miR-301a promotes cell proliferation by directly targeting TIMP2 in multiple myeloma

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Abstract: Background: Multiple myeloma (MM) is a plasma cell malignancy characterized by clonal proliferation of plasma cells in the bone marrow and microRNAs play a crucial role in its tumorigenesis and development. The purpose of this study was to investigate the biological functions of miR-301a in MM. Methods: Quantitative real-time PCR was used to detect the expression level of miR-301a. Cell proliferation was assessed by MTT assay. Flow cytometry was performed to evaluate cell apoptosis and cell cycle distribution. Moreover, luciferase reporter assay and western blot were conducted to determine the potential target of miR-301a in MM cells. Results: MiR-301a is significantly up-regulated in MM clinical bone marrow samples and cell lines compared with normal controls. Gain-of-function and loss-of-function studies in MM cell line U266 showed that miR-301a acts as an oncogene in MM by promoting cell proliferation and inhibiting apoptosis. Furthermore, a tumor suppressor gene, tissue inhibitor of metalloproteinases-2 (TIMP2) was identified as a direct target of miR-301a and knockdown of TIMP2 could mimic the effect of miR-301a in MM. Conclusions: MiR-301a promotes cell proliferation and inhibits apoptosis by directly targeting TIMP2 in MM, and miR-301a might represent a novel molecular in MM and may provide helpful therapeutic strategies for MM treatment.

Keywords: miR-301a, multiple myeloma, TIMP2, proliferation

Introduction

Multiple myeloma (MM), which accounts for nearly 1% of all cancers and for approximately 13% of all hematologic malignancies, is a plasma cell malignancy characterized by clonal proliferation of plasma cells in the bone marrow (BM) [1, 2]. The incidence of MM in the United States is 4 to 5 cases per 100,000 individuals and is reported to be increasing annually in both males and females, especially in the elderly [3, 4]. The main clinical features of MM are osteolytic bone lesions, hypercalcaemia, renal insufficiency, suppressed immunoglobulin production and increased BM angiogenesis [5]. Although the application of novel therapeutic agents including proteasome inhibitors and immune modulators leads to significant improvements in response rates, drug resistance is common and MM remains an incurable disease with a median survival time of 7 to 8 years [6, 7]. Therefore, identifying some novel molecular biomarkers involved in the tumorigenesis and development of MM is essential to achieve any improvement of treatment outcome of MM patients.

MicroRNAs (miRNAs) are short (18-25 nucleotide) non-coding single-stranded RNAs which bind to target messenger RNAs (mRNAs), usually in their 3’-untranslated regions (UTR), and inhibit their expression by either inducing their degradation or repressing their translation [8]. MiRNAs are involved in myriad biological processes including differentiation, proliferation, metabolism and apoptosis [9]. It was reported that deregulated miRNA expression plays a pivotal role in MM tumorigenesis and is associated with its tumor progression, molecular subtypes, clinical staging, prognosis, and drug response [10]. Moreover, a number of miRNAs are identified as oncogenes or tumor suppressors in the carcinogenesis and development of MM [11-13], highlighting the significant roles of miRNAs in the development of MM.

An increasing number of studies have shown that miR-301a is upregulated in several types of human cancers and its overexpression is associated with tumor progression and poor prognosis. For example, Chen et al. reported that miR-301a is significantly increased in pancreatic cancer (PC) and promotes PC cell prolif-
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expression by directly inhibiting Bim expression [14]. Wang et al. found that miR-301a is significantly upregulated in gastric cancer and promotes cell proliferation and invasion by targeting RUNX3 [15]. Yu et al. showed that miR-301a was over-expression in breast cancer tissues compared with adjacent non-tumor tissues, and upregulation of miR-301a is associated with tumor characteristics and poor prognosis [16]. However, the expression pattern and biological significance of miR-301a in MM remain unknown.

In the present study, we primary explored the expression level of miR-301a in MM and its effect on MM cells proliferation. We fist examined the expression level of miR-301a in plasma cells from MM patients and healthy donors by using quantitative real-time PCR. Then, we performed in vitro assays to determine the effect of miR-301a on MM cells proliferation, apoptosis and cell cycle distribution. Moreover, we identified one potential direct target of miR-301a correlate with its effect on MM cells. Our results suggested that miR-301a is a novel molecular involved in the development of MM and may provide new strategies for MM treatment.

Materials and methods

Clinical samples and cells

Bone marrow (BM) samples were obtained from 26 patients with newly diagnosed MM and 18 healthy donors (HD). CD138+ plasma cells were isolated (purity > 90%) from the bone marrow samples using the AutoMACS automated separation system (Miltenyi-Biotec) according to the manufacture's instruction. Written informed consents were obtained from all patients and healthy donors, and this study was approved by the ethics committee of our hospital.

The human MM cell lines (RPMI-8226, U266, MM1S, H929) were obtained from The American Type Culture Collection (ATCC). All MM cell lines and normal plasma cells from healthy donors (nPCs) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin and 100 U/ml of penicillin (all from Invitrogen, USA) at 37°C with 5% CO₂.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from patient samples and cell lines using Trizol reagent (Invitrogen) following the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized from total RNA (1 μg) in 20 μl reactions, using reverse transcriptase (Epicentre, Madison, WI). Expression levels of mature miR-301a were measured using a TaqMan microRNA assay (Applied Biosystems) by normalizing to U6. For analysis of TIMP2, a SYBR Green PCR kit (TaKaRa, Dalian, China) was used to quantify the TIMP2 mRNA levels and β-actin mRNA was amplified as an internal control. The relative expression levels of miR-301a or TIMP2 were determined by using the 2-ΔΔCT method. The primers for miR-301a were: forward 5'-GGCAGTGCAATAGTATTGT-3' and reverse 5'-TGGTGTCGTGGAGTGCTG-3'; the primers for TIMP2 were: forward 5'-AGCACCACCAAGAAGAG-3' and reverse 5'-GTGACCCAGTCCATCCAG-3'.

Transfection

Has-miR-301a mimics (miR-301a), negative control (miR-con), has-miR-301a inhibitors (anti-miR-301a) and inhibitor negative control (anti-miR-con) were synthesized by RiboBio (China). Small interfering RNA (siRNA) against TIMP2 and negative control (si-NC) were designed by Genepharma (China). The sequences of the si-TIMP2 were: sense 5'-GGCCUGAGAAGGAUAGATT-3', antisense 5'-UCUAUAUCCUUCUGGCCTT-3'. Cell transfection was performed by using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer’s protocol. Transfection efficiency was monitored by qRT-PCR.

MTT assay

For cell proliferation analysis, the transfected U266 cells were measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay according to the manufacturer’s protocol. Briefly, transfected cells were seeded at a density of 3 x 10³ cells per well into 96-well plates and maintained at 37°C in 100 μl of culture medium. Next, 10 μl of MTT (0.5 mg/ml, Sigma) was added to each well at different time (0 h, 24 h, 48 h, 72 h, 96 h) after seeding and cells were further incubated for 4 h at 37°C. Then, after removed the medium, 100 μl of DMSO (Sigma) was added to solubilize the crystals and the absorbance was measured at 450 nm.

Flow cytometry

Cell apoptosis was evaluated by using FITC-Annexin V Apoptosis Detection Kit (BD Biosciences).
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A

B

Figure 1. MiR-301a is upregulated in MM. A. Relative expression level of miR-301a examined by qRT-PCR in MM patients and healthy donors. B. Relative expression level of miR-301a examined by qRT-PCR in MM cell lines and normal plasma cells (nPCs). Data are mean ± SD of three independent experiments. *P < 0.05.

Luciferase reporter assay

The 3'-UTR of TIMP2 containing the predicted miR-301a binding site or the mutant TIMP2 3'-UTR was amplified by PCR and subcloned into psiCHECK-2 vector within XhoI and NotI restriction sites (Promega, WI, USA). For luciferase reporter assay, U266 cells were cultured in 24-well plates and transfected with wild-type (WT) or mutated (Mut) 3'-UTR of TIMP2, together with Has-miR-301a mimics (miR-301a) or negative control (miR-con). The luciferase activity was measured 48 h post-transfection using the Dual Luciferase Reporter Assay (Promega) according to the manufacture’s manual.

Western blot

Cells were washed twice with PBS and lysed in RIPA buffer containing a protease and phosphatase inhibitor mixture (Roche). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were incubated with the primary antibody overnight at 4°C, followed by incubation with the secondary antibody for 1 h at room temperature. Then, protein bands were detected by enhanced chemiluminescence reagents ECL (Millipore). The primary antibody against TIMP2 was purchased from Abcam and the β-actin antibody was obtained from Santa Cruz Biotechnology.

Statistical analysis

The results from at least three independent experiments were expressed as means ± SD. Statistical analysis was performed using SPSS 17.0 software. The significance of differences between groups was compared by Student’s t test (two groups) or one-way analysis of variance (ANOVA, more than two groups). P < 0.05 was considered statistically significant.

Results

miR-301a is upregulated in MM

To explore the expression level of miR-301a in MM, we performed quantitative real-time PCR (qRT-PCR) analysis on the bone marrow samples of 26 MM patients and 18 healthy donors. Our data showed that the expression level of miR-301a is significantly higher in MM patients than that in healthy donors (Figure 1A). We further examined miR-301a expression level in MM cell lines and normal plasma cells (nPCs). Consistently, we observed that miR-301a is markedly upregulated in MM cell lines in com-
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Comparison with nPCs (Figure 1B). These observations indicate the important role of miR-301a in the development of MM.

**MiR-301a promotes proliferation and inhibits apoptosis in MM cells**

To determine the role of miR-301a in MM cells, miR-301a was overexpressed or inhibited in U266 cells by transfection with has-miR-301a mimics (miR-301a) or has-miR-301a inhibitors (anti-miR-301a) and confirmed by qRT-PCR (Figure 2A, 2B). MTT assays showed that the proliferation of U266 cells was increased when transfected with has-miR-301a mimics while decreased when transfected with has-miR-301a inhibitors (Figure 2C, 2D). Then, we supposed that the effect of miR-301a on U266 cells proliferation may correlated with cell apoptosis and cell cycle distribution, and flow cytometry was performed. Our results showed that the apoptosis rate was significantly decreased in U266 cells with miR-301a overexpression and significantly increased with miR-301a inhibition (Figure 2E, 2F). For cell cycle analysis, miR-301a overexpression resulted in fewer cells arrest in G1/S phase and miR-301a inhibition has the opposite effect on cell cycle distribution (Figure 2G, 2H). These results collectively suggest that miR-301a promotes MM cells proliferation by inhibiting cell apoptosis.

**TIMP2 is a direct target of miR-301a in MM cells**

TargetScan 6.2 was used to explore the targets of miR-301a in MM cells and TIMP2 was predicted to be a potential target of miR-301a (Figure 3A). To confirm that, luciferase reporter assay was conducted. We found that miR-301a overexpression only significantly suppressed the luciferase activity of the WT 3′-UTR of TIMP2 in U266 cells (Figure 3B). Furthermore, the results of western blot indicated that miR-301a overexpression suppressed TIMP2 expression while miR-301a inhibition promoted TIMP2 expression (Figure 3C, 3D).

**Knockdown of TIMP2 promotes proliferation and inhibits apoptosis in U266 cells**

Since miR-301a promotes proliferation and inhibits apoptosis in MM cells and TIMP2 was predicted to be a direct target of miR-301a, we transfected U266 cells with si-TIMP2 to determine whether down-regulation of TIMP2 had a phenocopy of miR-301a overexpression. The expression of TIMP2 was confirmed by qRT-PCR (Figure 4A). Results from MTT assay showed that knockdown of TIMP2 significantly promoted U266 cells proliferation (Figure 4B). Flow cytometry indicated
Figure 3. TIMP2 is a direct target of miR-301a in MM cells. A. TargetScan prediction of a binding site for miR-301a in the 3'-UTR region of TIMP2. B. The relative luciferase activity was measured in U266 cells after co-transfected with miR-301a or miR-con with wild-type (WT) or mutated (Mut) 3'-UTR of TIMP2. C, D. Protein levels of TIMP2 in U266 cells transfected with miR-301a or anti-miR-301a measured by western blot. Data are mean ± SD of three independent experiments. *P < 0.05.

Figure 4. Knockdown of TIMP2 promotes proliferation and inhibits apoptosis in U266 cells. A. Relative expression level of TIMP2 examined by qRT-PCR in U266 cells after transfection with si-TIMP2 or si-NC. B. Cell proliferation of transfected U266 cells measured by MTT assay. C. Apoptosis rate of transfected U266 cells analyzed by flow cytometry. D. Cell cycle distribution of transfected U266 cells assessed by flow cytometry. Data are mean ± SD of three independent experiments. *P < 0.05.
that knockdown of TIMP2 resulted in decrease of apoptosis rate and fewer cells arrest in G1/S phase (Figure 4C, 4D). These results indicate that the effect of miR-301a on MM cells is involved in its suppression of TIMP2.

Discussion

Multiple myeloma (MM) is an age-dependent monoclonal tumor of BM plasma cells (PCs) and MM cells differ from healthy PCs because they retain the potential for a low rate of proliferation [17]. So, identifying some novel molecules involved in MM cells proliferation may lead to a better understanding of the pathogenesis and some new therapeutic strategies in MM. In recent years, numerous miRNAs have been reported to be associated with cell proliferation in various types of human cancers. For instance, miRNA-506 was showed to suppress cell proliferation by targeting FLOT1 in renal cancer cells [18]. MiRNA-449a was reported to inhibit proliferation and induce apoptosis by directly repressing E2F3 in gastric cancer [19]. However, Lu et al. indicated that miRNA-24-3p promotes cell proliferation and inhibits apoptosis in human breast cancer by targeting p27kip1 [20]. Although miRNAs were widely studied in many types of human cancers, the investigation of the roles of miRNAs in MM is largely lacking.

In this study, we observed that the expression level of miR-301a is significantly increased in MM clinical bone marrow samples and cell lines compared with normal controls, which indicates that miR-301a may play important role in the development of MM. Then, we conducted in vitro assays to investigate the biological functions of miR-301a on MM cells. We found that overexpression of miR-301a promotes cell proliferation, inhibits apoptosis and induces cell cycle transition from G1/S phase in MM cells. On the contrary, inhibition of miR-301a performed the opposite effect in MM cells. These findings suggest that miR-301a may function as a novel oncogene in MM and contribute to tumor progression of MM.

In addition, we identified a tumor suppressor gene, TIMP2, as a direct target of miR-301a by luciferase reporter assay and western blot. TIMP2 is a member of the tissue inhibitor of metallopeptidases (TIMPs) family, which can inhibit the activity of matrix metallopeptidases (MMPs) by binding with a 1:1 stoichiometry to the active site [21]. It was reported that TIMP2 suppress tumor cell proliferation and metastasis in many cancers including gastric, pancreatic and breast cancers [22-24]. In recent years, TIMP2 was showed to be regulated by several miRNAs in cancers. For example, Dai et al. observed that miR-200b represses TIMP2 expression at both the messenger RNA and protein levels in human endometrial cancer cell line HEC-1A cells [25]. Zhu et al. demonstrated that miR-106a regulates gastric cancer cells proliferation, migration and invasion by targeting TIMP2 [26]. Besides, Yang et al. found that miR-221/222 regulate cell proliferation, the cell cycle and apoptosis, in addition to, invasion, metastasis, and angiogenesis in glioma cell lines by targeting TIMP2 [27]. Here, we showed that TIMP2 is a direct target of miR-301a in MM, and demonstrated that knockdown of TIMP2 promotes proliferation and inhibits apoptosis in U266 cells. These results indicate that inhibition of TIMP2 expression could mimic the effect of miR-301a in MM.

Taken together, we demonstrated for the first time that miR-301a is significantly upregulated in MM clinical bone marrow samples and cell lines compared with normal controls. Moreover, overexpression of miR-301a promotes cell proliferation and inhibits apoptosis by directly targeting TIMP2 in MM. These results suggest that miR-301a may play as an oncogene in MM and might provide helpful therapeutic strategies for MM treatment in the future.

Disclosure of conflict of interest

None.

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