Mir-186 inhibits the proliferation and growth of multiple myeloma cells by targeting Jagged-1 expression

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Abstract

Purpose: To investigate the suppressive influence of mir-186 on multiplication and growth of multiple myeloma (MM), as well as the processes involved.

Methods: The U266 cells were divided into control, mir-186 overexpression, and inhibition groups. The latter two were transfected with mir-186 agent and antagonist, respectively. Cell viability, colony formation potential, cell cycle ratio, and Jagged-1 mRNA and protein levels were measured using various assays.

Results: Cell growth increased over time in all groups. However, mir-186 overexpression cells showed significantly decreased growth and colony formation capacity, relative to control, while the mir-186 inhibition cells showed significantly higher growth and colony formation capacity. The study revealed a higher proportion of G0/G1 stage cells and lower proportion of S-phase cells in mir-186 overexpression cells than in control cells. The opposite effect was seen in mir-186 inhibition cells. Jagged-1 protein and mRNA levels were significantly lower in mir-186 overexpression cells and higher in mir-186 inhibition cells than in control group. The Jagged-1 knockout group showed significantly higher Jagged-1 mRNA and protein levels than both the control and mir-186 overexpression groups (p < 0.05).

Conclusion: When overexpressed, mir-186 inhibits the growth of multiple myeloma cells by inhibiting cell colony-formation capacity and by regulating cell cycle through mir-186-induced regulation of the expression of Jagged-1. There is need for more research to confirm the clinical benefits of therapies based on these findings.

Keywords: mir-186, Targeted regulation, Jagged1, Inhibition, Multiple myeloma, Multiplication, Growth

INTRODUCTION

Multiple myeloma (MM) is a malignant disease of the blood. The tumor cells of MM arise from the plasma cells in the bone marrow which are used from the development of B lymphocytes to the final functional stage. The clonal proliferation of malignant plasma cells in the bone marrow causes bone marrow destruction, anemia, hyperviscosity, renal function damage, and other clinical manifestations. The incidence of MM is high, and it is seen mainly in the elderly in America and Australia [1,2]. At present, MM is an incurable disease. Thus, a global requirement for
identifying novel and efficacious therapeutic methods for this disease is imperative. It is known that miRNAs modulate cancer cell activities such as proliferation, apoptosis and metastasis, and act oncogenic factors in a variety of tumors [3]. The mir-186 was first found in human osteosarcoma cell lines and mouse eye tissues. It has been shown that mir-186 participates in, and regulates insulin cell differentiation and cardiovascular diseases, apart from its association with the pathogenesis of cancers [4]. Moreover, significantly abnormal mir-186 levels appear in MM patients [5].

In the present research, the influence of mir-186 on multiplication and growth of multiple myeloma U266 cells was investigated, as well as the associated mechanism.

EXPERIMENTAL

General information

Multiple myeloma U266 cells were provided by Wuhan Yipu Biotechnology Co. Ltd.

Methods

Cell culture

The U266 cells were cultured at 37 °C in RPMI-1640 containing 10 % fetal bovine serum and 1 % penicillin-streptomycin in a constant temperature cell culture chamber containing 5 % CO₂. The U266 cells were sub-cultured for an average of 2-3 days, and cells in logarithmic growth phase were used for the experiments.

Cell grouping and transfection

The U266 cells were divided into 3 groups: control group, mir-186 overexpression group and mir-186 inhibition group. Cells in good growth condition were inoculated into 6-cm Petri dishes. Then, 3 mL of lentivirus supernatant containing empty vector phu-6-mcs-pgk-egfp and mir-186 recombinant plasmid, was added to the mir-186 overexpression group. To cells in the control group was added 3ml of lentivirus supernatant containing empty vector phu-6-mcs-pgk-egfp, while mir-186 antagonist was added to the mir-186 inhibition group. Cell culture medium (2 mL) containing 10 % FBS was added and cultured for 48 h. Part of the cells in the mir-186 overexpression group was transfected with Jagged-1 plasmid without 3 'UTR, and this was named the Jagged-1 plasmid knockout group.

MTT assay for cell proliferation

A culture plate was taken out at baseline, 24 and 48 h, and 50 μL of MTT solution (5 mg/mL) was added to each well. After mixing and culturing for 4 h, the culture medium was discarded, and dimethyl sulfoxide was added (50 μL). After mixing, the optical density of the formazan solution was measured at 490 nm in an automatic microplate reader.

Colony-formation assay

After 48 h of cell transfection, logarithmic growth cells were washed with PBS and centrifuged for 5 min at 1000 rpm. The cells were inoculated at a density of 200 cells/well and cultured in the incubator for 14 days. Thereafter, the cells were PBS-rinsed, followed by ½ h fixation in 100 % methanol, and 30-min dyeing in crystal violet (2 mL). The cells were subjected to microscopic examination, and clone populations were recorded.

Determination of cell cycle distribution using flow cytometry

After 48 h of transfection, logarithmic growth cells were washed with PBS, centrifuged for 5 min at 1000 rpm, rinsed twice with PBS, fixed with 1 mL of 70 % ethanol, and centrifuged for 5 min at 1000 rpm. Then, 150 μL of propidium iodide and 5 μL of RNase were added. Flow cytometry was employed to determine the cellular proportion at all stages of the cell cycle in each group.

Real-time quantitative PCR for assay of expression of Jagged-1 mRNA in cells

After 48 h of transfection, total RNA in U266 cells at logarithmic growth phase was extracted with TRizol method. The concentration of RNA was measured using UV spectrophotometer, and the RNA was reverse-transcribed into cDNA in strict accordance with the operation instructions in the reverse transcription kit. The conditions were 70 °C for 1 h, 42 °C for 30 min, and 70 °C for 5 min. Then, 1 μL of cDNA was added to the reverse transcription reaction system under the conditions of 94 °C for 10 sec, 94 °C for 15 sec, 68 °C for 1 min, and 72 °C for 30 sec, and 40 cycles. The mRNA contents in the sample were determined with \(2^{-\Delta\Delta CT}\) procedure.

Western blot assay for protein expression of Jagged-1

After 48 h of transfection, total protein in U266 cells at logarithmic growth phase in the three groups was extracted with protein extraction kit.
(Sigma, USA), followed by SDS-PAGE, wet transfer to membrane, and blocking. Then, overnight incubation of the membranes with first immunoglobulins at 4 °C was done, after which the membranes were incubated with HRP-linked second immunoglobulins at laboratory temperature, followed by electrochemiluminescence. The expression level of Jagged1 protein was determined relative to that of GAPDH which served as internal reference.

**Statistics**

The SPSS ver. 20.0 software was used for analysis of results from this study. Measured data are shown as mean ± standard deviation (SD), and t-test was used for comparing groups. Counting data are shown as percentages, group comparison was performed using chi squared test. Significance was fixed at p < 0.05.

**RESULTS**

**Cell multiplication capacity**

The cell growth capacity in each group was increased with time. At 24h and 48h, the cell growth capacity was lower in the overexpressed mir-186 cells than in control, but cell growth capacity was better in mir-186 inhibition than in control cells (p < 0.05; Table 1).

**Colony-forming capacity of cells**

The potential for colony formation was lower in mir-186 overexpressed cells, when compared to that of controls, but it was lower in mir-186 inhibition cells than in control cells (p < 0.05; Figure 1).

![Figure 1: Colony forming capacity of cells in each group (CFU)](image)

**Table 1**: Comparison of cell growth capacity amongst the 3 groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Growth capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Control</td>
<td>0.21±0.05</td>
</tr>
<tr>
<td>mir-186 overexpression</td>
<td>0.22±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>mir-186 inhibition</td>
<td>0.21±0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>0.26</td>
</tr>
<tr>
<td>P-value</td>
<td>0.772</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05, vs. control; <sup>ab</sup>P < 0.05, vs. mir-186 overexpression cells

**Cell cycle distribution**

Relative to control cells, the G0/G1 phase cell population in mir-186 overexpressed cells was significantly higher, and cell population in S phase was significantly less. Relative to control, the cell population in G0/G1 phase in mir-186 inhibition cells was significantly lower, but there was a significantly higher S-stage cell population in mir-186 inhibition group (p < 0.05; Table 2).

**Jagged-1 mRNA expression**

There were significantly lower levels of Jagged-1 mRNA in mir-186 overexpressed cells than in control group, but they were significantly higher in the mir-186 inhibition group than in the control group (p < 0.05; Figure 2).

**Jagged-1 overexpression and miRNA-186 level**

The level of Jagged-1 mRNA was significantly higher in the Jagged-1 plasmid knockout cells than in the control and mir-186 overexpressed cells (Figure 3).

**Table 2**: Cell cycle distribution of cells in each group (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.16±10.24</td>
<td>38.41±8.79</td>
<td>9.43±2.15</td>
</tr>
<tr>
<td>mir-186 overexpression</td>
<td>67.85±11.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.84±4.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.31±1.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>mir-186 inhibition</td>
<td>45.22±8.67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>45.69±5.41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.09±2.01&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>25.470</td>
<td>64.530</td>
<td>0.180</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.834</td>
</tr>
</tbody>
</table>

<sup>a</sup>P < 0.05, vs. control; <sup>b</sup>p < 0.05, vs. mir-186 overexpression cells
DISCUSSION

Multiple myeloma (MM), a frequently-occurring cancer in the hematopoietic tissues, ranks 2nd in incidence in the world, and it seriously endangers the health and lives of the affected individuals [6]. In recent years, statistics have shown that the incidence of MM has been increasing yearly, especially amongst the younger population. Previous studies have revealed that genetic damage, tumor microenvironment and epigenetic regulation are crucial factors in MM development. The molecular mechanism involved in the epigenetic regulation of MM may provide new treatment strategies and early diagnosis, effective diagnosis and individualized treatment of the disease [7]. MicroRNAs (miRNAs) are one of the main ways of epigenetic regulation. Previous research revealed that unusual expression of miRNAs in MM is associated with chemotherapy resistance, tumor recurrence and changes in hematopoietic microenvironment.

MicroRNA-186 (mir-186) is significantly low expressed in a variety of tumor cells, and it inhibits tumor growth. A study reported that mir-186 was decreased significantly in non-small cell lung cancer cells, and overexpression of mir-186 inhibited the proliferation and migration of non-small cell lung cancer [8]. It has been shown that mir-186 inhibits the multiplication and invasiveness of carcinoma of the bladder, and inhibits the transformation of epithelial cells to mesenchymal cells, to certain extents [9]. In another study, mir-186 level was significantly enhanced in pancreatic ductal adenocarcinoma, thereby indicating proto-oncogene activity [10]. This further indicates that mir-186 may act as a proto-oncogene or tumor suppressor gene. In this study, the cell growth capacity of each group was increased with time. After 1 day and 2 days, the growth capacity in mir-186 overexpression cells was significantly decreased, but cell growth capacity of mir-186 inhibition group was significantly raised relative to that in control cells. The data suggest that when overexpressed, mir-186 significantly suppressed MM cell growth, and that mir-186 played the role of tumor suppressor gene.

Cell proliferation is an important biological feature of organisms, but abnormal proliferation is one of the main characteristics of tumor cells. Multiple myeloma cells with unlimited proliferation produce large quantities of monoclonal antibodies, suppress the proliferative capacity of normal polyclonal plasma cells and the secretion of polyclonal antibodies, and eventually lead to complications such as infection, bone injury, and renal function damage in MM patients [11]. In this study, there was significantly lower clone formation capacity in overexpressed mir-186 cells than in control cells, but it was higher in mir-186 inhibition cells than in control cells. Relative to control cells, the population of G0/G1 stage cells in mir-186 overexpression group was significantly higher, but the population S phase cells was significantly lower. In contrast, the number of cells in G0/G1 phase of the cell cycle was significantly lower in the mir-186 inhibition group than in control group, but S phase cell population was significantly higher. These data suggest that overexpressed mir-186 suppressed MM cell proliferation through inhibition of MM cell cloning capacity and regulation of the cell cycle. Similar results have been obtained in a previous study [12].

Jagged-1, a ligand of the Notch receptor, plays an important role in various hematopoietic systems. High expression level of Jagged-1 has been reported in MM cells [13]. It has been shown that the Notch signaling pathway promotes the proliferation of MM cells through the excitation ligand Jagged-1 [14]. This research has demonstrated that Jagged-1 mRNA was reduced in mir-186 overexpressed group, relative to the control group, but was significantly higher in mir-186 inhibition group than in the control.
group. The expression level of Jagged-1 was significantly higher in Jagged-1 knockout group than in control and mir-186 overexpressed groups. These data suggest that mir-186 directly targets Jagged-1. When overexpressed, mir-186 inhibited the multiplication and growth of MM cells through regulation of Jagged-1 mRNA. This is similar to results obtained in a previous study [15].

CONCLUSION

When overexpressed, mir-186 inhibits multiple myeloma cell growth by inhibiting its cloning capacity and regulating the cell cycle through mir-186-mediated regulation of Jagged-1 expression. However, there is need for more research to confirm the clinical benefits of therapies based on these findings.

DECLARATIONS

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Funding

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Xiaoqiang Zheng designed the study, supervised the data collection, and analyzed the data. Xiaoqiang Zheng prepared the manuscript for publication. Jinfeng Dong supervised the data collection, analyzed the data and reviewed the draft of the manuscript. Jinfeng Dong and Xiaoqiang Zheng contributed equally to this work and should be considered as co-first authors.

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