Let-7a suppresses cell proliferation via the TGF-β/SMAD signaling pathway in cervical cancer

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Abstract. Cervical cancer is the second most commonly diagnosed type of cancer among women after breast cancer. Recent research has addressed the role of microRNAs in cervical cancer. In the present study, we aimed to determine the effect of let-7a on the regulation of cell proliferation of cervical cancer and the related signaling pathway. Real-time RT-PCR was used to detect the expression of let-7a in the blood of cervical cancer patients and normal controls. The expression of let-7a was also assessed in cervical cancer cell lines: HeLa, SiHa and normal human immortalized keratinocytes HaCaT. Cell proliferation was tested by MTT assay, and cell apoptosis and cell cycle were examined by flow cytometric analysis in HeLa cells. Moreover, bioinformatic analysis, dual-luciferase reporter assay and western blotting were used to confirm the target gene for let-7a. In addition, the expression of TGF-β1, SMAD4 and p53 were assessed by western blotting and real-time PCR. Our studies showed that the expression of let-7a in cervical cancer was significantly reduced in cervical cancer patients compared with the expression in the normal control group. Cell proliferation of HeLa cells was inhibited by overexpression of let-7a. The cell cycle analysis showed that an increased population was arrested in the G2 phase in the let-7a mimic group when compared with that in the mimic control and untreated groups. In addition, the cell cycle-related factor p53 was increased in the let-7a overexpression group compared with that in the control and untreated groups. Furthermore, TGFB1 was confirmed to be a target of let-7a. Moreover, the expression of TGF-β1 and SMAD4 proteins was elevated in cervical squamous carcinoma and cervical adenocarcinoma tissues. However, the expression of TGF-β1 and SMAD4 was decreased in the let-7a-overexpressing cervical cancer cell lines (HeLa, SiHa and CaSki). Our data suggest that let-7a may play a role in the cell proliferation of cervical cancer by regulating the TGF-β/SMAD pathway, and may participate in the regulation of the occurrence and development of cervical cancer.

Introduction

Cervical cancer is the second most commonly diagnosed type of cancer among women and is a serious threat to women’s health worldwide. Approximately 20 million women die of cervical cancer each year (1). In recent years, significant advances have been made in understanding the mechanism of cervical carcinogenesis (2). However, the detailed mechanism of cervical carcinogenesis is still unknown to date. Therefore, identifying key factors in cervical cancer development may provide potential therapeutic targets for the prevention and treatment of cervical cancer. MicroRNAs (miRNAs) are a group of non-coding RNAs ~21-23 nt in length that negatively regulate gene expression by imprecisely binding to complementary sequences in the 3’ untranslated region (3’ UTR) of their target mRNAs (3,4). Increasing evidence shows that miRNAs are involved in many important biological processes, including development, cell proliferation, metabolism and signal transduction (5,6). In recent years, researchers have focused on the relationship between miRNAs and cancer. Altered expression of miRNAs has been found to be associated with the initiation and progression of various cancers (7-9). However, the potential involvement of miRNAs in cervical cancer remains to be fully elucidated.

As the earliest discovered miRNA in C. elegans, let-7 has been widely studied in a variety of tumors. It can regulate a variety of oncopgenes, such as K-Ras (9), EZH2 (10) and HMGA (11). Our previous study showed that let-7a suppresses the proliferation of lung cancer by inhibiting the K-Ras and c-Myc gene (12), and let-7a was found to participate in the regulation of diabetic nephropathy through the promoter regulation area rs1143770 polymorphism (13). These results suggest that let-7a is an active regulatory factor and plays an important role in a variety of diseases. In chip analysis of miRNAs, various miRNAs which displayed abnormal expression in cervical cancer included let-7 (14). This suggests that let-7 may participate in the development of cervical cancer. However, its mechanism is still unclear.
To the best of our knowledge, the TGF-β/SMAD signaling pathway plays a role in numerous types of cancers, including cervical cancer (15-17). The TGF-β/SMAD signaling pathway mediated by TGF-β1 is thought to be a key signaling pathway in the regulation of cell proliferation and induction of apoptosis (18). Previous research has revealed that the proliferation and invasion of cervical cancer may be inhibited by TGF-β1 (17). TGF-β binds to TGFBR1 resulting in phosphorylation of the carboxy-terminal serine residue of the SMAD intracellular messenger proteins, SMAD2 and SMAD3 (19). This phosphorylation results in oligomerization of SMAD2 and SMAD3 with SMAD4, a necessary step for nuclear translocation (20). Whether or not let-7a regulates the proliferation of cervical cancer by the TGF-β/SMAD signaling pathway requires further investigation.

In the present study, let-7a was decreased in cervical cancer as detected by real-time PCR. In addition, overexpression of let-7a inhibited the proliferation of cervical cancer cells and promoted cell apoptosis. Moreover, TGFBR1 was confirmed to be a target of let-7a by bioinformatic analysis, dual-luciferase reporter assay and western blotting. Furthermore, data showed that TGF-β1 and SMAD3 with SMAD4 were increased in cervical squamous carcinoma and adenocarcinoma patient tissues. Additionally, the expression levels of TGF-β1 and SMAD4 were reduced in the cervical cancer cells when let-7a was overexpressed. In addition, the expression of let-7a was increased when TGF-β1 was silenced in the cervical cancer cells. Taken together, our results indicate that let-7a may play an important regulatory role in cervical cancer through the TGF-β/SMAD signaling pathway.

Materials and methods

A total of 50 peripheral blood samples were collected and analyzed, including 25 incident cervical cancer cases and 25 healthy women. The patients were diagnosed as having cervical cancer according to pathologic findings. The age range of the patients was between 20 and 70 years. The control group was comprised of married females, 26.0-69.0 years of age, randomly selected during the same time period as the cases studied among healthy individuals without any history of cancer.

Paraffin-embedded tissues were obtained from patients with squamous carcinoma of the cervix (SCC) (n=20), adenocarcinoma of the uterine cervix (AUC) (n=15) and cervical intraepithelial neoplasia (CIN) (n=15). All patients were female Han Chinese from Chongqing Province, 30.0-67.0 years of age, from the First Affiliated Hospital of Chongqing Medical University.

Cell culture and transfection. The human cervical cancer cell lines (HeLa, SiHa and CaSki) and normal human immortalization keratinocyte cells (HaCaT) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO₂ at 37°C. HeLa, SiHa and CaSki cells were transfected with let-7a mimics (Shanghai GenePharma Corp., Shanghai, China) or a mimic control, short double-scrambled RNAs (dsRNAs) similar to Dicer-processed miRNAs, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then, the HeLa, SiHa and CaSki cells were divided into the let-7a group (transfected with let-7a mimics), control group (transfected with let-7a mimic control) and the untreated group (untransfected). HeLa cells were also transfected with TGF-β1 siRNA (Shanghai GenePharma Corp.) or siRNA control by Lipofectamine 2000, as previously described (21).

Real-time reverse transcription (RT)-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used to semi-quantify miRNAs according to the manufacturer's instructions. Briefly, 10 ng of total RNA was reverse transcribed using miRNA-specific looped RT primer, MultiScribe reverse transcriptase, RT buffer, dNTPs and RNase inhibitor in the GeneAmp 9700 PCR system (both from Applied Biosystems) under the following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min. Real-time PCR was performed on the resulting complementary DNA (cDNA) using miRNA-specific forward PCR primers, specific reverse PCR primer, miRNA-specific TaqMan probe and TaqMan Universal PCR Master Mix in a 7500 Real-Time PCR system (Applied Biosystems) as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. U6 served as an internal control (Applied Biosystems). Relative fold-change in let-7a expression was calculated using the ΔΔCT method (18) and the values are expressed as 2^-ΔΔCT.

MTT assay. HeLa cells were seeded at a density of 2x10³ cells/well in 96-well plates. After 1, 2, 3 or 4 days of incubation, 20 µl MTT (5 mg/ml) was added to each well and cultured for 4 h. The supernatant was removed, 150 µl of dimethyl sulfoxide (DMSO) was added and shaken for 5 min until the crystals were dissolved. The optical density (OD) 490 nm value was measured using an enzyme-linked immunosorbent assay reader. The data are expressed as the percentage of growth relative to that of cells in the untreated group. The inhibition rate of HeLa was calculated using the following formula: Inhibition rate of cells (%) = (1 - A490 value of let-7a group/A490 value of HeLa group x 100%. All experiments were performed in triplicate.

Flow cytometric assay. HeLa cells were harvested by trypsinization, washed in ice-cold phosphate-buffered saline (PBS), and fixed in 80% ice-cold ethanol in PBS. Before staining, the cells were pelleted using a chilled centrifuge and resuspended in cold PBS. Bovine pancreatic RNase (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 2 µg/ml and cells were incubated at 37°C for 30 min, followed by incubation with 20 µg/ml propidium iodide (PI; Sigma-Aldrich) for 20 min at room temperature to analyze the cell cycle, or incubation with 61 µl FITC-Annexin V and 20 µl PI with 300 µl binding buffer for 15 min at room temperature to analyze the apoptotic rate. The profiles of 1x10⁴ cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, Bedford, MA, USA).

Cloning and identification. To construct a plasmid containing the TGFBR1 3’ UTR fused to pmiR-RB-REPORT™ dual-luciferase vector (Guangzhou RuiBio Corp., Guangzhou, China), the 3’ UTR of the TGFBR1 gene was amplified using the following primers: forward, 5’-CGGCTCGAGGGTGTG
TTFAGGAGCTGGT-3’ and reverse, 5’-AATGCGGCGGC
CATAACAATTTTCCTTCGG-3’, and then cloned into a
pmiR-RB-REPORT™ vector with restriction endonucleases
XhoI and NotI to generate a pmiR-RB-REPORT™-
TGFBR1-3’ UTR wild-type. The potential binding sequences
of let-7a on the TGFBR1 3’ UTR were mutated by the
QuikChange™ Site-Directed Mutagenesis kit (Stratagene).
The recombinant plasmids were confirmed by restriction
enzyme digestion and DNA sequencing.

Dual-luciferase reporter assay. Luciferase assays were
performed according to the manufacturer's protocol. 293T
cells seeded into 96-well plates at a density of 4x10^3/well,
performed according to the manufacturer's protocol. 293T
Luciferase assays were Dual-luciferase reporter assay.

Immunohistochemistry. Cervical cancer tissues were embedded
in paraffin and sectioned at a thickness of 5 μm. After deparaftin-
zation of the sections, antigen retrieval was carried out by
boiling the samples in citrate buffer for 15 min at 92-98°C. To
be naturally cooled to room temperature, the sections were
washed thrice with PBS. Then, 10% normal goat serum was
added for 1 h at 37°C, and subsequently incubation was carried
out with human anti-rabbit TGF-β1 antibody (1:200 dilution)
and human anti-rabbit SMAD4 antibody (1:200) (both from
ProteinTech) in PBS at 4°C for overnight. The sections were
then incubated with goat anti-rabbit secondary antibodies
(IgG/HRP) for 15 min at 37°C using Non-Bio Two-Step
Histostain™ Plus kits and DAB staining (Zhongshan Golden
Bridge Biotechnology, Corp. Beijing, China). The nuclei were
counterstained with hematoxylin. The remaining procedures
were performed in accordance with the manufacturer's
instructions. The immunohistologic staining for TGF-β1 and
SMAD4 was observed by light microscopy.

Immunofluorescence. Cervical cancer tissues were embedded
in paraffin and sectioned at a thickness of 5 μm. After deparaftin-
zation of the sections, antigen retrieval was carried out by
boiling the samples in citrate buffer for 15 min at 92-98°C. In
order to be naturally cooled to room temperature, the sections
were washed thrice with PBS. Then 10% normal goat serum
was added for 1 h at 37°C. After that incubation was carried
out with human anti-rabbit TGF-β1 antibody (1:200 dilution)
and human anti-rabbit SMAD4 antibody (1:200) (both from
ProteinTech) in PBS at 4°C for overnight. After washing twice
with PBS, the sections were incubated with TRITC-conjugated
anti-rabbit IgG secondary antibody (1:50 dilution) (Zhongshan
Goldenbridge Biotechnology, Corp. Beijing, China) for
90 min at 37°C in the dark, washed thrice with PBS for 5 min
and finally sealed with 50% glycerin. Observations were
performed using confocal laser scanning microscopy (CLSM;
Leica, Wetzlar, Germany).

Results
Expression of let-7a is decreased in cervical cancer patient
serum and cells. To validate the alteration in expression of
let-7a in cervical cancer, the expression of let-7a was assessed
in the serum of 25 cervical cancer patients and 25 normal
controls by quantitative real-time PCR. The results showed
that let-7a was markedly decreased in the serum of cervical
cancer patients compared with that noted in the normal
controls (Fig. 1A). These findings corroborated the results of Shishodia et al (22), who found that let-7a expression was downregulated in cervical cancer tissues. These findings show that let-7a is consistently decreased in blood and cancer tissues of cervical cancer patients. Meanwhile, the expression of let-7a was examined in two cervical cancer cell lines. The results showed that let-7a was also decreased in the SiHa and HeLa cells compared with that in normal HaCaT cells (Fig. 1B). These data suggest that let-7a participates in the occurrence and development of cervical cancer.

Table I. Effects of let-7a on the proliferation of HeLa cells.

<table>
<thead>
<tr>
<th>Absorbance</th>
<th>Untreated group</th>
<th>Control group</th>
<th>let-7a group</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A490 (24 h)</td>
<td>0.19±0.02</td>
<td>0.23±0.02</td>
<td>0.13±0.02</td>
<td>31.58</td>
</tr>
<tr>
<td>A490 (48 h)</td>
<td>0.46±0.04</td>
<td>0.47±0.05</td>
<td>0.30±0.03</td>
<td>34.78</td>
</tr>
<tr>
<td>A490 (72 h)</td>
<td>1.12±0.15</td>
<td>1.07±0.09</td>
<td>0.62±0.05</td>
<td>44.64</td>
</tr>
<tr>
<td>A490 (96 h)</td>
<td>1.38±0.16</td>
<td>1.46±0.21</td>
<td>0.95±0.12</td>
<td>31.16</td>
</tr>
</tbody>
</table>

*p<0.05 vs. untreated group, **p<0.01 vs. untreated group, †p<0.05 vs. control group, ‡p<0.01 vs. control group. *Inhibition rate of the cells in the let-7a group vs. the untreated group.

Figure 2. The biological functions of let-7a in the HeLa cells. (A) Let-7a significantly inhibited the cell apoptosis of HeLa cells compared with that in control and untreated groups. (B) The effects of let-7a on the cell cycle distribution in the HeLa cells. (C) The effects of let-7a on p53 expression. Western blot analysis indicated that the protein level of p53 was increased in the let-7a mimic group cells compared with that in the mimic control and untreated group cells (†p<0.05 vs. the mimic control group; ‡p<0.01 vs. the mimic control group; *p<0.01 vs. the untreated group).
Overexpression of let-7a inhibits cell proliferation and promotes cell apoptosis in HeLa cells. Let-7a was overexpressed in the HeLa cells using the commercially available let-7a mimics. MTT assay showed that the inhibition rates of the let-7a group were 31.58, 34.78, 44.64 and 31.16% at 24, 48, 72 and 96 h, respectively (Table I). In addition, the cell proliferation was significantly inhibited in the let-7a group of HeLa cells compared with that in the control and untreated groups (all, p<0.05). This indicated that let-7a significantly inhibited the proliferative ability of the HeLa cells.

Furthermore, the cell apoptosis rates were tested by flow cytometry and were 3.71±0.21, 2.90±0.54 and 5.71±0.86% in the untreated, mimic control and let-7a group, respectively. The results showed that overexpression of let-7a significantly inhibited the cell apoptosis of HeLa cells compared with that in the control and untreated groups (p<0.05). This indicated that let-7a significantly inhibited the apoptotic ability of the HeLa cells.

Additionally, cell cycle analysis by flow cytometry showed that cells in the let-7a group were arrested in the G2 phase when compared with cells in the control and untreated groups (p<0.05). In addition, the percentage of cells in the let-7a group which remained in the G2 phase was increased by 9.33% compared with that in the untreated group, and increased by 5.28% compared with that in the control group (Fig. 2A). Meanwhile, western blot results showed that the key cell cycle factor p53 was increased in the let-7a group compared with that in the control and untreated groups (Fig. 2C). These data indicated that let-7a inhibited the cell proliferation of HeLa cells by affecting the cell cycle.

Let-7a negatively regulates TGFBR1 expression. To explore the mechanism of let-7a in cervical cancer, we aimed to ascertain the target genes. By bioinformatic analysis, a 7-nt match (nucleotides 2-8) to the seed region at the 5'-end of mature let-7a was present in the TGFBR1 3' UTR. By analyzing homology, the putative let-7a target site was highly conserved (Fig. 3A). Western blotting showed that the expression level of TGFBR1 was reduced significantly in the let-7a mimic group, compared with that in the control group in the HeLa cells (Fig. 3C). In addition, dual-luciferase reporter assay results showed that transcripts carrying the TGFBR1 3' UTR exhibited a significant reduction in luciferase activity in the let-7a mimic group. In contrast, the mimic control had no significant effect on the luciferase activity (Fig. 3B). Therefore, TGFBR1 was a target of let-7a.

let-7a regulates the TGF-β/SMAD signaling pathway in cervical cancer cells. The expression levels of TGF-β1 and SMAD4 were detected by immunohistochemistry in cervical squamous carcinoma and adenocarcinoma tissues. The results showed that TGF-β1 and SMAD4 protein levels were positive in the cervical squamous carcinoma and adenocarcinoma compared with levels in the adjacent mucosa (Fig. 4A).
Meanwhile, tissue specimens were immunofluorescence stained for protein presence with antibodies of TGF-β1 and SMAD4. The cervical cancer tissues showed a stronger TGF-β1 and SMAD4 presence than those of normal cervical tissues as demonstrated by the red fluorescence signals due to immunofluorescence (Fig. 4B).

let-7a mimics or control was transfected into cervical cancer cells (SiHa, HeLa and CaSki) by Lipofectamine 2000. Real-time RT-PCR displayed that let-7a was significantly enhanced in the let-7a group compared with that in the control and untreated groups (**p<0.01 vs. the control group; ##p<0.01 vs. the untreated group). The protein levels of TGF-β1 and SMAD4 were decreased in the let-7a mimic group compared with that in the control and untreated groups as detected by western blotting. Let-7a was significantly increased in the TGF-β1 siRNA group compared with that in the control and untreated groups as detected by real-time PCR (**p<0.01 vs. the control group; ##p<0.01 vs. the untreated group).

Figure 4. Effects of let-7a on the TGF-β/SMAD signaling pathway. (A) The expression levels of TGF-β1 and SMAD4 protein were augmented in cervical squamous carcinoma and adenocarcinoma as detected by immunohistochemistry. (B) The expression levels of TGF-β1 and SMAD4 proteins were increased in cervical squamous carcinoma and adenocarcinoma as detected by immunofluorescence. Images are shown at a magnification of x200. (C) Let-7a was significantly increased in the let-7a group compared with that in the control and untreated groups (**p<0.01 vs. the control group; ##p<0.01 vs. the untreated group). (D) The protein levels of TGF-β1 and SMAD4 were decreased in the let-7a mimic group compared with those in the control and untreated groups. (E) The protein level of TGF-β1 was decreased in the siRNA group compared with that in the control and untreated groups as detected by western blotting. (F) Let-7a was significantly increased in the TGF-β1 siRNA group compared with that in the control and untreated groups as detected by real-time PCR (**p<0.01 vs. the control group; ##p<0.01 vs. the untreated group).
Discussion

In humans, the let-7 family consists of 13 members, all sharing a common seed sequence. let-7 miRNA is involved in many physiological, as well as pathological processes. As known, let-7 target genes, such as HMGA2, RAS and LIN28 are oncogenes involved in cell cycle progression (23,24). The let-7 level was found to be low in a variety of primary and metastatic tumors, and its loss or downregulation was associated with increased cancer aggressiveness and poor clinical outcome (25). In the present study, we explore the expression level of let-7a in cervical cancer and studied the molecular mechanism of let-7a in regards to the proliferation of cervical cancer cells.

In the present study, real-time PCR was used to detect the expression of circulating let-7a in patient serum. The results showed that let-7a was decreased in the serum of cervical cancer patients. In addition, the expression of let-7a was reduced in three human cervical cancer cells. Additionally, it was reported that let-7a was decreased in the cancer tissues of cervical cancer patients (22). Collectively, the results displayed the downregulation of the expression of let-7a in patients and in cervical cancer cell lines.

To investigate the biological function of let-7a in cervical cancer, we assessed the cell proliferation, cell apoptosis and cell cycle in the HeLa cells. MTT assay showed that the inhibition rates of the let-7a group were 31.58, 34.78, 44.64 and 31.16%; at 24, 48, 72 and 96 h, respectively. In addition, the cell proliferation was significantly inhibited in the let-7a group in the HeLa cells compared with that in the control and untreated groups. This suggests that overexpression of let-7a inhibits the cell proliferation of cervical cancer. In addition, previous studies have shown that let-7a inhibited the cell proliferation in prostate and lung cancer (26,27). Therefore, let-7a may play a role in cell proliferation in various diseases. Our results also found that the cell apoptosis rates were 3.71±0.21, 2.90±0.54 and 5.71±0.86% in the untreated, control and let-7a groups. Moreover, it was reported that downregulation of the expression of let-7a markedly suppressed cell cycle progression and induced apoptosis in gastric cancer cells (28). This suggests that let-7a promotes cell apoptosis. Furthermore, cell cycle analysis showed that cells were arrested in the G2 phase in the let-7a group compared with that in the control and untreated groups. In addition, the percentage of cells arrested in the G2 phase in the let-7a group increased by 9.33% compared with that in the untreated group. This suggests that let-7a may inhibit cell proliferation of HeLa cells by G2 phase arrest. In addition, the key cell cycle factor p53 was increased in the let-7a group compared with that in the control and untreated group. The tumor suppressor p53 is a transcription factor that responds to various types of cellular stress, including DNA damage and oncogene activation. p53 regulates the expression of genes involved in a variety of cellular functions, including cell cycle arrest and apoptosis (29). These findings demonstrated that overexpression of let-7a suppressed the biological behavior of cervical cancer cells.

To explore the mechanism of let-7a in cervical cancer, we focused on the target gene for let-7a, TGFBR1. Our results showed that a highly conserved 7-nt match of mature let-7a was present in the TGFBR1 3’ UTR by bioinformatic analysis. In addition, dual-luciferase reporter assay showed that TGFBR1 exhibited a significantly reduction in the presence of let-7a mimics. In addition, western blot results showed that overexpression of let-7a decreased the expression of TGFBR1. Therefore, TGFBR1 is a target of let-7a. As known, TGFBR1 is an important element of the TGF-β/SMAD signaling pathway which has emerged as a central mediator of cancer progression due to its capability to regulate cell growth, differentiation and migration (30). Studies have also shown that repression of TGFBR1 enhanced the cell proliferation of lung cancer and cell migration and invasion of breast cancer (31,32). Moreover, our data showed that TGF-β1 and SMAD4 were both increased in cervical squamous carcinoma and adenocarcinoma patient tissues. In addition, western blot results showed that overexpression of let-7a reduced the expression of TGF-β1 and SMAD4. In addition, silencing of TGF-β1 protein increased the expression of let-7a. Therefore, let-7a had an effect on the TGF-β/SMAD signaling pathway in cervical cancer.

In conclusion, our results indicated that let-7a expression was downregulated in cervical cancer, and overexpression of let-7a affected cell proliferation, cell apoptosis and cell cycle through the TGF-β/SMAD signaling pathway in cervical cancer.

Acknowledgements

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References


