MicroRNA-362 is downregulated in cervical cancer and inhibits cell proliferation, migration and invasion by directly targeting SIX1

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Abstract. Cervical cancer is the second most common type of cancer in women accounting for 12% of all human cancers in the world. Mounting evidence demonstrates that microRNAs play important roles in the carcinogenesis and progression of cervical cancer. The aim of this study was to investigate the expression, roles and molecular mechanism of microRNA-362 (miR-362) in cervical cancer. According to the results, we found that expression level of miR-362 was significantly reduced in cervical cancer tissues and cell lines. Low miR-362 expression was correlated with FIGO stage, lymph node metastasis and vascular invasion in cervical cancer. Functional assays showed that restoration of miR-362 repressed cell proliferation, migration and invasion in cervical cancer. We also provided direct evidence that sineoculis homeobox homolog 1 (SIX1) was a direct target of miR-362 in cervical cancer, which was confirmed by bioinformatics analysis, luciferase reporter assay, qRT-PCR and western blot analysis. SIX1 was upregulated in cervical cancer and inversely correlated with miR-362 expression in cervical cancer. In addition, SIX1 knockdown could simulate the roles of miR-362 overexpression on cell proliferation, migration and invasion of cervical cancer. Moreover, rescue experiments indicated that restoration of SIX1 was sufficient to abolish proliferation, migration and invasion induced by miR-362 overexpression in cervical cancer cells. The newly identified miR-362/SIX1 pathway provides insight into cervical cancer progression, and may represent a novel therapeutic target.

Introduction

Cervical cancer, a common gynecological malignancy, is the second most common type of cancer in women after breast cancer accounting for 12% of all human cancers worldwide (1,2). It was estimated in 2015 that there would be ~530,000 new cases and 275,000 deaths due to cervical cancer on the basis of global cancer statistics (3). The primary cause of cervical cancer includes early sexual intercourse, promiscuity, and infection with ‘high-risk’ types of human papillomavirus (HPV) (4,5). Currently, predominant treatments for patients with cervical cancer are operation, chemotherapy, and radiotherapy (6,7). Although great efforts on the therapy and prevention of this disease, the prognosis remains unsatisfactory (8). The 5-year overall survival rate for cervical cancer patients with locally advanced disease and metastatic disease is ~30-50% and 5-15%, respectively (9). Therefore, it is important to fully understand the occurrence and development of cervical cancer to benefit and provide therapeutic strategies for patients with this disease.

Mounting evidence demonstrates that microRNAs (miRNAs) play important roles in the carcinogenesis and progression of cervical cancer (10-12). miRNAs are a large family of endogenous, non-coding and single-stranded RNA molecules with regulatory functions (13). The length of miRNAs are ~19-22 nucleotides (14). miRNAs modulate their target gene expression through directly interaction with the matched sites of target genes and triggering mRNAs degradation or translation inhibition (15-17). In this way, miRNAs participate in a great deal of diverse biological processes, including cell growth, development, apoptosis, survival, and metastasis (18-20). An increasing number of studies indicated that the abnormal expression of miRNAs are found in almost all human cancers (21-23), and could act as oncogenes or tumor suppressors mainly depending on the characteristics of their target genes (20). For example, miR-138 targets the oncogene MMP9, to inhibit cell invasion and metastasis of cervical cancer (24). miR-140 acts as an oncogene in cervical cancer via promoting cell growth and survival by targeting the tumor suppressor FOXO4 (25). These findings suggested that miRNAs could possess great potential as efficient therapeutic targets for treatments of cervical cancer.

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In this study, we examined miR-362 expression in cervical cancer tissues and cell lines. The association between miR-362 expression and clinicopathological features was also analyzed. In addition, the effects of miR-362 on cervical cancer cell proliferation, migration and invasion were investigated. Moreover, the molecular mechanism underlying the roles of miR-362 in cervical cancer was explored.

**Materials and methods**

**Ethical statement and tissue specimens.** This study was approved by the Ethics Committee of Beijing Chao-Yang Hospital, and was conducted following the principles laid down in the Helsinki Declaration. Fifty-eight pairs of cervical cancer tissues and matched adjacent normal tissues were collected from patients with cervical cancer who were operated at Department of Obstetrics and Gynecology, Beijing Chao-Yang Hospital. All tissues were frozen in liquid nitrogen and stored at -80°C until use.

**Cell lines, culture conditions and oligonucleotide transfection.** Human cervical cancer cell lines (HeLa, CaSki, C33A, SiHa) and an immortalized HPV-negative skin keratinocyte line (HaCaT) were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) or RPMI-1640 medium (Gibco) in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (v/v) fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). A light microscope (Olympus IX53; Olympus, Tokyo, Japan) was used to capture five fields per membrane. Each experiment was repeated at least three times.

**miR-362 target prediction and luciferase reporter assay.** The potential target genes of miR-362 were predicted using the TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org/microrna/). PGL3-SIX1-3’UTR Wt and PGL3-SIX1-3’UTR Mut were synthesized and confirmed by GenePharma. HEK293T cells were seeded into 24-well plates, after which they were transfected with miR-362 mimics or NC, along with PGL3-SIX1-3’UTR Wt or PGL3-SIX1-3’UTR Mut using Lipofectamine 2000. Forty-eight hours after transfection, the cell lysates were subjected to luciferase activity measurement by using Dual-Luciferase Reporter assays (Promega, Manheim, Germany), according to the manufacturer's protocol. All experiments were carried out in triplicate.

**Western blot analysis.** Primary antibodies used in this study were mouse anti-human monoclonal SIX1 antibody (1:1,000 dilution, sc-514441; Santa Cruz Biotechnology, CA, USA) and mouse anti-human monoclonal GADPH antibody (1:1000 dilution, sc-365062; Santa Cruz Biotechnology). Total protein was extracted from tissues, cell lines, and transfected cells using ice-cold RIPA buffer (Beyotime Institute of Biotechnology, Jiangsu, Haimen, China), and subjected to Bisinchonic Acid Protein assay kit (BCA; Thermo Fisher Scientific, Inc., Rockford, IL, USA) to measure its concentration. Equal proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes (Millipore, Bedford, MA, USA) and then blocked in 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated with specific primary antibodies at 4°C overnight, followed by incubation with corresponding horseradish peroxidase (HRP)- conjugated secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h. The specific
signals were visualized by using ECL chemiluminescent kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis. Data are presented as mean ± SD, and compared using SPSS version 13.0 software (Chicago, IL, USA). P-value <0.05 was considered statistically significant.

Results

miR-362 is downregulated in cervical cancer tissue and cell lines. In an attempt to explore the roles of miR-362 in cervical cancer, we first measured its expression in cervical cancer tissues and matched adjacent normal tissues using qRT-PCR. The expression levels of miR-362 were obviously reduced in cervical cancer tissues compared with those in matched adjacent normal tissues (Fig. 1A, P<0.05). We also detected miR-362 expression in cervical cancer cell lines. In comparison with an immortalized HPV-negative skin keratinocyte line HaCaT, miR-362 was significantly downregulated in cervical cancer cell lines HeLa, CaSki, C33A and SiHa (Fig. 1B, P<0.05). These results suggested that miR-362 may be involved in the regulation of malignant properties of cervical cancer.

Association between miR-362 expression and clinicopathological factors in cervical cancer. We further evaluated the association between miR-362 expression and clinicopathological features in cervical cancer. As shown in Table II, miR-362 expression was significantly correlated with FIGO stage (P=0.010), lymph node metastasis (P=0.034) and vascular invasion (P=0.039). However, there were no significant correlations between miR-362 expression and age (P=0.284), tumor size (P=0.315) or histological grades (P=0.439).

Effects of miR-362 overexpression on cell proliferation, migration and invasion in cervical cancer. To further investigate the roles of miR-362 in cervical cancer, miR-362 mimics were injected into HeLa and CaSki cells which expressed relatively lower miR-362 expression. After transfection at
48 h, qRT-PCR was performed to evaluate the transfection efficiency. As shown in Fig. 2A, miR-362 expression was markedly increased in miR-362 mimic-transfected HeLa and CaSki cells as compared with NC groups (P<0.05).

MTT assay was used to investigate the effect of miR-362 overexpression on cervical cancer cell proliferation. As presented in Fig. 2B, miR-362 mimics obviously suppressed the HeLa and CaSki cells proliferation compared with NC (P<0.05). Cell migration and invasion assay was adopted to evaluate the effect of miR-362 on cervical cancer metastasis. As shown in Fig. 2C, ectopic of miR-362 expression impaired the migration and invasion of both HeLa and CaSki cells (P<0.05). Collectively, miR-362 acted as a tumor suppressor on malignant proliferation, migration and invasion in cervical cancer cells, consequently it is involved in the progression of cervical cancer.

SIX1 is a direct target of miR-362. Based on bioinformatics analysis with TargetScan and miRanda, SIX1 was identified as a potential target of miR-362 (Fig. 3A). To further explore whether SIX1 was the direct target of miR-362, luciferase reporter assay was used. As shown in Fig. 3B, miR-362 significantly decreased the luciferase activities of PGL3-SIX1-3’UTR Wt (P<0.05) but not the PGL3-SIX1-3’UTR Mut in the HEK293T cells, suggesting direct regulation of miR-362 in the 3’UTR of SIX1.

To determine the regulation effects of miR-362 on SIX1 expression, qRT-PCR and western blot analysis were
performed in HeLa and CaSki cells after transfection with miR-362 mimics or NC. The results showed that miR-362 overexpression reduced SIX1 mRNA (Fig. 3C, P<0.05) and protein (Fig. 3D, P<0.05) expression in HeLa and CaSki cells. These results suggested that SIX1 was a direct target of miR-362.

SIX1 is inversely correlated with miR-362 expression in cervical cancer. Next, we measured SIX1 endogenous expression in cervical cancer tissues and matched adjacent normal tissues. The results of qRT-PCR and western blot analysis showed that SIX1 mRNA (Fig. 4A, P<0.05) and protein (Fig. 4B, P<0.05) was both significantly upregulated in cervical cancer tissues compared with matched adjacent normal tissues. In addition, the expression level of SIX1 in cervical cancer cell lines was also determined, and revealed that SIX1 protein level was higher in cervical cancer cell lines than that in HaCaT (Fig. 4C, P<0.05). Moreover, Spearman’s correlation analysis was performed to explore the association between miR-362 expression and SIX1 mRNA in cervical cancer tissues. As shown in Fig. 4D, SIX1 mRNA expression was inversely correlated with miR-362 expression in cervical cancer tissues (r=-0.7179, P<0.001).

Knockdown of SIX1 represses cell proliferation, migration and invasion in cervical cancer. In order to shed light on the roles of SIX1 in cervical cancer, MTT assay, cell migration and invasion assay were carried out in HeLa and CaSki cells transfected with SIX1 siRNA or NC siRNA. After transfection for 72 h, western blot analysis was performed to determine the SIX1 expression. As shown in Fig. 5A, SIX1 siRNA caused a significantly repression on SIX1 protein expression levels (P<0.05). Functional assays revealed that downregulation of SIX1 decreased the proliferation (Fig. 5B, P<0.05), migration and invasion (Fig. 5C, P<0.05) in HeLa and CaSki cells. These results indicated that SIX1 knockdown could simulate the roles of miR-362 overexpression on cell proliferation, migration and invasion.

Figure 3. SIX1 is a direct target of miR-362 in cervical cancer. (A) Predicted binding sites for miR-362 in the SIX1 3'UTR and the mutations in the binding sites. (B) Upregulation of miR-362 decreased the luciferase activities that carried Wt but not Mut 3'UTR of SIX1. (C) qRT-PCR detection of SIX1 mRNA in HeLa and CaSki cells transfected with miR-362 mimics or NC. (D) miR-362 overexpression downregulated SIX1 protein in HeLa and CaSki cells. *P<0.05.

Figure 4. SIX1 is inversely correlated with expression level of miR-362 in cervical cancer tissues. (A) qRT-PCR was performed to measure SIX1 mRNA in cervical cancer tissues and matched adjacent normal tissues. (B) Western blot analysis of SIX1 protein expression in cervical cancer tissues and matched adjacent normal tissues. (C) Western blot analysis of SIX1 protein expression in four cervical cancer cell lines and HaCaT. (D) Negative correlation was found between relative miR-362 and SIX1 mRNA expression in cervical cancer tissues. *P<0.05.
invasion of cervical cancer, suggesting SIX1 was a functional downstream target of miR-362.

Restoration of SIX1 abolished the tumor suppressive roles induced by miR-362 in cervical cancer cells. To confirm that miR-362 inhibited proliferation, migration and invasion by directly targeting SIX1, rescue experiments were performed. pcDNA3.1-SIX1 or pcDNA3.1 was transfected into HeLa and CaSki cells. The transfection efficiency was assessed using western blot analysis, and found that SIX1 protein was significantly upregulated in pcDNA3.1-SIX1 transfected HeLa and CaSki cells (Fig. 6A, P<0.05).

Rescue experiments indicated that restoration of SIX1 was sufficient to abolish proliferation (Fig. 6B, P<0.05), migration and invasion (Fig. 6C, P<0.05) induced by miR-362 overexpression in HeLa and CaSki cells. These results suggested that the inhibition of proliferation, migration and invasion of cervical cancer induced by miR-362 overexpression was at least partly through repression of the SIX expression.

Discussion

Accumulated evidence demonstrates that miRNAs play significant roles in the cervical cancer occurrence and development (26-28). These miRNAs may act as tumor suppressors or oncogenes through regulation of their target genes. Therefore, identification of cancer-specific miRNAs and their target genes in cervical cancer is critical for understanding their roles in tumorigenesis and tumor development, and could be investigated as novel therapeutic targets. However, so far, there...
is a paucity of data on the expression, functions and targets of miR-362 in cervical cancer. In this study, our results showed that miR-362 was significantly downregulated in cervical cancer. In addition, reduced miR-362 expression was correlated with FIGO stage, lymph node metastasis and vascular invasion, which strongly suggested that miR-362 may contribute to the progression of cervical cancer. Functional assays demonstrated that ectopic miR-362 inhibited proliferation, migration and invasion of cervical cancer cells. Moreover, SIX1 was validated as a direct and functional downstream target gene of miR-362 in cervical cancer. These findings suggested that miR-362 played tumor suppressive roles in cervical cancer carcinogenesis and progression.

Expression of miR-362 has been investigated in various types of cancer. For example, in breast cancer, miR-362 was found to be significantly upregulated in MDA-MB-231 and MCF7 cell lines compared with the control CCD-1095Sk cell line (29). Yang et al showed that expression level of miR-362 was higher in chronic myelocytic leukemia cell lines and fresh blood samples from chronic myelocytic leukemia patients (30). Ni et al reported that obvious upregulation of miR-362 was identified in hepatocellular carcinoma, and significantly associated with tumor progression (31). In gastric cancer, miR-362 expression was upregulated in tumor tissues and cell lines in comparison with that in normal gastric tissues and primary normal human gastric epithelial cells, respectively (32). These studies provided evidence to suggest that miR-362 was upregulated; however, in neuroblastoma, miR-362 expression level was reduced (33). In this study, we showed that miR-362 was significantly downregulated in cervical cancer. In addition, reduced miR-362 expression was correlated with FIGO stage, lymph node metastasis and vascular invasion. Therefore, miR-362 appeared to be involved in carcinogenesis and cancer progression, and played various roles depending on the type of cancer.

miR-362 has been investigated in the development of diverse human cancers. Ni et al demonstrated that miR-362 underexpression suppressed breast cancer cell proliferation, migration, invasion, induced G1 arrest and promoted apoptosis (29).

In chronic myelocytic leukemia, downregulation of miR-362 decreased tumor growth and upregulated GADD45α expression in a xenograft model (30). Previous study also

Figure 6. Upregulation of SIX1 abolishes the effects of miR-362 overexpression on cervical cancer cells. (A) SIX1 protein expression was upregulated in HeLa and CaSki cells after transfection with pcDNA3.1-SIX1. (B) SIX1 overexpression rescued the suppressive effect of miR-362 on HeLa and CaSki cell proliferation. (C) Restoration of SIX1 rescued the effect of miR-362 overexpression on the migration and invasion of HeLa and CaSki cells. *P<0.05.
observed that miR-362 knockdown inhibited hepatocellular carcinoma cell proliferation, clonogenicity, migration and invasion in vitro as well as tumor growth and metastasis in vivo (31). Wu and his colleagues revealed that upregulation of miR-362 repressed cell proliferation, migration and invasion of neuroblastoma in vitro, and decreased tumor growth of neuroblastoma in vivo (33). In gastric cancer, miR-362 knockdown inhibited cell proliferation, colony formation, and resistance to cisplatin-induced apoptosis (32). These findings suggested that miR-362 played important roles in these cancer types, and may therefore serve as a potential therapeutic target for the treatment of these cancers.

Identification of miR-362 target genes is important for understanding its role in tumorigenesis and tumor progression. Several target genes of miR-362 have been identified, including CYLD in hepatocellular carcinoma (31) and breast cancer (29), PI3K-C2β in neuroblastoma (33), and GADD45a in chronic myelocytic leukemia (30). To further explore the molecular mechanisms underlying the inhibitory effects of miR-362 in cervical cancer, bioinformatics analysis was carried out. Of many potential target genes for miR-362 predicted by algorithms databases, SIX1 was selected for further research. Luciferase reporter assay showed that miR-362 was able to directly target the 3'UTR of SIX1. The results of qRT-PCR and western blot analysis revealed that the restoration of miR-362 decreased SIX1 mRNA and protein expression in cervical cancer cells. In addition, the inverse correlation between miR-362 and SIX1 expression in cervical cancer tissues was further demonstrated showing that upregulation of miR-362 resulted in SIX1 downregulation. SIX1 knockdown was able to simulate the roles of miR-362 overexpression on cell proliferation, migration and invasion of cervical cancer. Furthermore, rescue experiments indicated that enforced SIX1 expression abolished the tumor suppressive roles of miR-362 on growth and metastasis of cervical cancer through directly targeting SIX1.

SIX1, a member of the homeodomain of the SIX families, is located on human chromosome 14q23 (34). Prior studies reported that the abnormal expression of SIX1 was found in breast cancer (35), colorectal cancer (36), esophageal squamous cell carcinoma (37), and pancreatic cancer (38). In cervical cancer, SIX1 expression was upregulated at both mRNA and protein level in tumor tissues compared with normal cervical tissues (39). Its expression was also higher in cervical cancer cell lines. Expression level of SIX1 was significant associated with clinical staging, differentiation and lymph node metastasis of cervical cancer (39,40). Research has demonstrated that SIX1 is related with development, progression, and prognosis of multiple tumors (38,41,42). Considering the importance and roles of SIX1 in cervical cancer, it could be developed as a therapeutic target for patients with cervical cancer. In this study, the results suggested that miR-362 inhibited cell proliferation, migration and invasion via negative regulation of SIX1. Thereby, miR-362 may be useful as a therapeutic target for suppression of human cervical cancer growth and metastasis.

In conclusion, this study is, to the best of our knowledge, the first study to demonstrate that miR-362 was involved in cell proliferation, migration and invasion in cervical cancer. The identification of candidate target genes of miR-362, such as SIX1, may provide an understanding of the potential carcinogenic mechanisms in cervical cancer.

References


