Original Article

miRNA-101 inhibits ovarian cancer cells proliferation and invasion by down-regulating expression of SOCS-2

Hong-Bin Zheng, Xiao-Gang Zheng, Bao-Ping Liu

Department of Nuclear Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

Received July 21, 2015; Accepted October 19, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: Objective: To investigate the expression of miRNA-101 in normal and malignant ovarian tissues and cells as well as its impact on the proliferation and invasion of human ovarian cancer H08910 and SKOV3 cell lines. Methods: Real time polymerase chain reaction (RT-PCR) was employed to detect the miR-101 and SOCS-2 expression in 20 separate ovarian cancer tissues and para-carcinoma tissues, human ovarian cancer cells (H08910 and SKOV3) and normal human ovarian epithelial cells (HUM-CELL-0088). After H08910 and SKOV3 ovarian cancer cells were respectively transfected with miR-NC (H08910/NC and SKOV3/NC) and miR-101 (H08910/miR-101 and SKOV3/miR-101), Western Blot was employed to detect the SOCS-2 expression in transfected cells. CCK-8 and clone formation and Transwell assays were employed to determine the proliferation and invasion ability of wild type and transfected ovarian cancer cells. Results: The expression of miR-101 in ovarian cancer tissues and cells was significantly lower than that in para-carcinoma tissues (t=19.12, P=0.002) and normal human ovarian epithelial cells (HUM-CELL-0088) (F=14.37, P=0.000), respectively. In contrast, the SOCS-2 expression in ovarian cancer tissues and cells was significantly higher than that in para-carcinoma tissues (t=25.03, P=0.000) and HUM-CELL-0088 cells (F=14.9, P=0.000) by Western Blotting analysis, respectively. Compared with wild type and empty vector transfected cells, the expression of SOCS-2 was significantly decreased in miR-101 transfected H08910 (t=10.9, P=0.001) and SKOV3 cells (t=21.03, P=0.000). The results of CCK-8, clone formation and Transwell assays revealed that the proliferation and invasion ability of ovarian cancer cells was markedly inhibited by the transfection of miR-101. Conclusion: miR-101 was validated to be reduced and SOCS-2 expression increased in ovarian cancer tissues and cells. The over expression of miR-101 can remarkably reduce the in vitro proliferation and invasion ability of ovarian cancer cells through the down-regulation of SOCS-2.

Keywords: miRNA-101, SOCS-2, ovarian cancer, cell proliferation

Introduction

Ovarian cancer is a gynecological tumor with the highest malignant degree and the second most lethal cause of malignancy-related death in women around the world [1]. For lacking of specific early symptoms and reliable methods of early imaging diagnosis, its diagnosis rate is very low at the early stage [2]. Therefore, operation and postoperative adjuvant chemotherapy are of important significance for its treatment. However, the effectiveness of chemotherapy is greatly restricted owing to the toxic and side effects of chemotherapeutics and the susceptibility of ovarian cancer to chemotherapeutic [3, 4]. Although revolutionary changes have been made by molecular targeting treatment for many malignant tumors, no effective targeted drug has ever been applied to treat ovarian cancer clinically [4-6]. Hence, in order to improve the treatment efficacy and prognosis in patients, it is very important to further investigate the genesis and development mechanism of ovarian cancer to find and develop an effective target for its treatment.

miRNA-101 is closely associated with the formation and development of human tumor. Its expression level is low in tumor tissues like bladder and gastric cancer, but high in normal ones. In tumor, miRNA-101 plays the role of tumor suppressor genes. miRNA-101 has two precursors, which are located in chromosome 1 and 9. However, its mature sequence is highly conservative in many species. Up to now, it has been reported that it is targeted at genes like MCL-1 (one member of BCL-2 family), as well as oncogenes FOS and EZH2. SOCS2, a member...
miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer

Table 1. The reaction condition of RNA reverse transcription

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template RNA</td>
<td>3 μg</td>
</tr>
<tr>
<td>5×MMLV RT Buffer</td>
<td>4 μl</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>0.75 μl</td>
</tr>
<tr>
<td>1 μM miR-RT primers</td>
<td>1.2 μl</td>
</tr>
<tr>
<td>40 U/μl Rnasin</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>200 U/μl MMLV Reverse Transcriptase</td>
<td>0.2 μl</td>
</tr>
</tbody>
</table>
| RNase-free water                     | The total system was added to 20 μl

of suppressor of cytokine signaling (SOCS) family, is a negative regulatory protein of cytokines. SOCS2 encodes 198 amino acids, including a C-terminal SOCS box, a central SH2 structural domain and an N terminal structural domain. It functions mainly by binding JAK proteins or phosphorylated tyrosine on cytokine receptors with the SH2 structural domain, thus inhibiting JAK/STAT-mediated cascade reactions. SOCS2 can widely act on multiple systems and influence their functions to some extent. Studies find that the aberrant methylation of SOCS2 is closely related to gene silencing during the formation and development of cancer. Both in gastric cancer cells and primary breast cancer cells, there are gene transcriptional silencing and aberrant methylation of SOCS2. All of these cells show growth inhibition.

MicroRNA (miRNA) is a group of endogenous small non-coding RNA molecules about 18~25 nt long and is involved in the regulation of about 30% of coding genes within human body. An increasing number of studies show that miRNA plays a very important role in the genesis and development of various malignant tumors [7, 8]. In this study, the expression of miR-101 in normal and ovarian cancer tissues and cells was compared, and, by transfecting ovarian cancer cells with miR-101, the impact of miR-101 over-expression on the proliferation and invasion ability of ovarian cancer cells was observed, so as to investigate its mechanism of action in the genesis and development of ovarian cancer.

Materials and methods

Cell culture

Human ovarian cancer cell lines H08910 and SKOV3 and normal human ovarian epithelial cells HUM-CELL-0088 were all purchased from American Type Culture Collection (ATCC) and stored in our department. These cells placed in DMEM culture medium supplemented with 10% fetal bovine serum (Invitrogen) were subcultured in an incubator with 5% CO₂ at 37°C.

Clinical data

The object of study was surgical specimens taken from 20 female patients diagnosed with ovarian cancer in our hospital who received operative treatment from June 2014 to February 2015. The age of these patients ranged from 39 to 66 years with a median age of 54 years. Among these operative specimens, ovarian cancer tissues and para-carcinoma tissues (normal ovarian tissues more than 5 cm away from the tumor’s margin) were taken, put into liquid nitrogen for storage within 10 min after separation and reserved for subsequent assays. Before operation, the patients were informed of the objective and methods and were asked to sign an informed consent form.

Major reagents

Total RNA Extraction Reagent (Trizol) was purchased from Dakewe Biotech Company, RNA RT Kit from Takara, primary antibodies and secondary antibodies used in Western Blotting from Cell Signaling, and Cell Counting Kit (CCK-8) from Beyotime Institute of Biotechnology.

RNA extraction

Above-mentioned samples stored in liquid nitrogen were taken and ground. Then, 1 ml Trzol was added. Next, subcultured H08910 and MRC-5 cells were collected, washed and centrifuged (1200 r/min×5 min) with PBS. Likewise, 1 ml Trzol was then added. After that, miRNA was extracted according to steps presented by miRNA Isolation Kit. Finally, the concentration and purity of the extracted RNA was determined with an ultraviolet spectrophotometer.

RNA reverse transcription

RNA extracted from the above steps was reversely transcribed to cDNA with PCR instruments. The reaction system was shown in the following Table 1 and the reaction conditions were 25°C×30 min, 42°C×30 min and 85°C×5 min.
miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer

Real-time fluorescence quantification pcr

PCR amplification was performed in ABI-7300 instruments for real-time fluorescence quantitative by adding relevant reagents and primers according to steps presented by Bi-Rad’s RT-qPCR kits, with cDNA as template and U6 as internal reference. The reaction conditions were 95°C×3 min and (95°C×12 s, 62°C×40 s) ×40 cycles. A relative quantitative analysis was conducted for the level of expression by using the 2^{-ΔΔCt} method.

Western blotting

Cell samples were collected and, after they being lysed by cell lysis buffer, protein samples were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Millipore). The resultant product was incubated with corresponding primary antibodies and secondary antibodies labeled with horse radish peroxidase (HRP). Then, DAB was used for color development and luminescence.

miRNA transfection

H08910 and SKOV3 cells at exponential growth stage were transfected with miR-NC and miR-101 by the way offered by lipo2000 Transfection Kit of Invitrogen. After 24 hours, the transfected cells were cryopreserved for subsequent assays.

Cell growth curve assay

Wild type H08910 and SKOV3 cells and ovarian cancer cells transfected with empty vectors (H08910/NC and SKOV3/NC) and miR-101 (H08910/miR-101 and SKOV3/miR-101) were spread out on a 48-well plate, with an initial density of 3000 cells per well. The viability of these cells was detected on day 2, 3, 4 and 5 according to steps presented by CCK-8 kit and a statistical analysis was performed.

Clone formation assay

After counting, ovarian cancer cells were spread out on a 6-well plate, with a density of 100 cells per well. The plate was placed into an incubator and cultured for 2 weeks. When clones were macroscopic, the culture process was terminated. Next, it was rinsed twice with PBS carefully, while cells were fixed by applying neutral methyl alcohol 4% for 15 minutes and stained with Wright-Giemsa’s compound stain for 30 minutes. Then, it was washed slowly with running water and air dried naturally. At last, the number of clones with more than 50 cells was counted under the microscope and the cloning efficiency was calculated according to the following formula:

\[ \text{Cloning efficiency} = \frac{\text{number of clones}}{\text{number of cells inoculated}} \times 100\% \]

Transwell

Wild type (WT) H08910 and SKOV3 cells and ovarian cancer cells transfected with empty vectors (H08910/NC and SKOV3/NC) and miR-101 (H08910/miR-101 and SKOV3/miR-101) were planted in the upper chambers of Transwell cell culture inserts (Corning) with a diameter of 8.0 μm, while 700 μL normal DMEM medium supplemented with 10% fetal bovine serum was added in the lower chambers. This system was cultured with 5% CO₂ for 24 hours at 37°C. After rinsing the lower chamber with PBS, cells growing in the chamber was fixed with 4% formalin and stained with 0.1% crystal violet solution. Then, the number of cells was counted by taking five random fields (amplification factor ×100) under optical microscope and the total amount was regarded as the number of cells in Transwell assay and compared with that obtained by other methods.

Statistical analysis

A statistical analysis was made by using SPSS11.0 software. For comparing the mean value of two groups, non-paired t test was adopted, while for comparing the mean value of three and more groups, one-way analysis of variance was adopted. In addition, inter-group comparison was made by Dunnett t test. And for the same study object, multiple measurement results obtained at different time points of the same indicator (cell viability detected by CCK-8 kit and the growth curve of mouse subcutaneous tumor) were compared statistically by variance analysis of repeated measurement data, with an inspection level α=0.05.

Results

Expression of miR-101 in ovarian cancer tissues and cells

The expression level of miR-101 relative to the housekeeping gene U6 in ovarian cancer tissues and para-carcinoma tissues of 20 patients
miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer

Figure 1. The expression of miR-101 in tissues and cells detected by RT-qPCR. A, B: The expression of miR-101 in ovarian cancer tissues and para-carcinoma tissues from patients with ovarian cancer. C: The expression of miR-101 in ovarian cancer cells H08910 and SKOV3 and normal ovarian epithelial cells HUM-CELL-0088.

Figure 2. Comparison of the expression of SOCS-2 in ovarian cancer tissues and para-carcinoma cells, ovarian cancer cells H08910 and SKOV3 as well as normal human ovarian epithelial cells HUM-CELL-0088 by Western Blotting. 1: Para-carcinoma tissues; 2: Tumor tissues; 3: HUM-CELL-0088; 4: H08910; 5: SKOV3.

Figure 3. Changes of the relative expression level of SOCS-2 in ovarian cancer cells H08910 and SKOV3 after miR-101 over-expression was detected by Western Blotting. 1: H08910; 2: H08910/miR-101; 3: SKOV3; 4: SKOV3/miR-101.

The results of statistical analysis indicated that the expression level of miR-101 relative to U6 in ovarian cancer tissues was 1.11±0.53, which was statistically significantly lower (t=19.12, P=0.002) than that in para-carcinoma tissues (5.05±2.03). In Figure 1C, it was shown that the expression level of miR-101 relative to U6 in ovarian cancer cells H08910 and SKOV3 was 1.05±0.24 and 1.45±1.34, respectively, statistically significantly lower (F=14.37, P=0.000) than that in normal ovarian epithelial cells (5.49±1.10).

Expression of SOCS-2 in ovarian cancer cells and normal human ovarian epithelial cells

The Figure 2 obtained by Western Blotting underwent gray scale scanning with Quality One and the relative expression level was presented as SOCS-2-to-β-actin gray scale ratio. Besides, a statistical analysis was conducted. Results indicated that the relative expression level of SOCS-2 in ovarian cancer cells was 1.04±0.12, notably higher (t=25.03, P=0.000) than that in para-carcinoma tissues (0.30±0.11), while the relative expression level of SOCS-2 in ovarian cancer cells H08910 and
miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer

SKOV3 was 1.16±0.09 and 1.27±0.18, respectively, notably higher (F=14.9, P=0.000) than that in normal human ovarian epithelial cells HUM-CELL-0088 (0.22±0.03).

The impact of over-expression of mir-101 on the proliferation and invasion of ovarian cancer cells in-vitro

As was shown in Figure 4A, after H08910 and H08910/NC cells were cultured for 5 days, their cell viabilities were 739.2±100.4% and 820.2±100.3%, respectively.
miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer

Figure 5. The impact of the over-expression of miR-101 on the invasion ability of ovarian cancer cells detected by Transwell assay.

Discussion

Ovarian cancer is a gynecological tumor with the highest malignant degree. For lacking of efficient means of early diagnosis, most patients have already been at the middle or advanced stage when diagnosed. And the tumor tissues may not be completely excised by any radical operation [1, 5]. Therefore, it is of great significance to further illuminate the pathogenesis of ovarian cancer to improve the prognosis of patients with the cancer.

miRNA plays an important role in such crucial life processes as embryonic development, cell cycle control, proliferation and differentiation and apoptosis [10]. It controls the expression of target genes by regulating the degradation and translation of mRNA [11, 12]. Bioinformatics researches show that miRNA participates in the regulation of about 30% coding genes within human body [7, 8]. More and more evidences indicate that there are various kinds of abnormal expressions of miRNA in human tumor and suggest that miRNA may play an important role in the genesis and development of malignant tumors by regulating or inhibiting the expression of oncogenes [13, 14].

SOCS-2 is an important member of suppressors of cytokine signaling and the expression product of oncogene socs-2 and participates in a series of important processes related to cell
miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer

growth, division and apoptosis [13, 14]. It is shown that abnormal expressions of SOCS-2 found in many malignant tumors [15-18] have yet not been reported in ovarian cancer.

In this study, it was demonstrated by qRT-PCR that the expression level of miR-101 and SOCS-2 in ovarian cancer tissues and cells significantly decreased and increased, respectively, compared with that in para-carcinoma tissues and normal cells. A study performed by Xiaoying Zhou et al. [19] suggested that miR-101 can inhibit the genesis and development of gastric cancer by inhibiting the expression of SOCS-2. Bioinformatics analysis confirmed that miR-101 had a binding site in the 3'UTR of SOCS-2 [19]. In order to verify the relation between the down-regulated expression level of miR-101 and the up-regulated expression level of SOCS-2 in ovarian cancer tissues and cells, this study successfully transfected ovarian cancer cells with miR-101 by lipo2000. The results manifested that in ovarian cancer cells with over-expressed miR-101, the expression level of SOCS-2 was up-regulated evidently compared with that in wild type tumor cells. Further studies indicated that the proliferation and invasion ability of ovarian cancer cells with over-expressed miR-101 was notably lower than that of wild type tumor cells.

It was shown that the expression of PGE2 increased in many malignant tumor tissues and cells, which was closely related to the genesis, development, invasion, metastasis and prognosis of these malignant tumors [1, 16, 17, 20]. Furthermore, in this study, it was found that, in ovarian cancer tissues and cells, the expressions of miR-101 and SOCS-2 were markedly negatively correlated with each other, and the proliferation and invasion ability of ovarian cancer cells both in-vitro and in-vivo with over-expressed miR-101 was notably lower than that of wild type tumor cells.

In this study, it was demonstrated by qRT-PCR that the expression level of miR-101 and SOCS-2 in ovarian cancer tissues and cells significantly decreased and increased, respectively, compared with that in para-carcinoma tissues and normal cells. A study performed by Xiaoying Zhou et al. [19] suggested that miR-101 can inhibit the genesis and development of gastric cancer by inhibiting the expression of SOCS-2. Bioinformatics analysis confirmed that miR-101 had a binding site in the 3'UTR of SOCS-2 [19]. In order to verify the relation between the down-regulated expression level of miR-101 and the up-regulated expression level of SOCS-2 in ovarian cancer tissues and cells, this study successfully transfected ovarian cancer cells with miR-101 by lipo2000. The results manifested that in ovarian cancer cells with over-expressed miR-101, the expression level of SOCS-2 was up-regulated evidently compared with that in wild type tumor cells. Further studies indicated that the proliferation and invasion ability of ovarian cancer cells with over-expressed miR-101 was notably lower than that of wild type tumor cells.

It was shown that the expression of PGE2 increased in many malignant tumor tissues and cells, which was closely related to the genesis, development, invasion, metastasis and prognosis of these malignant tumors [1, 16, 17, 20]. Furthermore, in this study, it was found that, in ovarian cancer tissues and cells, the expressions of miR-101 and SOCS-2 were markedly negatively correlated with each other, and the proliferation and invasion ability of ovarian cancer cells both in-vitro and in-vivo with over-expressed miR-101 was markedly lower than that of wild type tumor cells.

It was shown that the expression of PGE2 increased in many malignant tumor tissues and cells, which was closely related to the genesis, development, invasion, metastasis and prognosis of these malignant tumors [1, 16, 17, 20]. Furthermore, in this study, it was found that, in ovarian cancer tissues and cells, the expressions of miR-101 and SOCS-2 were markedly negatively correlated with each other, and the proliferation and invasion ability of ovarian cancer cells both in-vitro and in-vivo with over-expressed miR-101 was markedly lower than that of wild type tumor cells. All of these results suggest that miR-101 probably plays an important role in the genesis and development of ovarian cancer by regulating the expression of its target gene SOCS-2. Therefore, up-regulating the expression of miR-101 in ovarian cancer tissues and cells may become an effective method to treat ovarian cancer.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bao-Ping Liu, Department of Nuclear Medicine, The First Affiliated Hospital of Zhengzhou University, Jianshe East Road, Two Seven District, Zhengzhou, Henan Province, China. Tel: +86 18270287139; Fax: +86 18270287139; E-mail: liubaopingzzdx@163.com

References

miRNA-101 down-regulating expression of SOCS-2 in ovarian cancer


