit migration and invasion ability of cervical cancer cells through regulating EMT. Moreover, miR-630 expression could be regulated by E6/E7-p53 signaling pathway.

Keywords: E6/E7, p53, miR-630, migration and invasion, EMT

Introduction
Cervical cancer is one of the most common malignant tumors in the world, which is the fourth leading cause of cancer deaths and the third most commonly diagnosed cancer in females worldwide [1-3]. Persistent infection with high-risk HPV has a strong effect on the progression of cervical cancer [4]. HPV can encode two important oncogene proteins E6/E7, the disrupted expression of E6/E7 lead to increased genomic instability and alteration of cell genetic and epigenetics, these changes further activate oncogenes and inactivate tumor suppressor genes, thus inducing the occurrence of cervical cancer [5]. However, the pathogenesis of cervical cancer is a multi-stage process and the occurrence of cervical cancer is accomplished through the abnormal regulation of multiple genes which have synergistic or antagonistic effects on HPV/E6/E7 [6].

MiRNAs are small non-coding RNA molecules of 20-25 nucleotides which are found in plants, animals and some viruses. They generally bind to the 3'-untranslated region (3'UTR) of target messenger RNAs (mRNAs) and suppress the expression of mRNAs by inhibiting mRNAs’ translation or reducing the stability of mRNAs [7]. MiRNAs have been demonstrated to play important roles in cell differentiation, proliferation, apoptosis, invasion and migration...
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Accumulating evidence has illustrated that disorders of miRNA occur in numerous human cancers and they function as tumor promoters or suppressors [9], such as miR-106a, it was reported highly expressed in hepatocellular carcinoma and played a critical role in cancer invasion and metastasis [10]. MiR-21, acted as a tumor suppressor gene in carcinoma of colon by repressing cancer cell proliferation [11]. Participation of miRNAs in carcinogenesis and progression of cervical cancer have also been widely investigated [12]. For instance, the abnormal expression of miR-20b had a close relationship with cervical cancer cells' migration, invasion and proliferation [13].

MiR-630 is located at 15q14.1 of chromosome-linked miRNA cluster, which is an important component of DNA damage response (DDR), can cause subsequent modulation of several targets involved in DNA replication and apoptosis [14]. Currently, a study identified high expression of miR-630 could depress cell migration, invasion and the ability of colony formation in breast cancer [15]. By contrast, miR-630 was reported to be an oncogene that promoted cells' invasion and migration in bladder urothelial carcinoma, and stimulated the proliferation of renal cell carcinomas [16, 17]. However, the mechanism of deregulation of miR-630 in cervical cancer remained elusive.

In present study, we investigated the biological function of miR-630 in cervical cancer, and found that miR-630 can inhibit the migration, invasion via regulating EMT in cervical cancer cells. Moreover, we have confirmed the potential regulation of E6/E7-p53 on the expression of miR-630.

Materials and methods

**MiRNA microarray construction**

In Shanghai YiOu Biotechnology Co. Ltd (YiOu Biotechnology Co, Shanghai, China), a miRNA microarray of silencing E6/E7-group and control group in Caski and Ms751 cell lines were done according to manufacturer's instructions.

**Clinical tissue samples**

Ten freshly-frozen cervical cancer tissues and matched non-cancerous tissues were obtained from patients who underwent surgical resection at Fengxian Hospital. None of these patients had received radiotherapy, chemotherapy, hormone therapy or other related anti-tumor therapies before surgery. All samples were collected with participants' written informed consent, and the experiments were approved by the local ethics committee of the Fengxian Hospital.

**Cell culture**

Caski, Ms751, Hela and Siha cell lines were purchased from ATCC, Manassas, VA, USA. Siha, Hela and Ms751 were cultured in Eagle's Minimum Essential medium (GBICO, Beijing, China), Caski cultured in 1640 RPMI medium (HyClone, Beijing, China). All of them were maintained at 37°C in an incubator with a 5% CO₂ atmosphere supplemented with 10% fetal bovine serum (GBICO, Beijing, China).

**RNA isolation from cell lines and quantitative real-time PCR**

Total RNA was isolated using the Trizol on the basis of the manufacturer's instructions and analysis of mRNA levels was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green-based real-time PCR for all genes. For miRNA quantification, cDNA extraction was performed using miRNA Extraction Kit (Tiangen, Beijing, China) and Taqman miRNA assays (Life Technologies), reverse-transcription real-time PCR were performed for pri-miRNA and mature miRNA quantifications according to the manufacturer's instructions.

**Transfection**

According to the manufacturer's instructions of Lipofectamine 3000 (Invitrogen), stable transfections were constructed. All of the shmiRNAs and miRNA mimics were synthesized by Yazai biological company (Shanghai, China). Cells were infected with shmiR-630 lentiviral/miR-630-mimics lentiviral, the cells were harvested at 48 h post infection, and verified the silencing effects by RT-PCR.

**Cell viability assay (CCK8 assay)**

In 96-well Plates, 3×10³ cells control and silencing/overexpression cervical cancer cells were plated in per well with 100 μl of complete culture medium. Each group set up with five
blocing them in 1% BSA (Bovine Serum Albumin), incubating with primary antibodies against E-cadherin (1:1000, rabbit Abcam), N-cadherin (1:1000, rabbit Abcam), vimentin (1:1000, rabbit Abcam) and species-specific secondary antibodies IRDye680 anti-mouse (1:20000, LI-COR) and IRDye800 anti-rabbit (1:10000, LI-COR). β-actin (1:1000, rabbit Abcam) was used as a control to ensure the equal loading of protein.

ChIP assay

According to the manufacturer, chromatin immunoprecipitation assays were performed using the Chip express kit (Active Motif, Carlsbad, CA, USA). In brief, cells were cross-linked, lysed, sonicated and immunoprecipitation with the p53 antibodies and IgG isotype control. Protein A/G agarose magnetic beads and eluted collected the immunocomplexes. PCR-based quantification of 3000 base upstream region of the miR-630 promoter region was conducted.

Western blotting

Cells were harvested and lysed in 1× loading buffer (Roche Diagnostics, Indianapolis, IN, USA) added with 1:100 β-mercaptoethanol. SDS-PAGE can separate all the protein, and blocking them in 1% BSA (Bovine Serum Albumin), incubating with primary antibodies against E-cadherin (1:1000, rabbit Abcam), N-cadherin (1:1000, rabbit Abcam), vimentin (1:1000, rabbit Abcam) and species-specific secondary antibodies IRDye680 anti-mouse (1:20000, LI-COR) and IRDye800 anti-rabbit (1:10000, LI-COR). β-actin (1:1000, rabbit Abcam) was used as a control to ensure the equal loading of protein.

Statistical analysis

Statistical analyses were conducted using SPSS 21.0 software (Chicago, IL, USA) and GraphPad Prism5 (San Diego, CA) software. Data were presented as means ± SD, student’s t-test was used for comparison between groups. P value less than 0.05 was considered statistically significant.

Results

E6/E7 negatively regulated miR-630 expression and miR-630 was downregulated in cervical cancer tissues

E6/E7 can regulate a wide range of miRNA expression. A miRNA microarray analysis by knoc-
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Figure 1. E6/E7 negatively regulated miR-630 expression and miR-630 was downregulated in cervical cancer tissues. A. A schematic diagram of altered miRNAs by silencing of E6/E7. 8 miRNAs were significantly down-regulated in Caski and Ms751 cells with silencing of E6/E7 compared with control cells and 23 miRNAs were significantly up-regulated in both cell lines, B-E. The expression of miR-630 in Siha, Caski, Hela, and Ms751 cells with silencing of E6/E7, detected by RT-PCR and normalized with 18S expression. F. MiR-630 expression was downregulated in 10 paired cervical cancer tissues and normal tissues from Fengxian hospital via RT-PCR.

The down-regulated microRNA after E6/E7 knockdown

The up-regulated microRNA after E6/E7 knockdown

king down E6/E7 in Caski and Ms751 cells was performed. The results showed that 179 genes were down-regulated (fold change <1, \( P<0.05 \)) and 361 genes were up-regulated (fold change >3, \( P<0.05 \)) in Caski cells, 65 genes were down-regulated and 35 genes were up-regulated in Ms751 cells. 8 miRNAs genes including miR-5194, miR-5088-5p, miR-192-5p, miR-331-5p, miR-183-3p were down-regulated in both cell lines and 23 miRNAs genes...
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Figure 2. MiR-630 had no effect on cervical cancer cell proliferation. A. The expression of miR-630 in cell lysates from Siha, Hela, Caski and Ms751 cells was detected by RT-PCR and normalized with 18s expression. B. Silencing of miR-630 in Siha, Hela cells significantly decreased miR-630 expression, detected by RT-PCR and normalized with 18s expression. C. Overexpression miR-630 in Caski and Ms751 cells significantly increased miR-630 expression, detected by RT-PCR and normalized with 18s expression. D. The cell proliferation of Nc and shmiR-630 groups in Siha, Hela cells were determined by CCK8 assay at 0, 24, 48, 72, 96 h, respectively. E. Vector and mir-630-mimics groups in Caski, Ms751 cells were determined by CCK8 assay at 0, 24, 48, 72, 96 h, respectively. Values are means ± SD.

including miR-630, miR-29b-3p, miR-4758-3p, miR-6824-3p and miR-4436b-5p were up-regulated (P<0.05) (Figure 1A). These data confirmed that the expression of miR-630 was increased with silencing of E6/E7. Moreover, the expression of miR-630 was greatly elevated in Siha, Hela, Caski and Ms751 cells after silencing of E6/E7 by RT-PCR detection (P<0.05) (Figure 1B-E). We further measured the expression of miR-630 in 10 tumor tissues and normal tissues using quantitative real-time PCR, compared with normal tissues, the relative ex-
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expression of miR-630 was significantly reduced in tumor tissues (P<0.01) (**Figure 1F**).

**MiR-630 had no effect on cervical cancer cell proliferation**

Several representative cervical cancer cell lines, Siha, Hela, Caski and Ms751 were chosen for investigation. The expression of miR-630 in these cell lines was detected by RT-PCR (**Figure 2A**). MiR-630 relatively high expressed cell lines Siha and Hela were transfected with shmiR-630. The silencing effects of shmiR-630 in these two cell lines were validated by RT-PCR and the results showed that miR-630 mRNA expression levels were significantly decreased by shmiR-630 (**Figure 2B**). Meanwhile, miR-630 lowly expressed cell lines Caski and Ms751 were transfected with miR-630-mimics. The overexpression efficiency of the miR-630-mimics in these two cell lines were determined by RT-PCR and the results showed that miR-630 mRNA expression levels were significantly increased by transfecting miR-630-mimics (**Figure 2C**). To explore the effect of miR-630 on cervical cancer cell growth, the cells mentioned above were used to detect cell proliferation by Cell Counting Kit-8 (CCK8) assay. The results showed that silencing of miR-630 has no effect on the proliferation of Siha and Hela cells in vitro (P>0.05) (**Figure 2D**). Consistent with this, overexpression of miR-630 has no influence on the proliferation of Caski and Ms751 cells in vitro (P>0.05) (**Figure 2E**). Taken together, these data demonstrated that miR-630 has no effect on proliferation capacity of cervical cancer in vitro.

**MiR-630 suppressed cervical cancer cell invasion and migration in vitro**

We further examined the effects of silencing/overexpression of miR-630 on cervical cancer cell migration and invasion ability by wound healing assay, transwell assays with and without matrigel. Compared with the control group, the wound healing assay indicated that the relative migration distance of miR-630 silenced cells was significantly increased (P<0.01) (**Figure 3A**), while the relative migration distance of miR-630 overexpressed cells was obviously decreased (P<0.01) (**Figure 3B**). Furthermore, the transwell assays with and without matrigel showed that the migration and invasion rates increased when endogenous miR-630 was silenced by shmiR-630 (P<0.01) (**Figure 3C, 3D**). In contrast, ectopic expression of miR-630 significantly inhibited the migration and invasion of Caski and Ms751 cells (P<0.01) (**Figure 3E, 3F**).

In conclusion, these data showed that overexpression of miR-630 significantly suppressed the migration and invasion capacity of cervical cancer while silencing miR-630 promoted the migration and invasion capacity in vitro.

**MiR-630 played a suppressive role on cervical cancer cells’ invasion and migration in vivo**

To further investigate the function of miR-630 on tumor metastasis in vivo, pulmonary mouse models were established, ectopic expression of miR-630 Ms751 and the control cells were injected into nude mice via tail vein. After eight weeks, the mice were sacrificed and their pulmonary were examined. The general observation showed that the mice pulmonary with Ms751-miR630-mimics gained a significant reduction in the number of lung metastatic nodules compared with the control group (**Figure 4A**). Histological examination of the pulmonary tissue showed that the number of pulmonary metastatic nodules was lower in the mice inoculated with the Ms751-miR630-mimics cells.
We used on-line tools NCBI (http://www.ncbi.nlm.nih.gov/pubmed/) to analyze the promoter region of miR-630 and JASPAR (http://jaspar.genereg.net/) to analyze whether some binding sites for p53 exist in the promoter region of miR-630. Fortunately we found that p53 has a binding site located in the promoter region of miR-630, which indicated that E6/E7 may regulate miR-630 expression by restraining p53 (Figure 6A).

To further confirm the relationship between miR-630 and EMT in cervical cancer cells, the mRNA levels of Snail, Twist, Zeb1 and Zeb2, the key transcription factors that promote EMT, were determined by real-time PCR. Compared with the control group, the results showed that Snail, Zeb1 and Zeb2 were significantly increased in miR-630 silenced cells, while Twist remained unchanged (Figure 5E), and Snail, Zeb1 and Zeb2 were significantly reduced in miR-630 overexpressed cells (Figure 5F).

Obviously, miR-630 was closely associated with increased epithelial characteristics and decreased mesenchymal traits.

**MiR-630 could depress EMT in cervical cancer cells**

We further examined whether silencing/overexpression of miR-630 had any impacts on cervical cancer cell EMT. The expression level of E-cadherin, N-cadherin and Vimentin in cervical cancer cells were detected by RT-PCR and western blot. Interestingly, compared with the control group, the results showed that silencing of miR-630 led to increased expression in Vimentin and N-cadherin, and decreased E-cadherin expression, whereas adverse conclusions was found in miR-630 overexpression group (P<0.05) (Figure 5A-D). These results suggested that miR-630 can reverse EMT in cervical cancer cells.

To further confirm p53 can directly regulate miR-630 expression, the DNA-ChIP assay in Caski cells was applied, and 10 primers of miR-630 promoter region were constructed for the nucleic acid electrophoresis (Figure 6B). The results showed that p53 can bind to miR-630 promoter at 0 bp -300 bp regions (Figure 6C).

In addition, CHIP assay was performed once again after transfected sh-p53 in Caski cells and the consequence demonstrated that the
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Figure 5. MiR-630 could depress the epithelial-mesenchymal transition (EMT) in cervical cancer cells. A, C. Relative expression levels of E-cadherin, N-cadherin and Vimentin in Siha cells with mRNA silencing of miR-630 compared with Nc cells, detected by RT-PCR, normalized with 18s and western blot normalized with β-actin, respectively. B, D. Relative mRNA expression levels of E-cadherin, N-cadherin and Vimentin in miR-630 overexpressed Ms751 cells compared with vector cells, detected by RT-PCR, normalized with 18s and western blot normalized with β-actin, respectively. E, Snail, Zeb1 and Zeb2 were significantly increased in miR-630 silenced Siha cells, while Twist remained unchanged. F. Snail, Zeb1 and Zeb2 were significantly reduced in miR-630 overexpressed Ms751 cells, Twist remained unchanged. Data are means ± SD (*P<0.05, **P<0.01).

Discussion

Numerous studies have demonstrated that HPV-DNA integrated into the host cell after HPV infection, and then caused the altering expression of nearby miRNAs. For instance, oncogene E6 in HPV16 positive cervical cancer cells inhibited nearby miR-218 expression [18], HPV could control its own cell cycle through regulating the neighboring miR-145 expression [19]. In our study, we found that miR-630 expression was significantly up-regulated after the silencing of E6/E7, which suggested that there may be a negative relationship between E6/E7 and miR-630.

It has been reported that invasion and metastasis were more frequently occurring in advanced cervical cancer, which led to low sensitivity to chemotherapy drugs and poor prognosis in patients [20]. It was confirmed that high expression of miR-630 can suppress live cancer cells' invasion and migration through targeting the onco-gene Slug [21]. And miR-630 could improve the sensitivity of chemotherapy drugs in lung cancer [22]. Consistent with these existing studies, in our study, overexpression of miR-630 could significantly inhibit the invasion and metastasis ability of cervical cancer cell in both vivo and vitro.

Large number of studies have proved that miRNA play an important role in EMT [23]. MiR-200c could depress EMT process in breast cancer modulated by Zeb1 [24]. MiR-133a suppressed the migration and invasion of esophageal cancer cells by targeting the EMT regulator SOX4 [25]. As it is known that EMT is a biological process that epithelial cells transform into a...
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Figure 6. E6/E7 regulated mir-630 expression through p53 in cervical cancer. A. A schematic representation of the 50-bp region of a human miR-630 promoter (-76 to -127 bp). The underlined portions indicate potential binding sites. B. A Schematic structure of mir-630 promoter. The red and green rectangles indicate exon and intron of miR-630 respectively. The zone between black lines represents the primers used in ChIP analysis. C. ChIP assay was performed using chromatin from Caski cells. D. ChIP assay was performed from Caski cells after silencing of p53. E. The silencing effect of p53 by siRNA p53 in cells, detected by RT-PCR and normalized with 18s expression. F. Mir-630 expression detected by RT-PCR and normalized with 18s expression after silencing p53 by siRNA p53 in cells, P<0.01 (*P<0.05, **P<0.01).

mesenchymal phenotype through a particular program [26]. The main characteristics of EMT are down-regulation of epithelial cells markers, weakening of the intercellular junctions, which are more advantageous to cells’ motility, migration and expansion of the cells [27]. N-cadherin and Vimentin, the characteristics of mesenchymal marker, acquire elevated expression. EMT facilitates migration and invasion capacities of the tumor cells [28]. In present study, silencing miR-630 could reduce the expression of epithelial cells markers and increase the expression of mesenchymal markers, while, the over-

expression of miR-630 could decrease the expression of mesenchymal markers and up-regulate the epithelial cells markers’ expression, therefore, we revealed that miR-630 may participate in the occurrence of EMT.

Literatures reported that some miRNAs could be directly regulated by p53 at the transcriptional level [29], such as miR-215, miR-192, etc [30]. P53 is an important tumor suppressor gene which can regulate the cell cycle, maintain the stability of the genome, prevent the somatic mutation and carcinogenesis, induce
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cell apoptosis [31]. Therefore, the inactivation of p53 plays an important role on tumor’s formation. E6 and E7 proteins emerge in the high-risk HPV types and act as viral oncoproteins which are considered to be greatly associated with human cervical carcinogenesis. E6 and E7 promote the ubiquitin degradation of p53, thus blocking its effects [32]. In our study, according to the analysis of the datasets NCBI and JASPAR, the consequences of DNA-CHIP assay and cell experiments, we sufficiently proved that miR-630 could be positively regulated by p53 at the transcriptional level. Taken together, our data indicated that p53 directly regulate the expression of miR-630 at the transcriptional level and then inhibit the invasion and metastasis capacity of cervical cancer cells.

In summary, miR-630 could suppress the ability of invasion and migration of cervical cancer cells partly via inhibiting the EMT process. E6/ E7 regulate the expression of miR-630 at the transcriptional level by promoting the degradation of p53 protein. Through studying the function and underlying mechanism of miR-630 in cervical cancer, we probably revealed a novel regulatory axis in HPV inducing cervical cancer.

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Disclosure of conflict of interest

None.

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