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Inhibitory effects of miR-101 overexpression on cervical cancer SiHa cells

H. Liu[1], J. Zhao[2], J. Lv[3]

[1] Department of Gynecology, Rizhao People's Hospital, Rizhao
[2] Department of Gynecology, Maternity and Children's Hospital, Peking University First Hospital, Beijing
[3] Department of Laboratory Medicine, Rizhao People's Hospital, Rizhao (China)

Summary

Purpose of investigation: microRNAs (miRNAs), which can regulate cell biological behaviors such as proliferation and apoptosis as oncogenes or anti-oncogenes, are closely associated with cancer onset and progression. The aim of this study was to detect the expression changes of miR-101 in cervical cancer tissues and the effects on the biological functions of cervical cancer SiHa cells. *Materials and Methods:* Through transient transfection of SiHa cells with mature miR-101 sequences, the effects on apoptosis, proliferation, and cell cycle were evaluated by real-time PCR, CCK-8 assay, and flow cytometry. *Results:* Significantly less miR-101 was expressed in cervical cancer tissues than that in normal cervical tissues. miR-101 was overexpressed in SiHa cells through transient transfection of miR-101 mimics. CCK-8 assay and flow cytometry showed that miR-101 overexpression significantly inhibited cell proliferation, promoted apoptosis, and arrested them in the G_1/S phase. Real-time PCR exhibited that Mcl-1 and c-Fos mRNA expressions significantly decreased. *Conclusion:* miR-101 significantly reduced the viability of SiHa cells as a potential anti-oncogene.

Key words: microRNA-101; Cervical cancer; Apoptosis; Cell cycle; Proliferation.

Introduction

Widely existing in eukaryotic cells, microRNAs (miRNAs) are about 18-25 nt-long, evolutionarily conservative small non-coding RNAs, which can negatively regulate gene expression by binding 3'-UTR of target mRNA to affect the stability or to inhibit the translation [1]. Therefore, miRNAs are involved in multiple signaling pathways for tumor formation as either oncogenes or anti-oncogenes [2, 3]. miRNAs play important roles in cell differentiation, proliferation, apoptosis, metabolism, and tumor onset [4, 5], and there are miRNA expression disorders in most tumors [6, 7]; hence changes in miRNA expressions may be the biomarkers for tumor diagnosis, treatment, and prognosis [8].

Abnormal miRNAs expressions are closely related with the onset and progression of cervical cancer. For instance, miR-479 can inhibit the progression of cervical cancer by negatively regulating the proliferation of HeLa cells [9]. miR-200a may be associated with the prognosis of cervical cancer [10]. As a tumor-inhibiting miRNA, miR-101 has two precursors located on human chromosomes 1 and 9, but the mature sequences are highly conservative in all species. With downregulated expressions in prostate, breast, kidney, liver, and endometrial cancers, miR-101 is able to suppress the metastasis and invasion of tumor cells. However, the role of miR-101 in the onset of cervical cancer has rarely been studied.

In this study, to explore the role of miR-101 in the onset and progression of cervical cancer, as well as its clinical significance, the authors detected its expression changes in cervical cancer tissues. Through transient transfection of cervical cancer SiHa cells with mature miR-101 sequences, they evaluated the effects on apoptosis, proliferation, and cell cycle. Meanwhile, they predicted the target genes of miR-101 and postulated the possible molecular mechanism.

Materials and Methods

SiHa cells were cultured in MEM containing 10% FBS at 37°C in 5% atmospheric CO_2. Then cells in the logarithmic growth phase were inoculated onto six-well plates and transfected with miR-101 mimics and negative control (NC) at 20 μmol/L by using lipofectaminTM2000. The experiment was performed according to the instructions of lipofetaminTM2000.

qRT-PCR for miRNA was conducted according to the method of Chen *et al.* [11]. Briefly, a primer for reverse transcription with stem-loop miR-101 structure and a pair of corresponding PCR primers were designed (Table 1). RNAs were extracted from tissue samples or cells according to the instructions of Trizol. The reverse transcription system (20 μL) comprised 100 U M-MLV reverse transcriptase, 50 nmol/L primer with stem-loop structure, and 50 ng total RNA. Real-time PCR was carried out on a quantitative PCR system with corresponding primers and Maser SYBR

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Table 1. — *Primers used to perform qPCR of miR-101.*

Primer	Sequence (5'-3')
miR-101	Forward: GAAGATCTATATGGCCCATCTGAGGTG
	Reverse: CCCAAGCTTAAAAACCTCCCACCACGAAT
Mcl-1	Forward: TCAGCGACGGCGTAACAAACT
	Reverse: CAAACCCATCCCAGCCTCTT
c-Fos	Forward: ACGGCACTTTATATTGAC
	Reverse: TCCGGCTATTAAAATGAT
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT

Green I kit. PCR conditions: 95°C for ten minutes, 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 20 seconds, 40 cycles in total. Each tube contained 20 μL of the reaction solution, and three replicate samples were set. Normalization was performed by using U6 RNA as the internal reference. Relative expression (RQ) of target gene was calculated with the $2^{-\Delta\Delta CT}$ method. RQ = $2^{-\Delta\Delta CT}$ ($\Delta CT_{sample} = CT_{sample} - CT_{u6\ sample}$, $\Delta CT_{control} = CT_{control} - CT_{u6\ control}$, $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{control}$).

Cells were coated onto 96-well plates at the density of 0.5×10^5/well 24 hours after transfection. Three replicate wells were selected every day for detection. After removal of culture medium, 100 μL of serum-free culture medium and ten μL of WST-8 were added into each well to culture the cells at 37°C in 5% atmospheric CO_2 for two hours. Absorbance (A) of each well was detected at 450 nm with an enzyme-linked immunosorbent assay plate reader.

Cell apoptosis was detected according to the kit's instructions by flow cytometry. Cells were digested with EDTA-free trypsin and collected 48 hours after transfection, washed with PBS, re-suspended, and centrifuged at 2,000 r/min for five minutes to give $1\sim5\times10^5$ cells. Afterwards, the precipitate was resuspended with 500 μL of binding buffer, stained with five μL of Annexin V-FITC and five μl of propidium iodide (PI) and incubated at room temperature in dark for 15 minutes. The apoptotic rate was detected by flow cytometry. The experiment was conducted in triplicate.

Cells were digested with trypsin and collected 48 hours after transfection, centrifuged at 2,000 r/min for five minutes, washed with PBS, fixed overnight in pre-cooled 70% ethanol at 4°C, collected the other day, washed once with one mL of PBS, and incubated with 500 μL of PBS (containing 50 μg/ml PI, 100 μg/ml RNase A) at 4°C in dark for 30 minutes. Then the mixture was filtered through a 200-mesh screen and centrifuged, from which the supernatant was discarded. Finally, flow cytometry was carried out using a standard procedure.

miR-101 target genes were predicted as Mcl-1 and c-Fos based on the overlapped results of targetscan, microcosm, and microRNA targets. Meanwhile, the Mcl-1 and c-Fos mRNA expression levels were detected by real-time PCR.

All data were analyzed by SPSS13.0 and expressed as ± S.D. Inter-group comparisons were performed by *t*-test. $P < 0.05$ wa-considered statistically significant.

Results

miR-101 expressions in 20 cervical cancer tissue samples were detected by real-time PCR by using another 20 normal tissue samples as control. Figure 1 shows that miR-101 expression in cervical cancer tissues was significantly downregulated compared with that in normal tissues ($p < 0.05$). As suggested by Figure 1, miR-101 may function as an anti-oncogene. To verify the postulation, the authors overexpressed miR-101 in cervical cancer SiHa cells. Forty-eight hours after transfection with miR-101 mimics, miR-101 expression levels were detected by real-time PCR. Figure 2 shows that miR-101 expression level in SiHa cells significantly increased 48 hours after transfection with miR-101 mimics ($p < 0.05$).

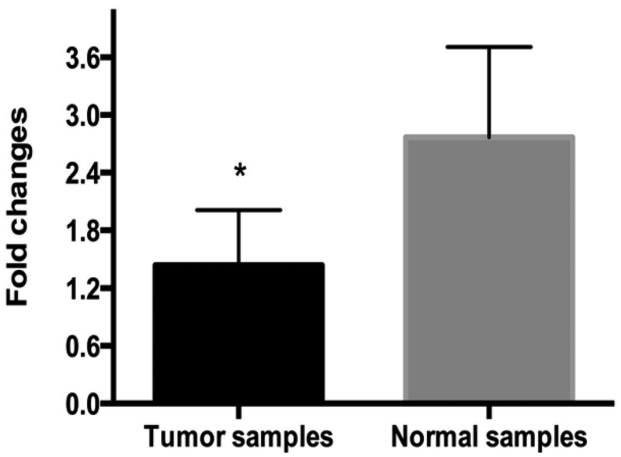

Figure 1. — Real-time PCR analysis of the expression level of miR-101 in cervical cancer samples. Downregulated relative level of miR-101 in 20 cervical cancer samples compared to the level in 20 normal samples, as measures by qPCR. Date represent ±s, derived from three independent experiments. *$p < 0.05$.

The effects of upregulated miR-101 expression on SiHa cell proliferation at different time points were evaluated with the CCK-8 method (Figure 3). Compared with the control group, the proliferation of SiHa cells overexpressing miR-101 significantly decelerated ($p < 0.05$).

Cell cycle and apoptosis were detected by flow cytometry 48 hours after transfection with miR-101 mimics, with those of NC and blank groups as references. Compared with the control group, SiHa cells in which miR-101 was overexpression were more prone to apoptosis (Figure 4A and 4B). In the meantime, the proportion of cells in the G_0/G_1 phase increased and that in the G_2/M phase decreased (Figure 4C and 4D).

miR-101 target genes were predicted based on targetscan, microcosm and microRNA Targets (Figure 5A) as Mcl-1 and c-Fos that mainly promoted cell survival, proliferation, and resistance to apoptosis. Meanwhile, Mcl-1 and c-Fos expression levels were detected by qRT-PCR. Figure 5B and 5C exhibit that Mcl-1 and c-Fos expression levels significantly decreased after miR-101 overexpression ($p < 0.05$).

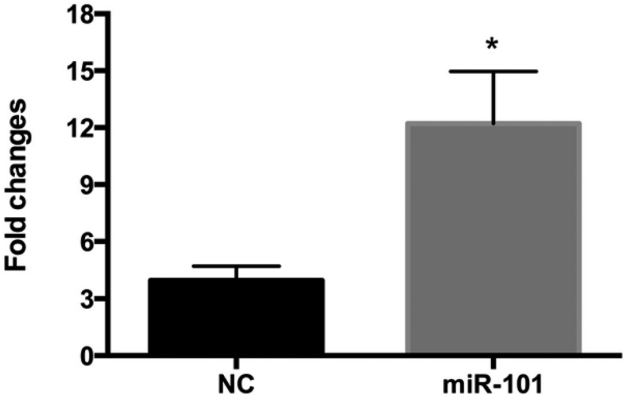

Figure 2. — Expression of miR-101 after transfection with miR-101 mimics for 48 hours in SiHa cells by qPCR analysis. The expression level of miR-101 is unregulated in the SiHa cells transfected with miR-101 mimics compared with the cells transfected negative control mimics. Data represent ±s, derived from three independent experiments. *$p < 0.05$.

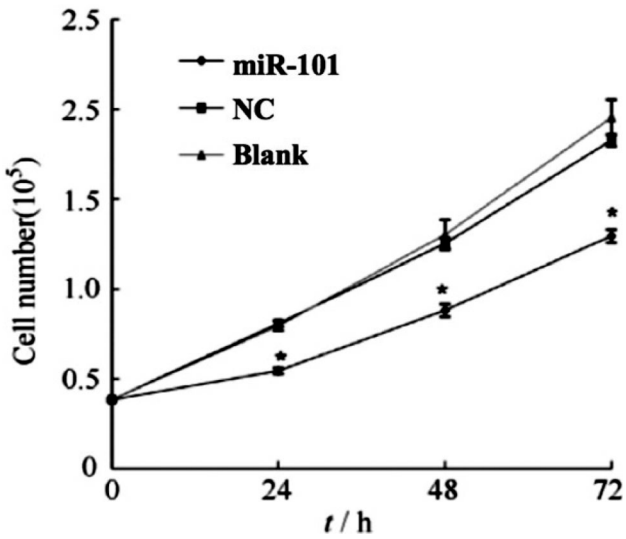

Figure 3. — CCK-8 analysis of SiHa cell proliferation. The three groups of SiHa cells were seeded on 96-well plates at a density of $5X10^3$ per well. The CCK-8 value (450 nm wavelength) was assayed for 24, 48 or 72 hours. The growth curves shows the proliferation of cells with miR-101 overexpression is suppressed compared with that of control cells. Data were obtained from three independent experiments. Values are ±s, *$p < 0.05$.

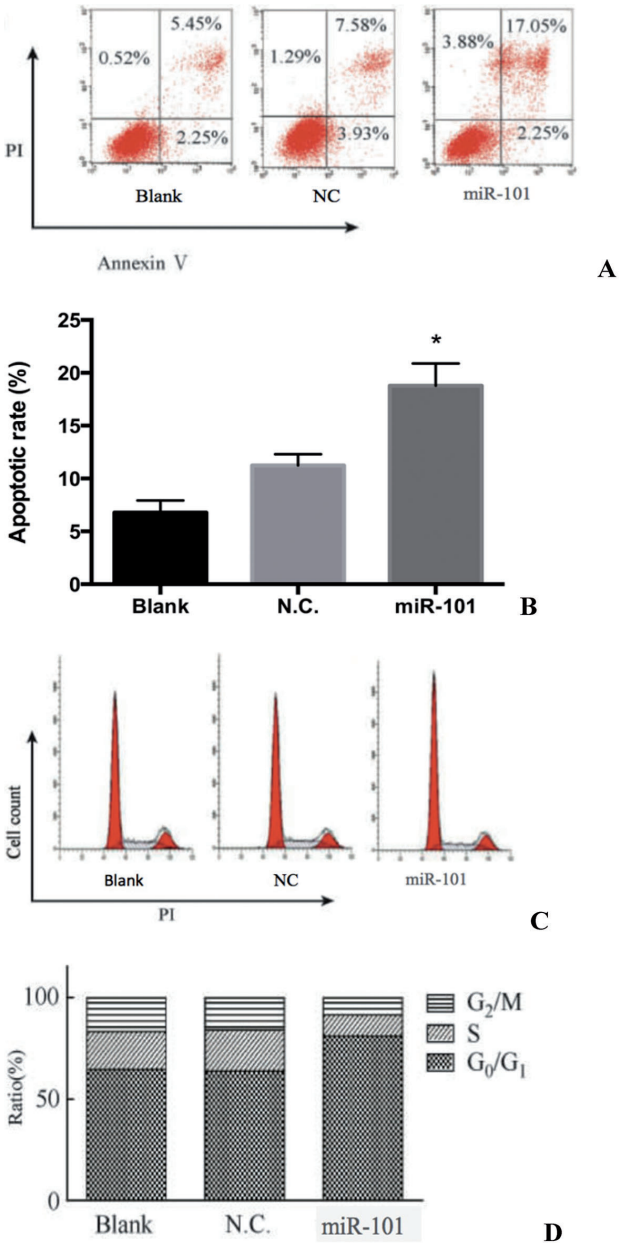

Figure 4. — FACS analysis of the effect of miR-101 on cell cycle and apoptosis. (A and B) Overexpression of miR-101 promote the apoptosis of SiHa cells after transfection with miR-101 mimics for 48 hours; (C and D) G2/M phase and S phase are decreased after transfection with miR-101 mimics for 48 hours. The data represent one of three independent experiments. Data were obtained from three independent experiments. Values are ±s, *$p < 0.05$.

Discussion

Cervical cancer occurs and progresses from cervical intraepithelial neoplasia, early invasive carcinoma, to advanced invasive carcinoma, which is a multi-gene, multi-step biological process that is closely associated with HPV infection, chromosomal mutation, and single nucleotide polymorphisms [12]. Clarifying the molecular mechanism for the onset and progression of cervical cancer provides theoretical evidence for finding new treatment strategies and determining prognosis.

Since cervical cancer has unique miRNA expression profiles, miRNAs may exert crucial regulatory effects on this

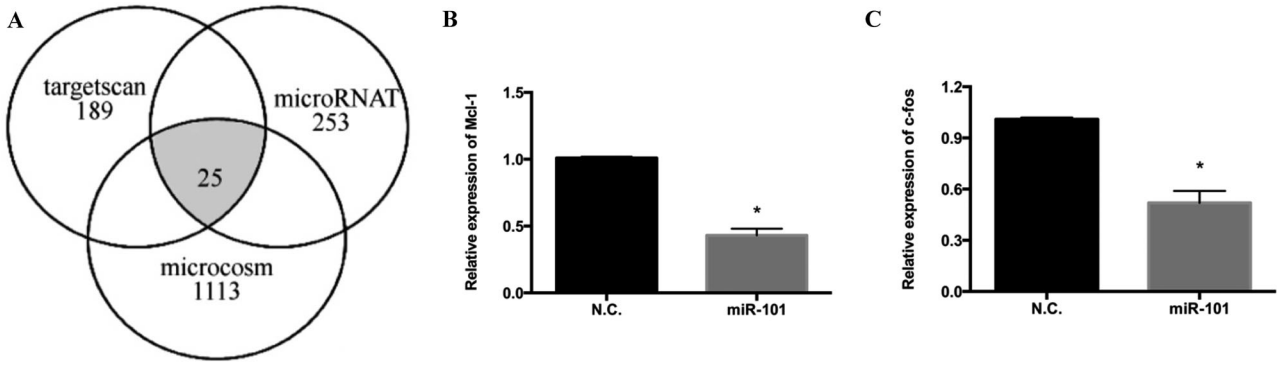

Figure 5. — Potential target genes of miR-101. (A) Venn diagram shows substantial overlap of core genes according to three algorithms; (B and C) represent the expressions of Mcl-1 and c-fos respectively. Data were obtained from three independent experiments. Values are ±s, $*p < 0.05$.

process. For example, miR-125b inhibits HPV infection and participates in the onset of cervical cancer [13]. miR-127 has been correlated with the lymphatic metastasis of this cancer [10]. Significantly downregulated in cervical cancer, miR-143 and miR-145 can suppress the growth of these cells [14]. Moreover, miRNAs may be correlated with the metastasis of cervical cancer. miR-200a cooperates with other regulatory genes to affect the metastasis and invasion of cervical cancer cells [10], and miR-9 is significantly upregulated in cervical cancer samples with lymphatic metastasis [10]. miR-124 is silenced through methylation upon cervical cancer, thus promoting its onset [15], which inspires studies on the regulatory mechanism for miRNA expression changes in cervical cancer.

As a tumor-suppressing miRNA, miR-101 has two precursors located on human chromosomes 1 and 9, but the mature sequences in all species are highly conservative. With downregulated expressions in prostate, breast, kidney, liver, and endometrial cancers, miR-101 can inhibit the metastasis and invasion of tumor cells [16-19]. Wang *et al.* reported that miR-101 expression was downregulated in gastric cancer. Similarly, miR-101 expression was downregulated and its target gene EZH2 was upregulated in patients with prostate cancer [20]. Su *et al.* found that miR-101, as an anti-oncogene, was involved in the onset of liver cancer [16]. Until now, the role of miR-101 in cervical cancer onset and the relevant mechanism have seldom been referred.

In this study, miR-101 expression was significantly downregulated in cervical cancer tissues compared with that in normal tissues. Upregulating miR-101 expression in SiHa cells significantly facilitated cell apoptosis, inhibited proliferation, and induced cell cycle arrest, indicating that miR-101 acted as an anti-oncogene. To unravel the mechanism by which miR-101 regulated the proliferation of cervical cancer cells and induced apoptosis, the present authors predicted its target genes by using three databases as Mcl-1 and c-Fos that were responsible for regulating cell cycle and survival. Mcl-1, as a member of the Bcl-2 family and a key anti-apoptosis protein located in the mitochondrial outer membrane, predominantly regulates the endogenous apoptosis pathway (mitochondrial pathway). In addition, Mcl-1 is closely associated with abnormal regulation of cell apoptosis and tumor onset [21, 22]. Oncogene c-Fos participates in invasive growth of tumor cells, degradation of extracellular matrix, angiogenesis, and cell migration. c-Fos can regulate the expressions of some angiogenic factors. For instance, it promotes angiogenesis in fibrosarcoma by activating angiogenic factor proliferin [23]. Furthermore, c-Fos expression was upregulated in ovarian, oral, and gallbladder cancers [24-26].

Conclusion

In conclusion, detecting miR-101 expression level in patients with cervical cancer provides evidence for treatment and prognosis determination, as well as allows targeted therapy. However, mechanism for the regulatory effects of miR-101 on cervical cancer onset remains unclear, and related signaling pathways of downstream target genes still need in-depth studies.

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Corresponding Author:
H. LIU, M.D.
Department of Gynecology
Rizhao People's Hospital
Rizhao 276826, Shandong Province (China)
e-mail: liuhongrph@126.com