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 the 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 keratinocyes 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, TGFBR1 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.

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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 oncogenes, 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 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 beween 30 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 keratinocye 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% CO_2 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 $2^{-\Delta\Delta Ct}$.

MTT assay. HeLa cells were seeded at a density of $2x10^3$ 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 $1x10^4$ cells were analyzed using a FACSCalibur 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'-GCGCTCGAGGGGTG

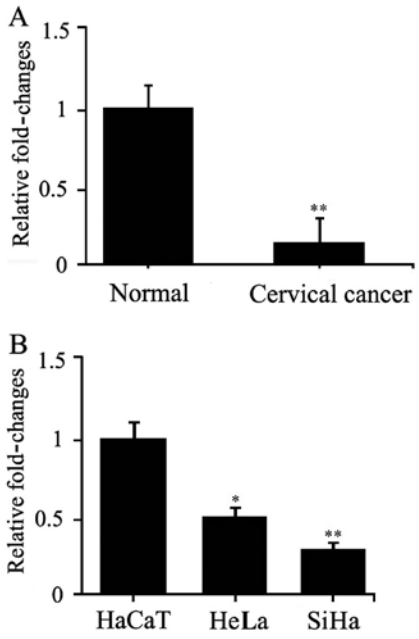

Figure 1. let-7a expression is downregulated in cervical cancer. (A) Expression of let-7a in serum was decreased in cervical cancer patients as detected by real-time RT-PCR (*p<0.05 vs. normal group). (B) Let-7a was also decreased in the SiHa and HeLa cells compared with that in normal HaCaT cells (*p<0.05 vs. the HaCaT group; **p<0.01 vs. the HaCaT group).

TTTAGGAGGCTGGT-3' and reverse, 5'-AATGCGGCCGC CATACAACTTTTCCTTCGG-3', and then cloned into a pmiR-RB-REPORT™ vector with restriction endonucleases *Xho*I and *Not*I 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, and then transfected with TGFBR1-3' UTR-wt (200 ng/ml), TGFBR1-3' UTR-mut (200 ng/ml) and let-7a mimics (50 nmol/l) with Lipofectamine 2000. After 48 h post-transfection, the cells were lysed and assayed for luciferase activities using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). The data recorded on the luminometer were normalized by dividing firefly luciferase activity with that of *Renilla* luciferase, and were analyzed and graphed.

Western blot analysis. After the above-mentioned treatment, cells were washed three times in ice-cold PBS (pH 7.4), and lysed with ice-cold RIPA buffer (Shanghai Beyotime Corp., Shanghai, China). The lysate was clarified by centrifugation at 15,000 x g for 30 min at 4˚C. The resulting supernatant was stored at -80˚C until further analysis. In addition, the protein content was determined using the BCA protein assay kit with BSA as the standard. Proteins (20 μg) were denatured at 100˚C in loading buffer for 10 min and separated by SDS-PAGE. After electrotransfer, the membrane was probed with antibodies against p53 (Abcam, Cambridge, UK), TGF-β1, SMAD4 (both from ProteinTech, Chicago, IL, USA) or GAPDH (Abcam). Then, the blots were incubated with a secondary antibody (Abcam). After washing, the bound antibody was visualized using an enhanced chemiluminescence assay kit (Tiangen Biotechnology, Corp. Beijing, China) according to the manufacturer's instructions.

Immunohistochemistry. Cervical cancer tissues were embedded in paraffin and sectioned at a thickness of 5 μm. After deparaffinization 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 deparaffinization 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).

Statistical analysis. All statistical tests were performed using SPSS software (SPSS, Inc., Chicago, IL, USA). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). p-values at <0.05 were considered statistically significant. All data are presented as mean ± standard deviation (SD).

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

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

Absorbance	Untreated group	Control group	let-7a group	Inhibition rate (%)[e]
A490 (24 h)	0.19±0.02	0.23±0.02	0.13±0.02[a,d]	31.58
A490 (48 h)	0.46±0.04	0.47±0.05	0.30±0.03[b,d]	34.78
A490 (72 h)	1.12±0.15	1.07±0.09	0.62±0.05[b,d]	44.64
A490 (96 h)	1.38±0.16	1.46±0.21	0.95±0.12[a,c]	31.16

[a]p<0.05 vs. untreated group, [b]p<0.01 vs. untreated group, [c]p<0.05 vs. control group, [d]p<0.01 vs. control group. [e]Inhibition rate of the cells in the let-7a group vs. the untreated group.

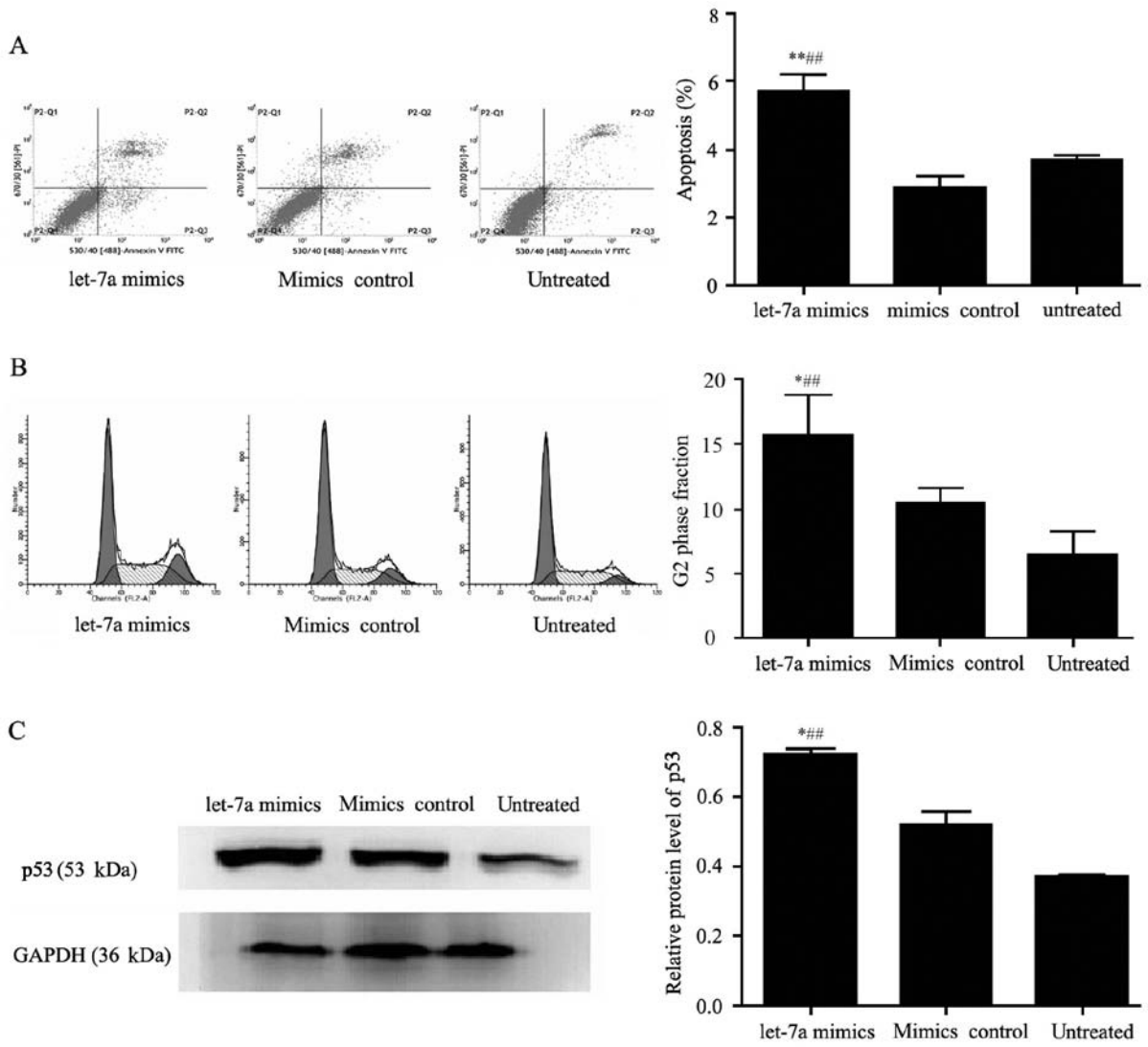

Figure 2. The biological functions of let-7a in the HeLa cells. (A) Let-7a significantly inhibited the cell apoptosis of HeLa cells compared with that in control and untreated groups. (B) The effects of let-7a on the cell cycle distribution in the HeLa cells. (C) The effects of let-7a on p53 expression. Western blot analysis iindicated 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).

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.

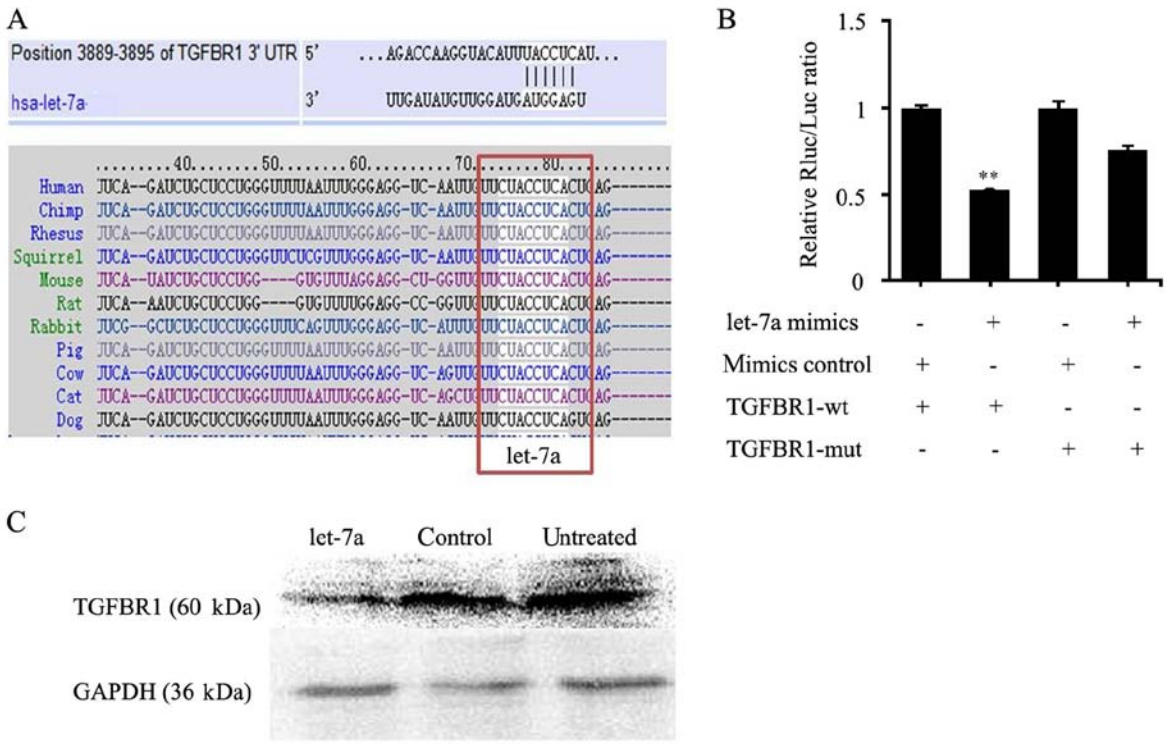

Figure 3. Let-7a regulates TGF-β1 by targeting TGFBR1. (A) Comparison of base pairs between mature let-7a and TGFBR1 3' UTR, and alignment of putative let-7a binding sites in TGFBR1 3' UTR from multi-species. Paired bases are indicated by a red line. In addition, the binding sites shown in this alignment are boxed with a red rectangle. (B) Luciferase activity of various reporters in the presence or absence of let-7a mimics in HeLa cells. The ratio of reporter *Renilla* lucifearse (Rluc) to control plasmid firefly luciferase (Luc) in relative luminescence units was normalized to the control value from the control transfection, in which TGFBR1-wt, TGFBR1-mut and let-7a mimics were co-transfected into HeLa cells. Luciferase activity was significantly decreased in the TGFBR1-wt + let-7a-mimic group compared with TGFBR1-wt + mimic control and TGFBR1-mut + mimic control groups ($^{**}p<0.01$ vs. TGFBR1-wt + mimics control). (C) Let-7a negatively regulated the expression of TGFBR1 in HeLa cells by western blotting. Overexpression of let-7a markedly inhibited the expression of TGFBR1 protein in the let-7a mimic group compared with the mimic control 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 ($p<0.01$) (Fig. 2A). These results demonstrated that let-7a had an effect on the cell apoptosis of 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. 2B). Meanwhile, western blot results showed 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).

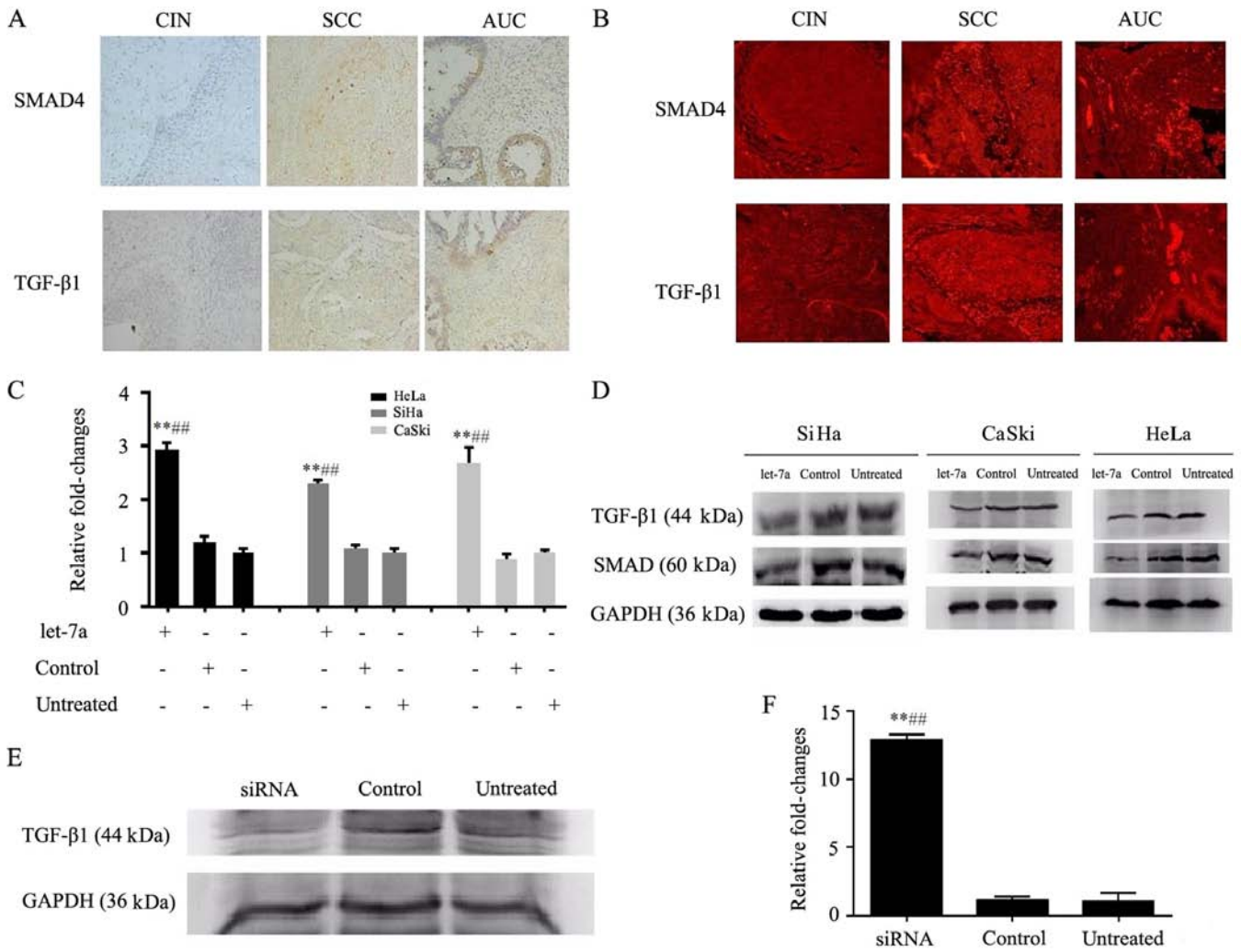

Figure 4. Effects of let-7a on the TGF-β/SMAD signaling pathway. (A) The expression levels of TGF-β1 and SMAD4 protein were augmented in cervical squamous carcinoma and adenocarcinoma as detected by immunohistochemistry. (B) The expression levels of TGF-β1 and SMAD4 proteins were increased in cervical 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).

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 ($p<0.05$) (Fig. 4C). This suggests that let-7a was successfully transfected into the cells.

TGF-β1 and SMAD4 are important factors in the TGF-β/SMAD signaling pathway. Thus, the expression levels of TGF-β1 and SMAD4 were assessed in the HeLa, SiHa and CaSki cells by western blotting. The results showed that TGF-β1 and SMAD4 proteins were decreased in the let-7a group, compared with that in the control and untreated groups ($p<0.05$) (Fig. 4D). This indicated that overexpression of let-7a decreased the expression of TGF-β1 and SMAD4 proteins in the cervical cancer cells.

Moreover, TGF-β1 siRNA was transfected into the HeLa cells and its expression was assessed by western blotting. The results showed that the expression of TGF-β1 was reduced in the TGF-β1 siRNA group compared with that in the control and untreated groups (Fig. 4E). No significant differences in the levels of TGF-β1 protein were observed between the control and untreated groups. This indicated that the expression of TGF-β1 was successfully suppressed in the HeLa cells. As expected, knockdown of TGF-β1 markedly increased the expression of let-7a in the HeLa cells as detected by real-time PCR ($p<0.05$) (Fig. 4F). These data further confirmed that the expression level of TGF-β1 protein was inversely correlated with let-7a.

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.

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References

1. Haie-Meder C, Morice P and Castiglione M; ESMO Guidelines Working Group: Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 21 (Suppl 5): v37-v40, 2010.
2. Castellsagué X, Díaz M, de Sanjosé S, Muñoz N, Herrero R, Franceschi S, Peeling RW, Ashley R, Smith JS, Snijders PJ, *et al*; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group: Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: Implications for screening and prevention. J Natl Cancer Inst 98: 303-315, 2006.
3. Aranha MM, Santos DM, Xavier JM, Low WC, Steer CJ, Solá S and Rodrigues CM: Apoptosis-associated microRNAs are modulated in mouse, rat and human neural differentiation. BMC Genomics 11: 514, 2010.
4. Schwarzenbacher D, Balic M and Pichler M: The role of microRNAs in breast cancer stem cells. Int J Mol Sci 14: 14712-14723, 2013.
5. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, *et al*: Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141: 672-675, 2008.
6. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, *et al*: Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 105: 10513-10518, 2008.
7. Ho AS, Huang X, Cao H, Christman-Skieller C, Bennewith K, Le QT and Koong AC: Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. Transl Oncol 3: 109-113, 2010.
8. Tsujiura M, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, Konishi H, Morimura R, Deguchi K, Fujiwara H, *et al*: Circulating microRNAs in plasma of patients with gastric cancers. Br J Cancer 102: 1174-1179, 2010.

9. Xia XM, Jin WY, Shi RZ, Zhang YF and Chen J: Clinical significance and the correlation of expression between *Let-7* and *K-ras* in non-small cell lung cancer. Oncol Lett 1: 1045-1047, 2010.
10. Kong D, Heath E, Chen W, Cher ML, Powell I, Heilbrun L, Li Y, Ali S, Sethi S, Hassan O, *et al*: Loss of let-7 up-regulates EZH2 in prostate cancer consistent with the acquisition of cancer stem cell signatures that are attenuated by BR-DIM. PLoS One 7: e33729, 2012.
11. Mayr C, Hemann MT and Bartel DP: Disrupting the pairing between *let-7* and *Hmga2* enhances oncogenic transformation. Science 315: 1576-1579, 2007.
12. He XY, Chen JX, Zhang Z, Li CL, Peng QL and Peng HM: The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice. J Cancer Res Clin Oncol 136: 1023-1028, 2010.
13. Zhou J, Peng R, Li T, Luo X, Peng H, Zha H, Yin P, Wen L and Zhang Z: A potentially functional polymorphism in the regulatory region of let-7a-2 is associated with an increased risk for diabetic nephropathy. Gene 527: 456-461, 2013.
14. Lee JW, Choi CH, Choi JJ, Park YA, Kim SJ, Hwang SY, Kim WY, Kim TJ, Lee JH, Kim BG, *et al*: Altered MicroRNA expression in cervical carcinomas. Clin Cancer Res 14: 2535-2542, 2008.
15. Fan DM, Tian XY, Wang RF and Yu JJ: The prognosis significance of TGF-β1 and ER protein in cervical adenocarcinoma patients with stage Ib~IIa. Tumour Biol 35: 11237-11242, 2014.
16. Chu GC, Dunn NR, Anderson DC, Oxburgh L and Robertson EJ: Differential requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo. Development 131: 3501-3512, 2004.
17. López-Muñoz H, Escobar-Sánchez ML, López-Marure R, Lascurain-Ledesma R, Zenteno E, Hernández-Vazquez JM, Weiss-Steider B and Sánchez-Sánchez L: Cervical cancer cells induce apoptosis in TCD4$^+$ lymphocytes through the secretion of TGF-β. Arch Gynecol Obstet 287: 755-763, 2013.
18. Bai X, He T, Liu J, Wang Y, Fan L, Tao K, Shi J, Tang C, Su L and Hu D: Loureirin B inhibits fibroblast proliferation and extracellular matrix deposition in hypertrophic scar via TGF-β/Smad pathway. Exp Dermatol 24: 355-360, 2015.
19. Feng XH and Derynck R: Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol 21: 659-693, 2005.
20. Pasche B, Pennison MJ, Jimenez H and Wang M: *TGFBR1* and cancer susceptibility. Trans Am Clin Climatol Assoc 125: 300-312, 2014.
21. Sun Y, Peng R, Peng H, Liu H, Wen L, Wu T, Yi H, Li A and Zhang Z: miR-451 suppresses the NF-kappaB-mediated proinflammatory molecules expression through inhibiting LMP7 in diabetic nephropathy. Mol Cel Endocrinol 433: 75-86, 2016
22. Shishodia G, Shukla S, Srivastava Y, Masaldan S, Mehta S, Bhambhani S, Sharma S, Mehrotra R, Das BC and Bharti AC: Alterations in microRNAs miR-21 and let-7a correlate with aberrant STAT3 signaling and downstream effects during cervical carcinogenesis. Mol Cancer 14: 116, 2015.
23. Nair VS, Maeda LS and Ioannidis JP: Clinical outcome prediction by microRNAs in human cancer: A systematic review. J Natl Cancer Inst 104: 528-540, 2012.
24. Shell S, Park SM, Radjabi AR, Schickel R, Kistner EO, Jewell DA, Feig C, Lengyel E and Peter ME: Let-7 expression defines two differentiation stages of cancer. Proc Natl Acad Sci USA 104: 11400-11405, 2007.
25. Serguienko A, Grad I, Wennerstrøm AB, Meza-Zepeda LA, Thiede B, Stratford EW, Myklebost O and Munthe E: Metabolic reprogramming of metastatic breast cancer and melanoma by *let-7a* microRNA. Oncotarget 10: 2451-2465, 2015.
26. Dong Q, Meng P, Wang T, Qin W, Qin W, Wang F, Yuan J, Chen Z, Yang A and Wang H: MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One 5: e10147, 2010.
27. Zhong Z1: Dong Z, Yang L, Chen X, Gong Z. Inhibition of proliferation of human lung cancer cells by green tea catechins is mediated by upregulation of let-7. Exp Ther Med 4: 267-272, 2014.
28. Song H, Xu W, Song J, Liang Y, Fu W, Zhu XC, Li C, Peng JS and Zheng JN: Overexpression of Lin28 inhibits the proliferation, migration and cell cycle progression and induces apoptosis of BGC-823 gastric cancer cells. Oncol Rep 33: 997-1003, 2015.
29. Lee JY, Kim HJ, Yoon NA, Lee WH, Min YJ, Ko BK, Lee BJ, Lee A, Cha HJ, Cho WJ, *et al*: Tumor suppressor p53 plays a key role in induction of both tristetraprolin and *let-7* in human cancer cells. Nucleic Acids Res 41: 5614-5625, 2013.
30. Akhurst RJ and Hata A: Targeting the TGFβ signalling pathway in disease. Nat Rev Drug Discov 11: 790-811, 2012.
31. Lei Z, Xu G, Wang L, Yang H, Liu X, Zhao J and Zhang HT: MiR-142-3p represses TGF-β-induced growth inhibition through repression of TGFβR1 in non-small cell lung cancer. FASEB J 28: 2696-2704, 2014.
32. Fang Y, Chen Y, Yu L, Zheng C, Qi Y, Li Z, Yang Z, Zhang Y, Shi T, Luo J, *et al*: Inhibition of breast cancer metastases by a novel inhibitor of TGFβ receptor 1. J Natl Cancer Inst 105: 47-58, 2013.