Original Article
MicroRNA-497 suppresses cell proliferation and induces apoptosis through targeting PBX3 in human multiple myeloma

Tianhua Yu1, Xuanhe Zhang2, Lirong Zhang3, Yali Wang1, Hongjuan Pan1, Zhihua Xu4, Xiaochuan Pang5

Departments of 1Blood Transfusion, 3Pathology, 4The 3rd Neurology, China-Japan Union Hospital of Jilin University, 126 Xiantai Street, Changchun 130033, China; 2Shihezi University, 221 North Fourth Road, Shihezi 832003, China; 5Department of Clinical Laboratory, The First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, China

Received November 20, 2016; Accepted November 29, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: Aberrant expression of microRNA-497 (miR-497) is implicated in development and progression of multiple types of cancers. However, the biological function and underlying mechanism of miR-497 in multiple myeloma (MM) remains unclear. Thus, we studied the potential biological roles of miR-497 in MM. The expression of miR-497 was examined in multiple myeloma and normal plasma cells by qRT-PCR. Biological functions of miR-497 were analyzed using cell proliferation, colony formation, cell cycle, apoptosis and luciferase assays in vitro, as well as via tumorigenicity in vivo analysis. Here, we observed reduced expression of miR-497 in MM plasma samples and cell lines. Ectopic expression of miR-497 dramatically suppressed cell proliferation and clonogenicity, as well as induced cell arrest at G0/G1 stage and apoptosis in vitro. Mechanistic investigation assays showed that Pre-B-cellleukemia transcription factor 3 (PBX3) was a novel and direct downstream target of miR-497. Interestingly, overexpression of PBX3 partially reverted the effect of miR-497 in MM cells. In xenograft model, overexpression of miR-497 inhibited tumorigenicity by repressing PBX3. These findings collectively suggested that miR-497 functioned as tumor suppressor in MM by directly targeting PBX3, supporting its utility as a novel and potential therapeutic agent for MM therapy.

Keywords: miR-497, multiple myeloma, PBX3, proliferation, apoptosis

Introduction
Multiple myeloma (MM) is a plasma cell malignancy characterized by the accumulation of clonal malignant plasma in bone marrow [1]. Although in the recent years novel research platforms and new therapeutics have been adopted, MM remains largely incurable by current therapeutic strategies [2, 3]. Therefore, it is urgently need to develop of novel treatment options for the treatment of patients with multiple myeloma.

MicroRNAs (miRNAs) are a class of small non-coding RNAs approximately 22 nucleotides in length that function as negative regulators of protein-coding genes by hybridizing to the sequences usually located at the 3' -untranslated region (UTR) of coding transcripts [4]. miRNAs are known to be involved in various biological processes including inflammation, embryonic development, hematopoiesis, immune responses and tumorigenesis [5, 6]. Growing evidence indicates that miRNAs play important roles in initiation, development, and progression of many cancers through the regulation of cell proliferation, cycle, apoptosis, differentiation and invasion [7, 8]. Deregulated expression of miRNAs has also been reported to be associated with high-risk multiple myeloma [9-11], thus eliciting interest for these molecules as diagnosis marker and antitumor therapeutic agents.

miR-497, located on human chromosome 17p13.1 [12], found in almost all human organs and tissues, such as the brain [13], breast [14], lung [15], gastric [16], and blood and so on [17]. The dysregulation of miR-497 has been reported in various tumor types [18]. For example, Han et al found that that reduced miR-497
miR-497 inhibits MM progression by targeting PBX3

expression enhanced cell proliferation, migration, and invasion by increasing MIF expression and MMP9 activity in ERα negative breast cancer [19]. Zhang et al showed that microRNA-497 suppressed the proliferation, migration and invasion of human bladder transitional cell carcinoma cells by targeting E2F3 [20]. Ge et al reported that overexpression of miR-497 in human osteosarcoma cells suppressed cell proliferation, colony formation, migration and invasion, and induced cell apoptosis and cell arrest at the GO/G1 phase of the cell cycle, as well as inhibited tumor growth in a nude mouse model [21]. However, the expression patterns as well as specific functions and underlying mechanisms of miR-497 in MM progression is still not known. Thus, we measured miR-497 expression in MM plasma and cell lines and studied biological functions and a possible molecular basis of miR-497 in MM.

Materials and method

Patients’ plasma preparation and cell lines

Plasma samples were obtained from patients with multiple myeloma and normal donors from China-Japan Union Hospital of Jilin University (Changchun, China). A total of 60 serum samples including 40 newly diagnosed symptomatic MM patients and 20 healthy donors were enrolled in this study. This study was approved by the Ethics Committee of Jilin University (Changchun, China), and written informed consent was obtained from all participants.

Multiple myeloma cell lines: H929, MM1S, and RPMI8226 (all from American Type Culture Collection (ATCC, VA, USA) and normal plasma cells (nPCs) were grown in RPMI-1640 medium (GibcoBRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GibcoBRL) at 37°C in a humidified air atmosphere with 5% CO2.

RNA extraction and real-time quantitative RT-PCR

Total RNA or miRNA was extracted using RNeasy kit (Qiagen, USA) or miRNA Easy kit (Qiagen) according to manufacturer’s instructions. RNA purity and concentration was determined with a spectrophotometer (ND-1000; Nano-Drop Technologies). cDNAs were synthesized using miScript II RT kit (QIAGEN). Quantitative PCR were performed using miScript SYBR Green PCR kit (QIAGEN) in an ABI-7900HT machine following manufacturer’s instructions. The primers of miR-497 and U6 (as an internal control) were used in this study from QIAGEN. Relative miRNA expression normalized to U6 was carried out using the 2-ΔΔCt method. The primers of miR-497 and U6 (as an internal control) were used in this study from QIAGEN. The primers of PBX3 and GAPDH (as a internal control) were used in this study as previously described [22]. Relative PBX3 mRNA expression normalized to GAPDH was performed using 2-ΔΔCt method.

Cell transfection

miR-497 mimic or corresponding negative control (miR-NC) were brought from Ambion (Carlsbad, CA, USA). siRNAs against PBX3 (si-PBX3) and the corresponding scramble control (si-NC) were purchased from GenePharma (Shanghai, China). PBX3 code sequence was amplified by PCR and inserted into pCDNA3.1 vector, and named as pCDNA3.1-PBX3 that was used as PBX3 overexpression plasmid. These molecular production were transfected into the MM1S cells using oligofectamine-2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol.

Cell proliferation, colony formation, cycle and apoptosis assays

Cell proliferation was measured using an 3-(4, 5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide (MTT) assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Absorbance was measured at 570 nm using a spectrophotometer once a day for 3 consecutive days. The experiments were performed in triplicate and repeated three times.

For colony formation assay, transfected cells were seeded into a 6-well plate at density of 500 cells/well and cultured for two weeks in RPMI 1640 medium containing 10% FBS. Colonies were fixed and stained with 0.1% crystal violet (1 mg/ml), and counted. All experiments were performed in triplicate wells.

For cell cycle assay, briefly, cells were harvested 48 h after transfection, and then were fixed in 70% cold ethanol overnight at 4°C. Cells were stained with 50 μg/mL propidium iodide...
miR-497 inhibits MM progression by targeting PBX3

Cell cycle was determined with a FACS Calibur system (BD Biosciences) and data were analyzed with ModFit 3.0 software (BD Biosciences).

Cell apoptosis were determined 48 h after transfection using an Annexin V-FITC/propidium iodide apoptosis detection kit (BD Biosciences) in a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest software (BD Biosciences).

Luciferase reporter assay

The potential miR-497-binding sites in PBX3 3' untranslated region (3'UTR) were predicted by TargetScan7.1 and miRanda (www.microRNA.org). Sequences containing wild-type or mutant seed region of PBX3 were synthesized and inserted into XbaI site of a pGL3-basic vector (Promega) and termed PBX3-Wt-3'UTR and PBX3-Mut-3'UTR, respectively. Cells in 24-well plates were co-transfected with miR-497/miR-NC, PBX3-Wt-3'UTR/PBX3-Mut-3'UTR, and pRL-TK vector using Lipofectamine 2000. Luciferase activity levels were measured using a dual-luciferase reporter assay system following the manufacturer's instructions (Promega).

Western blot analysis

Total protein was extracted from cell pellets using CytoBuster Protein Extraction Reagent (Merck Millipore, Darmstadt, Germany). Total protein concentration were measured with a BCA Protein Assay Kit (Beyotime Biotechnology, Jiangsu, China). Equal amounts of proteins (30 μg) from each sample was separated via 10% SDS-PAGE and transferred onto the polyvinylidene difluoride membrane (PVDF, Sigma, USA). After blocked by incubation in 5% nonfat dry milk in PBST (0.05% Tween-20 in PBS), membranes were probed with antibody against PBX3 and GAPDH (Signalway Antibody, Baltimore, MD, USA) at 4°C overnight. Then the membrane was incubated with the goat-anti-mouse IgG conjugated to horseradish peroxidase antibody (Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h. Proteins band were visualized with chemiluminescent detection system (ECL, Thermo Scientific, Rockford, IL, USA) Plus Western Blot Detection Reagents (GE Healthcare, USA).

In vivo tumorigenicity

Stable miR-497 expressing cells and miR-NC expressing cells were generated by transfected with miR-497 mimic or miR-NC. MM1S cells (2 × 10^6) stably expressing miR-497 or miR-NC were injected subcutaneously into the dorsal right flank of 6-week-old female Balb/c nude mice (N = 5 mice/group, Animal Center of Jilin University, Changchun, China). Tumors volume were measured once every week from the first injection until mice sacrifice using the following formula: V = (L × W^2)/2 (V, volume; L, length; W, width of tumor). After 5 weeks, the mice were sacrificed, and the tumors were removed, weighted and stored at -80°C for further analysis. All animal experiments were performed according to the animal experimental guidelines of Jilin University.
miR-497 inhibits MM progression by targeting PBX3

Figure 2. miR-497 inhibits proliferation, colony formation, induced cell cycle at G0/G1 phase and apoptosis of MM cells. A. The expression level of miR-497 was detected in MMS1 cells after transfected with miR-497 mimic or miR-NC by qRT-PCR. B-E. Cell proliferation, colony formation, cell cycle and apoptosis were determined in MMS1 cells after transfected with miR-497 mimic or miR-NC. All *P<0.05, **P<0.01.
miR-497 inhibits MM progression by targeting PBX3

**Figure 3.** PBX3 is a direct target of miR-497 in MM cells. A. Schematic of the construction of wild-type or mutant-type PBX3 3’UTR vectors is shown. Wt: Wild-type; Mut: Mutant-type. B. Relative luciferase activities were analyzed in MMS1 cells cotransfected with wild-type or mutant-type PBX3 3’UTR vector and miR-497 or miR-NC. Wt: Wide-type; Mut: Mutant-type. C, D. PBX3 expression on mRNA level and protein level was determined in MMS1 cells after transfected with miR-497 mimic or miR-NC. GAPDH was used as loading control. All *P<0.05, **P<0.01.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., city, state) and The SPSS 19 software package (SPSS Inc; Chicago, IL, USA). A Student’s t-test was used to evaluate the significance of differences between two groups. ANOVA was employed to the significance of differences more than two groups. All data are presented as the mean ± SD (standard deviation) from at least three times independently experiment. *P<0.05 was considered statistically significant.

**Results**

**miR-497 is downregulated in MM tissues and cell lines**

We first examined the basal level expression of miR-497 in plasma cells from sixty MM patients and twenty normal donor samples (nPCs). qPCR analysis revealed that the expressions of miR-497 were significantly lower in MM plasma sample than those normal plasma cell samples (Figure 1A). Then, we evaluated the expression levels of miR-497 in four MM cell lines (H929, MM1S, RPMI8226 and U266) and normal donor plasma cell samples (nPCs). As shown in Figure 1B, miR-497 was significantly downregulated in four MM cells compared with nPCs. MM1S cell line exhibiting the lowest miR-497 levels was selected for further study (Figure 1B). These results suggest that low expression of miR-497 may play a role in progression of MM.

**miR-497 inhibits MM cell proliferation and induces apoptosis in vitro**

To investigate possible biological function of miR-497 in MM, miR-497 was ectopically overexpressed in MM1S cells, and the effects on cell proliferation examined. As presented in Figure 2A, miR-497 expression was significantly increased in MM1S cells transfected with miR-497 mimic, compared with cells transfected with miR-NC. MTT data showed that upregulation of miR-497 by transfection with miR-497 significantly impaired cell proliferation of MM cells (Figure 2B). The colony formation assay disclosed a similar trend, with significantly reduced clonogenic ability of cells transfected with miR-497 mimic, compared with cells transfected with miR-NC (Figure 2C). Since cell proliferation was closely associated with cell cycle, thus FACS analysis was performed to examine the effects of miR-497 on cell cycle distribution. A higher percentage of cells transfected with miR-497 were present in the G0/G1 phase and a lower percentage in the S phase, compared with those transfected with miR-NC (Figure 2D). We next investigated the role of miR-497 in apoptosis by performing Annexin V/propidium iodide (PI) staining. The proportion of apoptotic cells was significantly increased in cells transfected with miR-497 mimic, compared with cells transfected with miR-NC (Figure 2E). Our results collectively indicate that miR-497 inhibited cell growth and induced apoptosis in MM cells.
miR-497 inhibits MM progression by targeting PBX3

PBX3 is a direct target of miR-497 in MM cells

To further investigate the molecular mechanisms underlying miR-497-mediated regulation of MM cell growth, we searched for potential targets of miR-497 using TargetScan and miRanda algorithms. Among the predicted genes, PBX3 was selected as a candidate target of miR-497 (Figure 3A) in view of its involvement in the proliferation and survival of cancer cells [23, 24]. To further confirm PBX3 as a direct target of miR-497, luciferase activity assays were carried out. The result showed that overexpression of miR-497 significantly reduced the luciferase activity of cells transfected with wild-type PBX3-3'UTR, but had no effect on mutant-type PBX3-3'UTR (Figure 3B), validating binding of miR-497 to PBX3 3'UTR. Subsequent experiments demonstrated that miR-497 overexpression significantly inhibited PBX3 expression at both mRNA and protein levels (Figure 3C and 3D). Based on the collective results, we propose that miR-497 inhibits PBX3 expression in MM by directly targeting its 3'UTR region.

miR-497 regulates cell proliferation and apoptosis via the suppression of PBX3

The above results suggested that PBX3 was a direct target of miR-497 in MM cells, thus, we wonder whether miR-497 exerted its function through the regulation of PBX3 in MM cells. siRNA targeting PBX3 (si-PBX3) was transfected into MM1S cells to knockdown endogenous PBX3 expression, and qRT-PCR and western blot analyses were performed to confirm the reduced PBX3 levels (Figure 4A and 4B). In addition, we also showed that the knockdown of PBX3 significantly inhibited the proliferation, colony formation, and induce cell arrest at G0/G1 phase and apoptosis of the MM1S cells, which resembled the suppressive effects of miR-497 overexpression in MM cells (Figure 4C-F). To further elucidate whether PBX3 is a functional target of miR-497 in MM cells, we performed gain-of-function analyses by transfection of PBX3 plasmids lacking 3'UTR into miR-497-expressing MM1S cells. qRT-PCR and western blot analyses showed that MM1S cells transfected with PBX3 overexpression plasmid could restore PBX3 expression (Figure 5A and 5B). The result also showed that the restoration of PBX3 expression was able to partially counteract the effects of miR-497 on cell proliferation, colony formation, cycle and apoptosis in MM cells (Figure 5C-F). These data indicated that miR-497 exerts suppressive role in MM by repressing PBX3.

MiR-497 inhibits tumor growth in vivo by targeting PBX3

To further determine whether miR-497 inhibits tumor growth in vivo, MM1S cells transfected
miR-497 inhibits MM progression by targeting PBX3

Figure 5. Reintroduction of PBX3 reverses the effects of miR-497 in MM cells. A, B. PBX3 expression on mRNA level and protein level was determined in MMS1 cells transfected with miR-497 mimic or miR-NC and with/without overexpression PBX3 plasmid. C-F. Cell proliferation, colony formation, cycle and apoptosis were determined in MMS1 cells transfected with miR-497 mimic or miR-NC and with/without overexpression PBX3 plasmid. All *P<0.05, **P<0.01.

Discussion

Abnormal expression of various miRNAs has been reported to be involved in the development and progression of MM, acting as oncogenes or tumor suppressors [9-11]. For example, Saha et al reported that that miR-29a functioned as a tumor suppressor that played an important role during PRIMA-1met-induced apoptotic signaling by targeting c-Myc [25]. Yang et al showed that miR-137 and miR-197 expression was downregulated in MM plasma and cell lines, and could induce apoptosis and suppress tumorigenicity by targeting MCL-1 in MM [26]. Liu et al found that ectopic expression of miR-186 significantly inhibited MM cell growth in vitro and in vivo, and induced cell cycle G0/G1 arrest by repressing Jagged1 [27].

The present study showed that miR-497 expression was significantly lower in multiple myeloma cell lines and multiple myeloma plasma compared with normal plasma cells. Transfection of miR-497 mimic in MM1S cells significantly inhibited MM cell growth in vitro and in vivo, and induced cell cycle G0/G1 arrest by repressing Jagged1 [27]. The present study showed that miR-497 expression was significantly lower in multiple myeloma cell lines and multiple myeloma plasma compared with normal plasma cells. Transfection of miR-497 mimic in MM1S cells significantly inhibited cell proliferation and colony formation, and induced cell cycle at G0/G1 phase and apoptosis in vitro, as well as suppressed tumorigenesis in vivo, which is in accordance with previous reports of other tumor types [19-21]. To the best of our knowledge, this is the first study to report the biological function of miR-497 in MM, which contribute to improve our understanding of the mechanisms underlying MM progression and development.

miR-497, a member of miR-15 family (miR-15a, miR-15b, miR-16-1/2, miR-195, miR-424 and
miR-497 inhibits MM progression by targeting PBX3

miR-497 has been reported to be downregulated and act as a tumor suppressor in multiple cancers such as gastric cancer [16], breast cancer [14], non-small cell lung cancer [15], prostate cancer [28] and ovarian cancer [29]. On the contrary, in glioma [30], leukemia [31] and colorectal cancer [32], miR-497 expression was upregulated and function as oncogene. However, the detail biological function and underlying mechanism of miR-497 in MM remains unclear. Thus, to validate the potential role of miR-497 in the development and progression of MM, in vitro cell and in vivo animal experiments were carried out. Data show that miR-497 expression was downregulated in MM plasma and cell lines, and that overexpression of miR-497 can inhibit cell proliferation, colony formation, induce cell cycle at G0/G1 phase and apoptosis in vitro, and can suppress tumorigenesis in vivo. These findings suggested that miR-497 function as tumor suppressor in MM.

Several target genes regulated by miR-497 in different cancers have been reported [15, 16, 18-20]. Here, we showed that PBX3 is a direct target of miR-497 as evidenced by the fact that ectopic expression of miR-497 reduced luciferase activity of the wide-type PBX3-3’UTR and miR-497 downregulated PBX3 expression on mRNA level and protein level. PBX3 (Pre-B-cellleukemia transcription factor 3) belongs to the human PBX family of transcription factors [33]. Previous studies indicated that PBX3 played crucial role in carcinogenesis [23, 24]. The proto-oncogene PBX3 has been showed to be upregulated in gastric cancer [34], colorectal cancer [23], prostate [24] and leukemic [35]. Moreover, PBX3 was regulated in different cancer by other miRNAs, such as miR-33a-3p [22] and miR-495 [36]. Of none, recently a study showed that miR-320a induces a marked reduction in MM cell proliferation and an increase in apoptosis via direct binding and inhibition of PBX3 [38]. Here, we identified PBX3 as a direct target of miR-497 in MM. We also found that downregulation of PBX3 phenotypically copied miR-497-induced phenotypes, whereas overexpression of PBX3 reversed the effects of miR-497 on cell proliferation, colony formation, cycle, apoptosis in MM cells. In a mouse xenograft model, miR-497 overexpression inhibited tumorigenicity by repressing PBX3. These results suggest that miR-497 exerts tumor suppressor roles in MM by targeting PBX3.

Figure 6. miR-497 suppresses tumor growth in vivo. A. Growth curves for tumor volumes in xenografts of nude mice. B. Photographs of tumor tissues. C. Tumor tissues weight. D. The expression level of miR-497 was detected in tumor tissues from different group using qRT-PCR. E, F. PBX3 expression on mRNA level and protein level was measured in tumor tissues from different group. All *P<0.05, **P<0.01.
miR-497 inhibits MM progression by targeting PBX3

497 may function as tumor suppressor in MM. These findings implied that miR-497 might be a potential therapeutic target for MM.

Disclosure of conflict of interest

None.

Address correspondence to: Zhihua Xu, Department of The 3rd Neurology, China-Japan Union Hospital of Jilin University, 126 Xiantai Street, Changchun 130033, China. E-mail: xuzhihua495@sina.com; Xiaochuan Pang, Department of Clinical Laboratory, The First Hospital of Jilin University, 71 Xinmin Street, Changchun 130021, China. E-mail: pangxiao-ochun1119@126.com

References


miR-497 inhibits MM progression by targeting PBX3


