Let-7b-5p regulates proliferation and apoptosis in multiple myeloma by targeting IGF1R

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Multiple myeloma (MM) is the most common cause of death from hematological malignancy worldwide, and recent studies have revealed that let-7b-5p can play an inhibitory role in tumorigenesis. However, the role of let-7b-5p in MM still remains unclear. The aim of this study was to elucidate the molecular mechanisms by which let-7b-5p acts as a tumor suppressor in MM. Here, quantitative real-time polymerase chain reaction results showed that the expression of let-7b-5p was remarkably reduced in MM tissues and MM cell lines (RPMI-8226 and U266 cells). Furthermore, over-expression of let-7b-5p significantly suppressed RPMI-8226 cell proliferation and induced S/G2 cell cycle arrest and apoptosis. Luciferase reporter assay results demonstrated that insulin-like growth factor receptor 1 (IGF1R) was negatively regulated by let-7b-5p at the post-transcriptional level. The mRNA and protein levels of IGF1R in RPMI-8226 cells were down-regulated by let-7b-5p. Furthermore, the cell phenotype altered by let-7b-5p inhibitor can be rescued by IGF1R silencing (si-IGF1R). Taken together, our results demonstrated that let-7b-5p functions as a tumor suppressor in MM, suggesting that let-7b-5p may be a potential therapeutic target for MM.

Keywords multiple myeloma; let-7b-5p; proliferation; apoptosis

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Introduction

Multiple myeloma (MM) is a post-germinal center B-cell malignancy characterized by tumor cell infiltration of the bone marrow and osteolytic lesions in the vicinity of the tumor cells [1,2]. MM is the second most common hematological malignancy worldwide [3]. Although conventional chemotherapy, together with autologous stem-cell transplantation, can modestly prolong patient survival to 4–5 years, the available treatments fail to cure MM disease in most cases. MM pathogenesis is a multistep process with chromosomal changes, as well as transcriptional and epigenetic events occur at different stages during the process of the disease [2,4]. Recent studies have illustrated that the aberrant expression of microRNAs (miRNAs) in MM is associated with genetic abnormalities and can regulate critical genes associated with MM pathogenesis [5–8], suggesting potential relevant clinical applications in this disease.

The miRNAs are small noncoding single-stranded RNAs, which negatively regulate the expression of 60% human genes through base-pairing with the 3’-untranslated region (3’-UTR) of target mRNAs at a post-transcriptional level [9]. Recent studies have demonstrated that miRNAs functioned as oncogenes or tumor suppressors, and they are involved in cell proliferation, differentiation, apoptosis, and migration in various types of human tumors [10–12]. Let-7b-5p belongs to the let-7 family and plays an important regulatory role in the cell cycle and differentiation. A recent study showed that let-7b-5p was significantly down-regulated in MM [8]. However, the biological role of let-7b-5p in the carcinogenesis of MM is still unclear.

In this study, we demonstrated a significant down-regulation of let-7b-5p in MM tissues and RPMI-8226 cells. It was found that let-7b-5p over-expression inhibited cell proliferation and promoted cell apoptosis determined by methylthiazolletetrazolium (MTT) and flow cytometer assays, respectively. In addition, insulin-like growth factor receptor 1 (IGF1R), over-expressed in MM tissues, was validated as a target of let-7b-5p through luciferase assay and western blot analysis. In summary, our studies indicated that let-7b-5p could act as a tumor suppressor by targeting IGF1R in MM, which suggested that it has potential diagnostic and therapeutic value for MM.
Materials and Methods

Patient samples, cell lines, and transfection
Twenty-four MMs, including IgG and IgM isoforms, were collected from patients at Shaanxi Provincial People’s Hospital between June 2012 and April 2014, who ranged in age from 32 to 86 years with a mean of 60.5 years. Nine control cases whose age ranged from 38 to 82 years with a mean age of 57 years underwent routine physical examinations. Bone marrow samples were snap-frozen in liquid nitrogen until RNA extraction. Both tumor and normal tissue samples were confirmed by pathological examination. No local or systemic treatment had been conducted before operation. The study was approved by the Hospital Ethical Committee, and informed consent was obtained from each patient prior to the study.

RPMI-8226 cells and U266 cells were obtained from the American Type Culture Collection (Rockville, USA) and grown in RPMI 1640 medium. HEK293T cells were obtained from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco’s Minimum Essential Medium. All the media were supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Carlsbad, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma, St Louis, USA) at 37°C in a 5% CO₂ atmosphere. Plasma cells were isolated from five healthy volunteers as the control. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol.

Plasmid constructions
pcDNA™6.2-GW-miR vector (Invitrogen) was used to construct let-7b-5p plasmid. EcoRI and HindIII sites were inserted into the multiple cloning site of the vector, and then the let-7b-5p precursor was chemically synthesized (Beijing AuGCT DNA-SYN Biotechnology Co. Ltd, Beijing, China) and cloned into pcDNA™6.2-GW-miR vector between the EcoRI and HindIII sites. IGF1R was first predicted as the target gene of let-7b-5p through bioinformatics analysis (http://www.targetscan.org/). Specified fragments of IGF1R were chemically synthesized, and then the luciferase-UTR reporter constructs were generated by introducing the wild-type (WT)/mutant-type (MT)-IGF1R 3’-UTR, with a putative let-7b-5p binding site into pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, USA) between the XhoI and SacI sites. All sequences of let-7b-5p precursors and WT/MT-IGF1R were shown in Table 1.

Quantitative real-time polymerase chain reaction analysis
Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The concentration and quantity of RNA were assessed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA). The ratio of A260 : A280 was used to indicate the purity of total RNA. Complementary DNA was synthesized from RNA, using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). miRNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR) were

Table 1. Primers and oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-let-7b-5p-sense</td>
<td>AATTCCGGGGTGAGGTAGTTGTGTTGTTTCAGGCGACGTGATG</td>
</tr>
<tr>
<td></td>
<td>TTGGCCCTCAGAAGATAACTATAACCTACTGCCCTCTGGA</td>
</tr>
<tr>
<td>pre-let-7b-5p-antisense</td>
<td>AGCTTCAGGGGAAGGCAGTAGTTGTATAGTTATCTTCCGAGGGCA</td>
</tr>
<tr>
<td></td>
<td>CATCACGTGCCCTGAACACACACACTACTCACCTCACCAGG</td>
</tr>
<tr>
<td>IGF1R-WT-sense</td>
<td>CCCCCCAACATTTATCTACCTCAC</td>
</tr>
<tr>
<td>IGF1R-WT-antisense</td>
<td>TCGAGTACAGTGGTAGATATAAAGTTGTTGGGAGCT</td>
</tr>
<tr>
<td>IGF1R-MT-sense</td>
<td>CCCCCCAACATTATGATGAGGA</td>
</tr>
<tr>
<td>IGF1R-MT-antisense</td>
<td>TCGAGT CTCCATC ATAAAGTTGTTGGGAGCT</td>
</tr>
<tr>
<td>let-7b-5p-RT</td>
<td>GTCTGATCTCAGTGCTGTCCTGAGTGAGTCAGGAATTCAGCTGACTGAGCA</td>
</tr>
<tr>
<td>let-7b-5p-F</td>
<td>ATCCACGTGCTGCTGCTG</td>
</tr>
<tr>
<td>let-7b-5p-R</td>
<td>TGCTTGAGGTAGTTGTTG</td>
</tr>
<tr>
<td>IGF1R-F</td>
<td>TGAGATACATCCAGCAGCATCA</td>
</tr>
<tr>
<td>IGF1R-R</td>
<td>GGGCTTAAGGTCTGTCCTGTC</td>
</tr>
<tr>
<td>U6-RT</td>
<td>GTGCTATACAGTGCTGAGTGGTTGGTCAGCA</td>
</tr>
<tr>
<td>U6-F</td>
<td>TGGCGGTGCTGCTTCCGACG</td>
</tr>
<tr>
<td>U6-R</td>
<td>CCACTTCTGCTGAGGTGC</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>AGTGTGACGTGGACATCGGCAA</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>ATCCACATCTGCTGAGGTG</td>
</tr>
</tbody>
</table>
performed using the special primers for let-7b-5p and U6. qRT-PCR was performed using a SYBR Green Reagent (TaKaRa) on the FTC-3000™ system. All primers used are shown in Table 1. β-Actin and U6 were used to normalize mRNA and miRNA, respectively. The relative expression levels of the genes were calculated using the 2^-ΔΔCt method [13]. All assays were performed in triplicate.

Cell proliferation assay
RPMI-8226 cells were seeded at 3000 cells/well in 96-well plates with 100 μl of RPMI-1640 medium, supplemented with 10% fetal bovine serum without antibiotics, and cultured overnight. Then the cells were transfected with let-7b-5p ctrl, let-7b-5p, let-7b-5p inhibitor, inhibitor ctrl, si-IGF1R, and si-ctrl, respectively. The cell proliferation assay was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24, 48, and 72 h after transfection. A 20 μl of MTT solution was added and incubated for 4 h at 37°C, then the supernatant was discarded and 150 μl dimethyl sulfoxide was added to the wells. The absorbance at 492 nm was measured using a microplate spectrophotometer.

Cell cycle analysis
At 48 h post-transfection, RPMI-8226 cells were harvested by trypsinization and centrifuged at 1200 rpm for 10 min. The cells were washed three times with cold phosphate buffer saline (PBS) and fixed in 70% ice-cold ethanol overnight at 4°C. Then, cells were resuspended in PBS containing 20 μg/ml propidium iodide (PI) and 10 U/ml RNaseA and incubated at room temperature for 30 min. The cell cycle was then analyzed by a BD Calibur flow cytometer (Franklin Lakes, USA).

Cell apoptosis assay
The extent of apoptosis was evaluated by the Annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech, Nanjing, China). RPMI-8226 cells were transfected with let-7b-5p, inhibitor, si-IGF1R, or negative control. At 48 h transfection, cells were harvested in cold PBS, stained with 5 μl Annexin V-FITC and 5 μl PI at room temperature in the dark for 15 min, and then analyzed using a flow cytometer.

Dual-luciferase assay
PmirGLO-IGF1R-3'-UTR-WT/MT vector was co-transfected with let-7b-5p or let-7b-5p ctrl into HEK293T cells using Lipofectamine 2000. Forty-eight hours after transfection, luciferase activities were evaluated using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. Renilla luciferase served as an internal control, and the normalized firefly luciferase activity was obtained by firefly luciferase activity/Renilla luciferase activity.

Western blot analysis
RPMI-8226 cells transfected with let-7b-5p, inhibitor, si-IGF1R, or negative control were lysed using RIPA buffer and supplemented with protease inhibitor (Invitrogen). The proteins were subject to 8%–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in tris-buffered saline withTween-20 (TBST) for 2 h and incubated with anti-IGF1R antibody (#9750; Cell Signaling Technology, Beverly, USA) and anti-β-actin antibody (#sc-47778; Santa Cruz, Santa Cruz, USA) at 4°C overnight. After being washed three times with TBST, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. Relative protein expression was normalized to β-actin level in each sample. Quantifications of western blots were performed using ImageJ software.

Statistical analysis
Data were presented as the mean ± SEM. The difference between groups was analyzed using Student’s t-test. Spearman’s correlation analysis was used to analyze the correlation between let-7b-5p and IGF1R expressions. All data were analyzed using SPSS 13.0 software, and all experiments were carried out in triplicate. P < 0.05 was considered statistically significant.

Results
Let-7b-5p was down-regulated in MM tissues and cell lines
The mRNA expression levels of let-7b-5p in MM tissues and RPMI-8226 cells were measured using qRT-PCR analysis. As shown in Fig. 1A, the level of let-7b-5p was 0.34 ± 0.24, significantly lower in MM tissue samples compared with normal bone marrow tissues (0.97 ± 0.07) (P < 0.001). This was similar to the suppressed expression level of let-7b-5p in RPMI-8226 cells and U266 cells (P < 0.001; Fig. 1B), suggesting that let-7b-5p might be a potential suppressor gene in MM.

Let-7b-5p inhibited growth of RPMI-8226 cells
To determine the effects of let-7b-5p on cell growth in vitro, the over-expression plasmid let-7b-5p or the antisense oligonucleotide of let-7b-5p (let-7b-5p inhibitor) were transfected into RPMI-8226 cells.
As shown in Fig. 2A,B, the expression of let-7b-5p was significantly increased or decreased in RPMI-8226 cells transfected with let-7b-5p or inhibitor, respectively. According to the results of the MTT assay, RPMI-8226 cells transfected with let-7b-5p displayed significantly slower growth rate than that in control cells (P < 0.05; Fig. 2C), but promoted cell growth by transfecting with let-7b-5p inhibitor
Figure 1. Let-7b-5p was down-regulated in both multiple myeloma (MM) tissues and cells  (A) Decreased let-7b-5p expression in MM tissues compared with normal bone marrow tissues. (B) Significant loss of let-7b-5p expression in RPMI-8226 cells and U266 cells in comparison with normal plasma cells. 2$^{-\Delta \Delta C_t}$ method was used to analyze the data. Data were presented as the mean ± SEM. **P < 0.001 vs. control.

Figure 2. Over-expression of let-7b-5p inhibited growth and induced S/G2 arrest in RPMI-8226 cells  (A) The expression level of let-7b-5p in RPMI-8226 cells transfected with let-7b-5p was increased compared with let-7b-5p control group. (B) RPMI-8226 cells transfected with let-7b-5p inhibitor was decreased compared with let-7b-5p inhibitor control group. (C,D) MTT assay showed the absorbance of cells transfected with let-7b-5p or let-7b-5p inhibitor control decreased compared with the let-7b-5p control or let-7b-5p inhibitor group at 24, 48, and 72 h. (E,F) Flow cytometry demonstrated an accumulation of RPMI-8226 cells at S phase when transfected with let-7b-5p or let-7b-5p inhibitor control, compared with the let-7b-5p control or let-7b-5p inhibitor group. All experiments were carried out in triplicate. *P < 0.05, **P < 0.01 vs. control.
Let-7b-5p induced apoptosis of RPMI-8226 cells

To evaluate the effects of let-7b-5p on MM cell survival, the cell apoptosis assay was performed by flow cytometry analysis in RPMI-8226 cells. At 48 h post-transfection, cells were collected and analyzed. As shown in Fig. 3, the apoptotic rate of RPMI-8226 cells transfected with let-7b-5p was significantly increased compared with the let-7b-5p control or let-7b-5p inhibitor group. All experiments were carried out in triplicate. Data were presented as the mean ± SEM. *P < 0.05 vs. control.

(P < 0.05; Fig. 2D). Let-7b-5p control or inhibitor control had no effect on cell growth. Whether slower cell growth rate was due to perturbation of cell cycle progression was determined. As shown in Fig. 2E, let-7b-5p over-expression could trigger an accumulation of cells at S phase, and a decrease in the percentage of cells in G2 phase (P < 0.05). The phenomenon was opposite when RPMI-8226 cells were transfected with let-7b-5p inhibitor (P < 0.05; Fig. 2F). These results strongly suggested that let-7b-5p participated in arresting cells at S/G2 phase and thus inhibited growth of RPMI-8226 cells.

Let-7b-5p suppressed MM progression by targeting IGF1R

To further determine whether silencing of IGF1R could attenuate the suppressive effect of let-7b-5p on MM cells, the effect of IGF1R interfering RNA (si-IGF1R) was validated by qRT-PCR (P = 0.001; Fig. 5A). Then the MTT assay, cell cycle, and apoptosis analysis were performed, and the results showed that si-IGF1R inhibited cell proliferation (P < 0.05; Fig. 5B), increased the accumulation of cells at S phase (P = 0.03; Fig. 5C), and promoted the cell apoptosis (P = 0.019; Fig. 5D), when compared with control siRNA-transfected cells. Importantly, the effects of let-7b-5p inhibitor on RPMI-8226 cells were reverted by co-transfection of si-IGF1R. These data suggested that let-7b-5p suppressed MM progression partially by targeting IGF1R.

Let-7b-5p was inversely correlated with IGF1R expression in MM tissues

qRT-PCR assay was used to measure the endogenous expression level of IGF1R in MM tissues. The results showed that IGF1R mRNA was significantly increased in MM tissues compared with normal bone marrow tissues (P < 0.001; Fig. 6A). Furthermore, it was also found that the expression of IGF1R was reversely associated with the expression of let-7b-5p in MM tissues (P = 0.002, r = -0.601; Fig. 6B).

Discussion

Recent reports suggested that aberrant expression of miRNAs is a common event in human cancers and is associated with the pathogenesis of most malignancies [14,15], including MM [16]. Among them, let-7b-5p is one of the most frequently studied miRNAs in human cancers,
including malignant melanomas [17], lung cancer [18], and gastric cancer [19]. Ma et al. [20] showed that the level of let-7b expression in benign breast disease was higher than in breast cancer specimens determined by in situ hybridization, indicating that let-7b could inhibit growth and facilitate differentiation of benign breast disease by inhibiting the expression of basigin. Chen et al. [21] also showed that let-7b was reduced in lung cancer tissues, and in vitro experiments showed that let-7b could suppress cell proliferation and promote cell apoptosis of tumor cells via post-transcriptional repression of CYP2J2 (cytochrome P450 epoxygenase 2J2) and let-7b significantly inhibited the tumor phenotype by targeting CYP2J2 in vivo. Moreover, it has been reported that let-7b over-expression inhibited cyclin D1 protein expression, and knockdown of cyclin D1 expression significantly increased cisplatin-induced G1 arrest and apoptosis [22].

In this study, we found that the expression of the let-7b-5p was significantly down-regulated in clinical MM tissues and RPMI-8226 cells. Let-7b-5p over-expression suppressed the cell growth and promoted cell apoptosis of RPMI-8226 cells. Taken together, we supposed that let-7b-5p might be a novel tumor suppressor in MM.

IGF1R is a tyrosine kinase receptor and is composed of two extracellular α-subunits that contain the ligand binding sites and two β-subunits with tyrosine kinase activity contained within their cytoplasmic domains. Phosphorylation of IGF1R-β subsequently activates the downstream PI3 K/ Akt and MAPK/ERK signaling pathways, which regulate cell proliferation, differentiation, and survival [23,24]. In recent years, growing evidence has been emerging that IGF1R may be involved in tumorigenesis in many cancers, including lung and breast cancers [25]. Li et al. [26] indicated that IGF1R/IGF1R signaling induces tumor-associated lymphangiogenesis and contributes to lymphatic metastasis of colorectal cancer through in vitro and in vivo ways. IGFIR also promoted prostate cancer growth by stabilizing α5β1 integrin and inhibited the nasopharyngeal cell apoptosis [27,28]. In addition, the aberrant expression of IGF1R was frequently regulated through targeting 3'UTRs of target mRNAs to induce their degradation or translational repression. Yuan et al. [29] reported that over-expression of miR-140 suppresses non-small-cell lung carcinoma cell proliferation, migration, and invasion through targeting IGF1R. miR-486 could reduce both proliferative and migratory capacity as well as induce apoptosis in lung cancer cell lines [30]. miR-150* and miR-630 could promote apoptosis in pancreatic cancer cells by targeting IGF1R [31]. Here, we demonstrated that let-7b-5p inhibited IGF1R expression, and confirmed that IGF1R was identified as an important downstream target of let-7b-5p in MM cells. We also found that silencing of IGF1R...
induced cell growth inhibition, S/G2-phase arrest, and promoted cell apoptosis, which was similar to the phenotypes induced by let-7b-5p over-expression and reversed the effects of let-7b-5p inhibitor. Furthermore, IGF1R was increased in MM tissues and negatively correlated with the expression of let-7b-5p. Taken together, these results suggested that let-7b-5p might inhibit cell growth and promote cell apoptosis by targeting IGF1R in MM.

In conclusion, we demonstrated that let-7b-5p was significantly decreased in MM tissues and cell lines. Over-expression of let-7b-5p inhibited the cell proliferation, caused S/G2 phase arrest, and promoted cell apoptosis by targeting IGF1R in RPMI-8226 cells. Therefore, our findings suggested that let-7b-5p is a tumor suppressor that may inhibit carcinogenesis and the development of MM. Therefore, let-7b-5p may be a potential diagnostic and therapeutic target for MM.
References


