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ORIGINAL ARTICLE

Up-regulation of *Let-7a* Expression Induces Gastric Carcinoma Cell Apoptosis *In Vitro*

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Key words: gastric carcinoma; microRNA; apoptosis

Objective To investigate effect of *let-7a* gene over-expression on apoptosis of gastric cancer cell lines SGC-7901.

Methods The stable *let-7a* gene over-expressing SGC-7901 cells, SGC-7901/*let-7a* cells, were established using shRNA lentiviral vector methods. Real-time RT-PCR analysis was used to evaluate the expression level of *let-7a* mRNA. Cells apoptosis was assessed by flow cytometry.

Results RT-PCR analysis revealed *let-7a* expression in SGC-7901/*let-7a* cells was significantly increased. Cellular apoptosis assay showed that over-expression of *let-7a* could increase apoptosis of SGC-7901 cells ($P=0.002$).

Conclusion Up-regulating *let-7a* expression promoted apoptosis in SGC-7901 cells.

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MICRORNAS (miRNAs) are a class of naturally occurring small non-coding RNAs that regulate gene expression by targeting mRNAs for translational repression or cleavage.¹⁻² They could regulate a large fraction of protein-coding genes, and as high as 30% of all genes could be the targets of miRNAs.³ As a new layer of gene regulation mechanism, miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation, and apoptosis.⁴⁻⁵ In our previous study, it was found that decline of *let-7a* was one of the earliest biochemical events associated with the progression of gastric mucosa cancerization.⁶ We also found the effect of *let-7a* over-expression on proliferation

and cell cycle of gastric cancer.⁷⁻⁸ By using Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) we successfully identified ten differential proteins, which may be associated with the function of *let-7a* and provide the basis to comprehensively investigate the functional mechanisms of *let-7a* in gastric carcinoma.⁹ Thus, we performed this study to determine the influence of *let-7a* over-expression on cell apoptosis of gastric carcinoma *in vitro*.

MATERIALS AND METHODS

Cell culture

Human gastric cancer SGC-7901 and human embryonic kidney (HEK) 293T cells purchased from China Center for Type Culture Collection (Wuhan, China) were grown in

Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Plasmid construction

The DNA sequence of *let-7a* was obtained from GenBank (ID No. NM_0062). The primers (forward: 5'-AGGATCCAAAGGTGGTGGTAAGAGGGTGAT-3', reverse: 5'-AGTCGACA-TAAGACAAGAAGCAAAAGGTTT-3') were designed to amplify the entire coding region of *let-7a*, which were synthesized by Sangon Biotech (Shanghai, China). After amplification, 2 µl of the amplified product was separated by 1% agarose gelatin gel electrophoresis with 1×Tris-borate with EDTA (TBE) buffer.

The amplified fragment and plasmid Pwpxl-MOD2 (Shanghai Institute of Biochemistry and Cell Biology) were digested with *Bam*HI and *Sal*I. The digested fragments were isolated by gel purification agarose. The *let-7a* coding fragment was inserted into the corresponding restriction sites of plasmid Pwpxl-MOD2 to form a new vector that was named Pwpxl-MOD2-*let-7a*.

Using packaging plasmids to produce shRNA lentivirus from lentiviral vectors

Briefly, 20 µg Pwpxl-MOD2-*let-7a* (transfer plasmid) and 15 µg pMDlg-Prre, 10 µg pRsv-REV, 7.5 µg pMD2G (packaging plasmids) were co-transfected into HEK 293T cells using calcium phosphate co-precipitation. Viral supernatant was harvested 72 hours after transfection and filtered through a 0.45-µm cellulose acetate filter, then ultracentrifuged at 50 000 ×g at 4°C for 2 hours. The shRNA lentivirus were resuspended in serum-free DMEM, divided into aliquots and frozen at -70°C. The control lentiviral vector containing supernatant was obtained by transfection of plasmid Pwpxl-MOD2 into HEK 293T cells in the same way. The functional titer of virus in supernatant was determined with *in vitro* transduction and flow cytometry (FCM) analysis.¹⁰

Transducing SGC-7901 cells with shRNA lentivirus

SGC-7901 cells were transduced with viral supernatant at a multiplicity of infection of 5. Briefly, the transduced SGC-7901 cells were maintained in culture medium for 4 to 5 passages until green fluorescent protein (GFP) was stably expressed. Sequentially, the transduced cells were trypsinized and diluted to a concentration of 10 cells/ml. An aliquot of 100 µl cells was seeded in 96-well plate. After 10 days of incubation, the single colony strongly expressing GFP was

visualized under fluorescent microscopy and selected for further use. The stable *let-7a* gene over-expressing SGC-7901 cells, SGC-7901/*let-7a* cells, between passages 10 and 20 were used in the experiments. SGC-7901 cells transduced with control lentiviral vectors were used as the negative control, which was named SGC-7901/EV cells. The parental SGC-7901 cells were used as the blank control.

Real-time RT-PCR analysis

Total RNA was isolated from cultured cells of each group. *let-7a* expression was quantified with real-time RT-PCR. 18sRNA was used as an endogenous control. The reaction was performed in a volume of 25 µl containing oligonucleotide primers (2 µl each), cDNA template (0.5 µl) and SYBR Green mix (25 µl) in duplicate (TaKaRa Bio Inc., Japan). The amplifications were performed in the ABI Prism 7000 Sequence Analyzer (Applied Biosystems). The relative expression level of the *let-7a* gene was calculated by comparing the threshold cycle (Ct) values of experimental cells to that of the blank control. The Ct values for each reaction were determined using Taq-Man SDS analysis software (Richa Agarwala). For each sample tested, the duplicate Ct values were averaged. Accordingly, $\Delta Ct = (\text{mean Ct value of SGC-7901/let-7a cells}) - (\text{mean Ct value of 18sRNA})$, and $\Delta\Delta Ct = \Delta Ct_{\text{SGC-7901/let-7a}} - \Delta Ct_{\text{SGC-7901}}$. The relative gene expression in a particular sample was then given by the relative amount of the target: $2^{-\Delta\Delta Ct}$ value.¹¹

Cell apoptosis determined by FCM

Cell apoptosis was detected with the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Flow Cytometry Assay Kit (Invitrogen Carlsbad, CA, USA) using FCM. After different treatments at the indicated times, SGC-7901/*let-7a*, SGC-7901/EV and SGC-7901 cells were washed and resuspended in binding buffer (2.5 mmol/L CaCl₂, 10 mmol/L HEPES, pH 7.4, and 140 mmol/L NaCl) before being transferred to a 5 ml tube. The cells were incubated in the dark with 5 µl each of Annexin V-FITC and PI for 15 minutes. Binding buffer was then added to each tube, and the samples were analysed using a Beckman Coulter Epics XL flow cytometer. Annexin V-FITC (-)/PI (-) staining indicated live cells, Annexin V-FITC (+)/PI (-) staining indicated cells that were in the early stages of apoptosis, and Annexin V-FITC (+)/PI (+) staining indicated cells that were in the late stages of apoptosis or necrosis. All experiments were performed three times.

Statistical analysis

Data were analyzed using the SPSS 10.0 software for

Windows XP. Data were expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) was performed to compare the difference among groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Over-expression of *let-7a* in SGC-7901 cells transduced with *let-7a* lentiviral vector

Fluorescent microscopy indicated more than 98% transduced cells strongly expressed GFP (Fig. 1). FCM analysis showed that the functional titer of virus was 5.0×10^8 TU/ml.

Relative expression levels of *let-7a* mRNA in the SGC-7901, SGC-7901/EV, and SGC-7901/*let-7a* cells were 1.00 ± 0.07 , 1.00 ± 0.07 , and 9.00 ± 0.09 , respectively. The copies of *let-7a* in SGC-7901/*let-7a* cells were 9-fold higher than those in SGC-7901 cells and SGC-7901/EV cells, respectively ($F = 10295.535$, $P = 0.000$). However, expression level of *let-7a* mRNA showed no significant difference between SGC-7901 and SGC-7901/EV cells ($P > 0.05$).

Effect of *let-7a* over-expression on cell apoptosis in human SGC-7901 cells

The apoptosis rate of SGC-7901/*let-7a* cells was $12.0\% \pm 3.1\%$, which was significantly higher than that of SGC-7901 cells ($1.9\% \pm 1.0\%$; $F = 27.142$, $P = 0.002$). However, the apoptosis rate of SGC-7901/EV cells was $2.5\% \pm 0.7\%$, which was not significantly different from that of SGC-7901 cells ($F = 4.019$, $P > 0.05$) (Fig. 2).

Figure 1. SGC-7901 cells were transduced with *let-7a* lentiviral vectors at a multiplicity of infection of 5 (A) and control lentiviral vector (B), respectively. $\times 200$ The brightly stained cells are green fluorescent protein positive cells.

Figure 2. Cell apoptosis of SGC-7901/*let-7a* (A), SGC-7901/EV cells (B), and SGC-7901 cells (C).

The cells were stained with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), and the percentages of apoptotic cells (annexin V+ cells) were then assayed using flow cytometric analysis. The lower left quadrant of the flow cytometry histograms indicates the percentage of live cells; the upper left quadrant indicates the percentage of PI positive, necrotic cells; the lower right quadrant shows the percentage of Annexin-V-FITC positive, apoptotic cells; the upper right quadrant reveals the percentage of PI and Annexin positive, late apoptotic cells. The data are the mean of the three different experiments.

DISCUSSION

Historically, *let-7* was found to bind to the 3' untranslated region of *lin-41* and *hbl-1* (*lin-57*), thereby inhibiting their translation in nematodes,¹²⁻¹⁶ and controlling the developmental transition from the L4 stage into the adult stage.¹²⁻¹⁴ In our previous study, deregulation of

let-7a was found in gastric carcinoma, and effects of inducing cell-cycle arrest, inhibiting cell growth were shown by over-expression *let-7a* in SGC-7901 cells.⁶⁻⁹ Yu *et al*¹⁷ demonstrated that vector-based *let-7* could significantly increase the expression of *let-7* in breast self-renewing tumor-initiating cells and inhibit their proliferation. Transient over-expression *let-7* was demonstrated to be a potential growth suppressor in human colon cancer cells.¹⁸ These

results led us to test whether *let-7a* might have a general ability to induce gastric carcinoma cell apoptosis. In this study, we constructed human immunodeficiency virus type 1-derived lentivirus encoding a specific shRNA of *let-7a* to infect the human gastric carcinoma cells. And the *let-7a* lentiviral vector positive cells were selected to evaluate the apoptosis.

In SGC-7901/*let-7a* cells, *let-7a* abundance was 9-fold of that in the controls, suggesting an efficient and specific increasing of *let-7a* gene expression by lentiviral-vector-mediated gene augmentation. Current studies have also investigated whether over-expression *let-7a* may increase gastric carcinoma cell apoptosis *in vitro*. Our results clearly showed that *let-7a* over-expression in SGC-7901 cells could induce apoptosis, indicating that up-regulation of *let-7a* can suppress tumor progression. The findings strengthened the proposal of a role for *let-7a* in the control of cell survival in tumor. *Let-7a* gene provides a therapeutic target to suppress such morbid growth. The long-term goals of our program are to exploit the potential use of *let-7a* as an approach for the management of gastric carcinoma *in vivo*.

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