European Review for Medical and Pharmacological Sciences 2013; 17: 3002-3006

Suppression of RNA interference on expression of c-myc of SKOV3 ovarian carcinoma cell line

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Abstract. – AIM: To investigate suppression of RNA interference (RNAi) on expression of c-myc of SKOV3 ovarian carcinoma cell line.

MATERIALS AND METHODS: The c-myc -siRNA was designed and synthesized, then transfected to SKOV3 ovarian carcinoma cell lines. The cell lines were divided into four groups, including the blank control group, the siRNA transfection group, the mock transfection group and the negative control group. The expression level of c-myc mRNA and protein were detected by RT-PCR and Western blotting, respectively. The growth and proliferation of SKOV3 ovarian carcinoma cell lines were observed with CCK-8 assay.

RESULTS: After transfected with c-myc -siRNA, the expression level of c-myc mRNA and protein were down-regulated, the growth and proliferation of SKOV3 ovarian carcinoma cell line were inhibited in the siRNA transfection group. There were significant differences between the siRNA transfection group and the blank control group ($p < 0.05$). The silencing efficiency was 77.78%, the protein suppression rate was 67.78%, and the inhibition ratio was 56.35% by CCK-8 assay in siRNA transfection group.

CONCLUSIONS: The down-regulation of c-myc expression of SKOV3 ovarian carcinoma cell line by c-myc -siRNA can lead to the suppression of cancer cell proliferation. The small interfering RNAs technique can inhibit the proliferation of carcinoma cell by oncogene silencing.

Key Words:
RNA interference, Gene silencing, Ovarian cancer, C-myc.

Abbreviations

CCK-8 = Cell Counting Kit-8; RNAi = RNA interference; dsRNA = double-standed RNA; siRNA = silencing RNA; RPMI-1640 = Roswell Park Memorial Institute-1640; FBS = fetal bovine serum; RT-PCR = Real Time Polymerase Chain Reaction; DEPC = diethylpyrocarbonate; RIPA = Radioimmunoprecipitation Assay; SDS-PAGE = Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis; HRP = horseradish peroxidase; ECL = Electrochemiluminescence

Introduction

Ovarian cancer is one of the three most common malignant tumors in female reproductive system, average onset time of which is about 50 years-old[1]. The early symptoms are not significant, only the abdomen will be slightly obesity, patients may feel abdominal distension and gastrointestinal discomfort after eating[2]. And hydrops abdominis will be shown, when ovarian cancer comes to later stage[3]. Almost 2/3 patients are diagnosed in an advanced stage; therefore, the therapeutic efficacy of ovarian cancer is poor and the mortality is very high[4]. In recent years, it is confirmed, by studying the mechanism of apoptosis, anti-apoptotic factors play an important role in tumor cell growth and drug resistance. C-myc gene is an important member of the myc gene family, it is not only a translocation gene, but also a regulate gene[5,6]. C-myc can regulate the cell cycle, integrate cell proliferation with metabolism and stimulate glutamine catabolism[7]. C-myc would permit the cell to proliferate indefinitely and cellular immortality, promote cell division, participate in the apoptotic and occurrence and development of a variety of tumor[8,9]. RNA interference (RNAi) is gene silencing induced by double-stranded RNA (dsRNA)[10]. The mechanism is to inhibit gene expression by blocking translation or transcription of specific genes. When inducing the dsRNA which is homologous to endogenous mRNA coding region into cell, the degradation of this mRNA will cause gene silencing[11,12]. Combining the RNAi with c-myc, researchers have discovered that human breast cancer can be suppressed[13]. Although this topic has been already developed in other cancer lines, the ovarian cell line (SKOV3) so far is lacking with this method. Therefore, in this experiment, taking SKOV3 ovarian carcinoma cell line as a model, the inhibited effect of proliferation of ovarian cancer can be observed by trans-

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fecting the c-myc siRNA to SKVO3 cells. We want to find new ways for the clinical treatment of ovarian cancer.

Materials and Methods

Sources of Materials

SKOV3 ovarian cancer cell line (Shanghai Cell Bank, Chinese Academy of Sciences).

Methods

Cell Culture

SKOV3 ovarian cancer cell line was cultivated in the RPMI-1640 medium including 10% fetal bovine serum (FBS), demanding 37°C, 5% CO_2 and saturated humidity.

Short Interfering RNA (siRNA) Design

The sequence of antisense strand is 5'-CCTCTCGAGGCAGGAGGG-3' (made by Shanghai BioAsia Bio-Technology Co., Ltd) and bases are modified by thiophosphoric acids. Sequence of nonsense oligonucleotide is 5'-ATGCCCCTCAACGTT-3', the study design method refers to the reference[14]. RPMI-1640 medium, TRIzol RNA kit and Lipofecter Liposomal Transfection Reagent lipofectamine 2000 were products of GIBCO Company (Carlsbad, CA, USA). DDP was purchased from Shandong Qilu Pharmaceutical Co., Ltd. RT-PCR kit was form AKARA Biothechnology (Dalian) Co., Ltd. Methyl thiazolyl tetrazolium (MTT), dimethyl sulfoxide and trypan blue were products of Sigma Aldrich Co., St Louis, MO, USA). C-myc rabbit anti human polyclonal antibody and immunohistochemical kits were purchased from Beijing Zhongshan Biotechnology Company.

SiRNA Transfection

Extracting logarithmic phase cell at one day before transfection, and cells were inoculated into the 96-mesh board with each pore 1.5×10^4 cells. 60 mm culture flask cell was adjusted to 1.0×10^6/bottle. Cancer cells were cultured at 37°C, 5% CO_2, and saturated humidity conditions up to the convergence rate arriving at 70%-80% in 96-mesh board and 60 mm culture flasks, and then synchronized by 0.2% FBS for 24 h. Transfection steps were according to the RNAi manual of Shanghai BioAsia Bio-Technology Co., Ltd., adding 0.3 μg siRNA and 0.9 μg RNAi-Mate to 96-mesh board with the final volume of 100 μL, adding 10 μg siRNA and 30 μg RNAi-Mate to 60 mm culture flask with the final volume of 5 mL. Cancer cells were cultured at 37°C, 5% CO_2 and saturated humidity conditions for 48h, and then using Real time PCR and Western blotting to analysis c-myc mRNA and protein content of each group, CCK-8 was to detect the proliferation of cancer cell lines.

Experimental Classification

Blank control group: without transfection (without siRNA and transfer agent); transfection group: add siRNA targeting c-myc and transfer agent siRNA-Mate; mock transfection group: only add isodose transfer agent siRNA-Mate; negative control group: add siRNA for negative control and transfer agent siRNA-Mate.

RT-PCR

Total cellular RNA was extracted in each group (6 samples of each group) using TRIzol, and followed by cDNA synthesis. PCR reaction system: the total volume is 50 μL (final concentration of primers is 0.2 μmol/L), including the Real time PCR MasterMix 25 μL, primers (10 μmol/L each 1 μL, 3 μL cDNA template, 20 μL water without DEPC). PCR conditions: 50°C 2 min, 95°C 10 min, 95°C 15 s, 60°C 1 min, 45 cycles. PCR products were put in 2% agarose gel electrophoresis, making use of gel imaging system to scan photograph and observe the size and quality of stripe.

Western Blotting

RIPA lysis buffer cracked each group cells respectively (6 samples of each group), and the supernatant was collected after centrifugation. Proteins were isolated by 8% SDS-PAGE, which incubated with anti-c-myc polyclonal antibodies overnight. The membrane was washed and HRP-labeled second antibody conventionally incubated proteins for 1h. ECL chemiluminescence reagent reacted for 1 min for darkroom exposure imaging. Protein expression bands were scanned in gray scale (optical density (OD) value) scanning, using automatic image analysis system to quantitative analysis. Inhibition ratio of c-myc protein expression = (1 - c-myc OD value of experimental group/c-myc OD value of blank control group) 100%.

Inhibition Rate Measured by CCK-8 Assay

Routine culture cell lines were made into cell suspension, which were added into 96-mesh board, cultured at 37°C for 24h and treated with

synchronization for 24h. SiRNA was transfected according to the aforementioned method, 8 meshes for each group. And cells were cultured at 37°C for 48h. At last, 10 μL CCK-8 was added in per mesh and cultured at 37°C for 3h. We took OD value at 450 nm by ELISA Reader, calculating inhibition ratio. The inhibition rate = (1 - OD value of experimental group/OD value of control group) 100%.

Statistical Analysis

Building a database using Excel, each set of data expressed as mean ± standard deviation () for statistical analysis using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Date were analyzed by one-way anova under completely randomized design to compare sample means among many groups, using the SNK-q test for pairwise comparisons ($\alpha = 0.05$).

Results

Results of RT-PCR

Electrophoresis analysis showed that the amplification products were target genes, without hybrid bands (Figure 1). After interfered by c-myc-siRNA, c-myc mRNA expression levels of transfection group were significantly decreased compared with the control group. The difference was statistically significant ($q = 20.48$, $p < 0.05$), and the silencing efficiency was 77.78% (Table I).

Results of Western Blotting

C-myc protein expression levels of transfection group decreased significantly compared with blank control group, and the difference was statistically significant ($q = 22$, $p < 0.05$), protein inhibition efficiency was 67.78% (Figure 2).

Results of CCK-8

Cell proliferation of transfection group was limited, compared with the control group, the difference was statistically significant ($p < 0.05$), while the differences of negative control group, mock transfection group and blank control group were not statistically significant ($p > 0.05$). The inhibition rate was 53.65% (Table II).

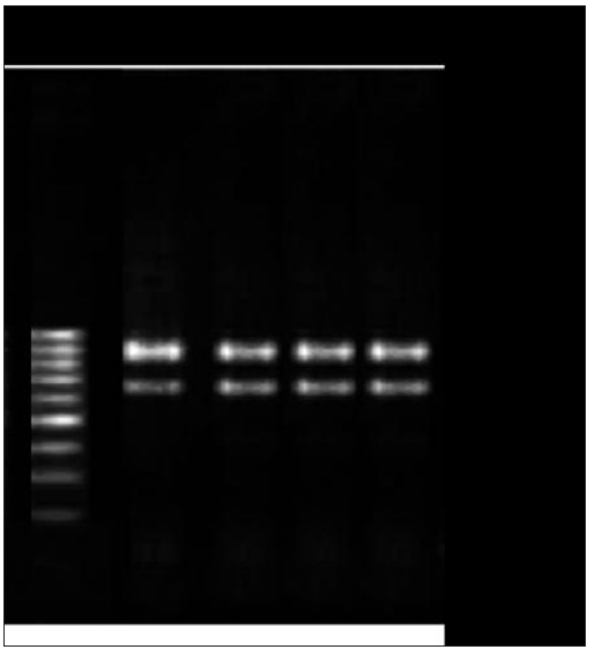

Figure 1. RT-PCR electrophoretogram of SKOV3 ovarian cells c-myc mRNA. M: Marker, 1: transfection group 2: blank control group, 3: mock transfection group, 4: negative control group.

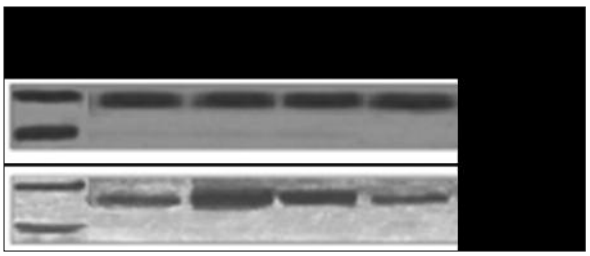

Figure 2. Western blotting electrophoretogram. M: Marker, 1: blank control group, 2: mock transfection group, 3: negative control group.

Table I. C-myc mRNA expression levels and OD values after transfection ($\bar{x} \pm s$).

Groups	N	c-myc/β-actin	OD
Blank control group	6	0.98 ± 0.05	477465. ± 3889.32
Transfection group	6	0.76 ± 0.01	2153.52 ± 1889.74
Mock transfection group	6	0.93 ± 0.05	4663.28 ± 3364.37
Negative control group	6	0.92 ± 0.05	4675.26 ± 3749.55
F		125.73*	199.35*

*$p < 0.05$.

Table II. OD value tested by CCK-8 after transfection.

Groups	N	OD	Statistical treatment	
			Ratio	q
Blank control group (1)	6	1.47 ± 0.23	(1):(2)	0.02
Mock transfection group (2)	6	1.45 ± 0.19	(1):(3)	0.72
Negative control group (3)	6	1.47 ± 0.21	(1):(4)	9.93*
Transfection group (4)	6	0.62 ± 0.17		
F		44.32*		

*$p < 0.05$.

Discussion

RNAi is a specific dsRNA binding with the homologous mRNA specifically in the cell, at the same time activating the endogenous RNA endonuclease[15]. Its homologous mRNA is degraded into small fragments of 21-23 nucleotides, so that the corresponding gene will be silencing[16]. Through targeting apoptosis, angiogenesis, organization invasion and metastasis genes, genes related to tumor occurrence and development can be determined. RNAi also was regarded as a new means for cancer treatment.

The results showed that after c-myc-siRNA interfering SKOV3 ovarian cancer cell lines, c-myc mRNA and protein expression levels of transfection group decreased significantly compared with control group, and cell proliferation was limited. It indicates that the designed siRNA specifically leads to the reduction of c-myc mRNA levels, resulting in gene silencing. And CCK-8 test results showed that there was no significant difference between negative control group, mock transfection group and blank control group. C-myc protein and gene level have no change, indicating that no cytotoxic effects have caused by siRNA or transfection reagent and gene silencing is specific and effective. This study proved c-myc siRNA significantly inhibitory effect to the expression of c-myc SKOV3 ovarian cancer cell from the mRNA and protein level. The mechanism is (1) siRNA (short interfering RNA) binds with c-myc mRNA directly, inhibiting it from transporting to the ribosome[17,18]. (2) siRNA with negatively charged combines with complementary c-myc mRNA to activate RNase, and then RNA of RNA/DNA hybrid double-stranded could be specifically cut[19,20]. In a word, the RNAi may suppress ovarian cancer by inhibiting the expression of c-myc, which can regulate cell proliferation.

Conclusions

The study reveals that the siRNA targeting c-myc not only effectively inhibits c-myc gene expression at the transcriptional level, but also specific inhibits proliferation of SKOV3 ovarian cancer cell line *in vitro*. The results suggest that c-myc plays an important role in regulating cell proliferation in ovarian cancer cells, and targeting c-myc RNA interference is an effective treatment for ovarian cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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