Aberrant expression of miR-153 is associated with the poor prognosis of cervical cancer

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Abstract. Previous studies have demonstrated that microRNAs (miRNAs) are frequently dysregulated in tumors and are associated with the initiation and progression of various types of cancer. miR-153 has been previously shown to have an anti-tumor effect in the majority of cancer types. However, to date, the expression status and function of miR-153 in cervical cancer (CC) remains unclear. In the present study, the expression of miR-153 in CC tissues and cell lines was examined, revealing that the expression of miR-153 was markedly downregulated in the CC tissues and cell lines investigated, when compared with matched noncancerous tissues and normal cervical epithelial cell line. Furthermore, ectopic expression of miR-153 by miR-153 mimic inhibited cell proliferation; however, transfection with the miR-153 inhibitor promoted the cell proliferation in CC cell lines. Finally, the results showed that the downregulation of miR-153 was associated with poor 5-year over survival in CC patients and it could be regarded as an independent biomarker to predict the prognosis of CC patients. Collectively, these results indicated that miR-153 may function as a tumor suppressor in CC, and it may be a potential novel therapeutic target for CC.

Introduction

Cervical cancer (CC) is one of the most malignant female reproduction cancers in human, as well as the fourth most common cause of cancer-related morality in the world (1,2). Globally, it was estimated that there were about 530,000 new diagnosed cases each year (1). In the meantime, approximately 135,000 causes were found in China, accounting for about 25% of all the new CC causes each year (3). Despite advances in the diagnosis and treatment methods for CC in recent years, unfortunately, the prognosis of CC patients remains poor (4–6). Therefore, exploring the molecular mechanisms related to the tumor initiation and progression of CC is of vital importance and might provide new biomarker to predict and improve the prognosis of CC patients.

Increasing researches have demonstrated that microRNAs (miRs) are a class of small, non-coding RNAs that play critical roles in various biological cellular processes, including cell proliferation, migration, differentiation, and apoptosis (7-9). In recent decades, dysregulation of miRs have been implicated in the development and malignant progression of human cancers and might be used as potential diagnostic or therapeutic targets for cancers (10-12). To date, over 2,500 miRs have been identified in human genome (13). Among numerous cancer-related miRs, miR-153 was recently found to be dysregulated in human cancers including breast cancer (14), ovarian cancer (15), osteosarcoma (16), esophageal squamous cell carcinoma (17), gastric cancer (18), pancreatic cancer (19), non-small-cell lung cancer (20), and colorectal cancer (21). Significantly, miR-153 expression was found upregulated in colorectal cancer but downregulated in the rest cancer types mentioned above (14-21), which implies that miR-153 plays a dual role in the progression of human cancers. However, up to now, the role of miR-153 in regulating human CC cells remains unexplored. Importantly, miR-153 was found dysregulated in gynecological tumors including breast cancer and ovarian cancer (14,15). Therefore, it is worthwhile to investigate the clinical significance of miR-153 in CC.

In the present study, we found miR-153 was significantly downregulated in CC tissues and cell lines. Ectopic the expression of miR-153 inhibited cell proliferation in CC cell lines. Moreover, the downregulation of miR-153 in CC patients was associated with tumor size and lymph node metastasis. Furthermore, we revealed that the downregulation of miR-153 predicts the poor prognosis of CC patients. Taken together, our data revealed that miR-153 plays a tumor suppressor role...
in the progression of CC and may provide a novel potential therapeutic target for CC.

Materials and methods

Clinical tissue samples. This study was approved by the Ethics Committee of The Central Hospital of Wuhan. A total of 93 pairs of CC tissues and matched noncancerous tissues were collected at the Central Hospital of Wuhan between March 2008 and September 2011. The written informed consent has been obtained from all the enrolled patients. These enrolled patients did not receive any anti-tumor treatments prior to surgical resection. Tissues were immediately snap-frozen in liquid nitrogen after surgical resection, and stored in liquid nitrogen before usage. Clinicopathological parameters of the enrolled patients were collected at the beginning of the follow-up period and summarized in Table I. The overall survival time was calculated as the time between the date of surgery and the date of mortality or last follow-up for 60 months.

Cell lines and cell culture. The human CC cell lines including C-33A, HeLa, and SiHa were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), and 100 U/ml penicillin and 0.1 mg/ml streptomycin. The human cervical epithelial cell line End1 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Keratinocyte-Serum Free medium (K-SFM; Invitrogen; Thermo Fisher Scientific, Inc.) with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, and additional calcium chloride 44.1 mg/l (final concentration 0.4 mM). Cells were maintained in humidified incubator at 37°C containing 5% of CO₂.

Cell transfection. The miR-153 mimic (5'-UUGCAAUAGU CACAAAAGUGAUC-3'), miR-153 inhibitor (5'-AUCACU UUUUGUGUCAUGCA-3') and negative control (miR-con) (5'-UAGCUUUACAGACUGAUGUGA-3') were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The cells were seeded into 6-well plates at a density of 3x10⁴ cells/well. The synthetic miRNAs were transfected to the cultured cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the instructions provided by the manufacturer. Further analyses were performed 24 h post transfection.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the cultured cell lines was isolated using TRIzol reagent (Beyotime Institute of Biotechnology, Haimen, China). RNA was reverse transcribed to cDNA using TaqMan miRNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-PCR was performed using PrimeScript miRNA RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The following procedures were used: 1 cycle at 95°C for 2 min, 40 cycles at 95°C for 30 sec and 58°C for 40 sec. U6 snRNA was used as an internal control to normalize the expression of miR-153. The primers for miR-153 and U6 snRNA used in this study were purchased from Guangzhou RiboBio Co., Ltd. and the detailed sequences were as follows: miR-153: Forward, 5'-TTGCATAGTCACAAAAGTGAT-3'. Reverse, 5'-CAG TCGCTGTCGTGGAAT-3'; U6 snRNA: Forward, 5'-CTC GCTTGGGCGACGACATACT-3', Reverse, 5'-ACGCTT CAGCAATTTCGCTGTC-3'. The relative expression levels were calculated using the 2^(-ΔΔCq) method (22).

Cell proliferation assay. To assess the cell proliferation rate, MTT method was employed. Briefly, the cells were cultured in 96-well plate at the density of 3x10⁴ cells/well. At indicated time points (0, 24, 48, 72 h), 10 µl MTT solution (5 mg/ml; Beyotime Institute of Biotechnology) was added to each well and incubated for additional 3 h. Then, the supernatant was discarded followed by adding 200 µl DMSO to dissolve the violet formazan crystals. After incubation for 2 h, the optical density was measured at 570 nm using the Multiskan Spectrum equipment (Thermo Fisher Scientific, Inc.).

Statistical analysis. SPSS v16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. To determine the significance of two groups and multiple groups, Student’s t test and one-way ANOVA were conducted respectively. Chi-square test was used to analyze the correlation between the expression of miR-153 and clinicopathological features. The Kaplan-Meier curve and log-rank test was used to analyze the overall survival of CC patients. Univariate and multivariate analyses with Cox proportional hazards model were used to identify the independent predictors for the prognosis of CC.

Table I. Associations between microRNA-153 expression and clinicopathological features.

| Variable                        | Cases (n) | High (n=25) % | Low (n=68) % | P-value |
|---------------------------------|-----------|--------------|--------------|---------|
| Age (years)                     |           |              |              |         |
| >50                             | 45        | 12 (26.7)    | 33 (72.3)    | 0.662   |
| ≤50                             | 48        | 13 (27.1)    | 35 (71.9)    |         |
| Lymph node metastasis           |           |              |              |         |
| Negative                        | 39        | 11 (28.2)    | 28 (71.8)    | 0.039   |
| Positive                        | 54        | 14 (25.9)    | 40 (74.1)    |         |
| Tumor size                      |           |              |              |         |
| ≥4 cm                           | 55        | 16 (29.1)    | 39 (70.9)    | 0.018   |
| <4 cm                           | 38        | 9 (23.7)     | 29 (76.3)    |         |
| Clinical stage                  |           |              |              |         |
| I-II                            | 47        | 10 (21.3)    | 37 (78.7)    | 0.093   |
| III                             | 46        | 15 (32.6)    | 31 (67.4)    |         |

miR-153, microRNA-153.
Data were presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*MiR-153 is downregulated in CC tissues and cell lines.* We measured the expression of miR-153 in CC cell lines (C-33A, SiHa, and HeLa) and normal cervical epithelial cell line End1 by RT-qPCR. As presented in Fig. 1A, the expression of miR-153 was significantly reduced in CC cell lines investigated compared with that in normal cervical epithelial cell line (all P<0.01). To further confirm the significance of miR-153 expression in CC, the expression level of miR-153 in 93 paired CC tissues and adjacent normal tissues was measured by the same method. We found the miR-153 expression was significantly lower in CC tissues compared with adjacent normal tissues (P<0.01; Fig. 1B), which was consistent with the observations on CC cell lines and normal cervical epithelial cell line. These 93 CC patients were then classified into two groups based on the expression level of miR-153: Namely high miR-153 expression group and low miR-153 expression group. The 75th percentile of 2^ΔΔCq was used as the cut-off point (0.72) for patients with high or low miR-153 expression (25).

*MiR-153 inhibits the proliferation of CC cell lines in vitro.* To explore the role of miR-153 in CC cells, miR-153 expression level was altered with miR-153 mimic and inhibitor. RT-qPCR showed that miR-153 expression was significantly enhanced in all the investigated CC cell lines transfected with miR-153 mimic compared with those transfected with miR-con (all P<0.01; Fig. 2A). Conversely, the miR-153 inhibitor transfection could reduce the expression of miR-153 in the CC cell lines investigated (all P<0.01; Fig. 2A). Cell proliferation rate was measured using MTT assay, the cell proliferation of CC cell lines transfected with miRNAs was also measured by the same method. As shown in Fig. 2C, we found the CC cells transfected with miR-153 mimic showed obvious growth inhibition, while those transfected with miR-153 inhibitor showed obvious growth stimulation (all P<0.05).
Clinical significance of miR-153 expression in CC. To investigate the clinical significance of miR-153, we assessed the association between miR-153 expression and clinical-pathological features. The results revealed that low miR-153 expression was associated with tumor size (P=0.018), lymph node metastasis (P=0.039) but not related to age (P=0.662), clinical stage (P=0.093) in CC patients (Table I). The Kaplan-Meier curve and log-rank test was employed to analyze the effect of miR-153 expression on 5-year overall survival of the 93 enrolled CC patients. We found the patients with high miR-153 expression (n=25) live longer than those with low miR-153 expression (n=68) (P=0.037; Fig. 3). Additionally, multivariate analysis indicated that miR-153 expression was an independent predictor for the overall survival in CC patients (P=0.025; Table II). Taken together, there was a strong correlation between low miR-153 expression and poor outcomes in CC patients.

Discussion

miRNAs function mainly through complementary binding to the 3'-UTR of target gene which can induce the degradation of its mRNA, or suppress gene transcription to reduce the expression levels of its target genes (24-26). Many studies have confirmed that miRNAs were critical players in the initiation and progression of human cancers including CC (27,28). For CC patients, the overall survival has significantly improved in the past decade due to the usage of HPV vaccination and improvement of therapeutics methods but it is still undesirable (4,5). Therefore, it is of great importance to understand the molecular mechanisms underlying the progression of CC, which are important for developing novel therapeutic strategies for patients with CC.

Although documented evidence indicates that miR-153 can function as either oncogene or tumor-suppressor in cancers, the role of miR-153 in regulating CC cells remains unexplored (14-21). In the present study, the miR-153 expression was found to be downregulated in CC tissues and cell lines. The cell proliferation rate analysis results demonstrated that the cell proliferation rate in CC cells was higher than in normal cervical epithelial cell line. To further elucidate the effect of miR-153 on the progression of CC, the synthetic miRNAs were introduced into the CC cell lines. We discovered that the overexpression of miR-153 diminished but the downregulate miR-153 expression increased the proliferation rate in the CC cells investigated. The above findings indicated that miR-153 functions as tumor-suppressor in CC and inhibits CC progression partly through cell proliferation inhibition. The progression of CC is a complex pathological process in which proliferation and apoptosis of CC cells serves an important role (29). The proliferation and apoptosis of CC cells are regulated by multiple molecules, including phosphatase and tension homolog (PTEN) (30), miR-21 (31), miR-940 (32), and DJ-1 (33). Therefore, the aberrant expressed status of miR-153 and the involvement of miR-153 in CC proliferation process highlighted the importance of miR-153 in CC.

Then, we analyzed the 5-year overall survival rate of the recruited CC patients. We found the low expression of miR-153 indicates a poor 5-year survival. Following, we found the low miR-153 expression was related to tumor size and lymph node metastasis. The univariate and multivariate analyses demonstrated that low miR-153 expression was an independent predictor for poor overall survival of patients with CC.

In conclusion, we provided evidence that miR-153 exert its tumor suppressor potential by inhibiting cell proliferation. Meanwhile, there was a strong correlation between low miR-153 expression and poor outcomes in CC patients, indicating miR-153 could potentially serve as a therapeutic target for CC.

### Table II. Univariate and multivariate analyses of overall survival.

| Variable                  | Univariate analysis | Multivariate analysis |
|---------------------------|---------------------|----------------------|
|                           | HR      | 95% CI   | P-value | HR      | 95% CI   | P-value |
| miR-153                   | 2.062   | 1.080-3.936 | 0.028 | 2.080   | 1.096-3.949 | 0.025 |
| Age                       | 1.798   | 0.870-3.716 | 0.113 | -       | -         | -       |
| Lymph node metastasis     | 1.981   | 1.103-3.873 | 0.046 | 2.001   | 1.030-3.889 | 0.041 |
| Tumor size                | 2.021   | 1.047-3.904 | 0.036 | 2.043   | 1.065-3.922 | 0.032 |
| Clinical stage            | 1.896   | 0.945-3.804 | 0.072 | -       | -         | -       |

HR, hazard ratio; CI, confidence interval; miR-153, microRNA-153.
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