

## Original Article

# MiR-20a promotes cell proliferation by targeting SRCIN1 in human multiple myeloma

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**Abstract:** MicroRNAs (miRNAs) play an essential role in cancer development. While miR-20a has been shown to play important roles in several cancers, the specific functions of miR-20a in the pathogenesis of multiple myeloma (MM) remain unknown. In the present study, we focused on the influence of the miR-20a on the MM progression. Our results showed that miR-20a was significantly upregulated in MM cell lines and patient MM cells. Upregulated expression of miR-20a significantly promoted NCI-H929 cells proliferation, decreased NCI-H929 cells population in G0/G1 phase and inhibited NCI-H929 cells apoptosis. Furthermore, SRCIN1 was identified as a direct target of miR-20a and overexpression of SRCIN1 rescued cells from miR-20a induced MM cell proliferation. Overall, our data collectively indicated that miR-20a acted as a tumor oncogene and that its upregulation in cancer might contribute to MM progression, indicating that miR-20a could be a potential therapeutic target in the treatment of MM.

**Keywords:** MicroRNAs, miR-20a, multiple myeloma, proliferation

## Introduction

Multiple myeloma (MM) is a lethal disease of antibody-secreting bone marrow plasma cells (PCs) that accounts for 10% of all hematological neoplasia with worldwide increasing incidence [1]. MM usually develops from an asymptomatic premalignant stage called monoclonal gammopathy of undetermined significance (MGUS). This benign condition can progress to myeloma or related malignancies at a rate of ~1% per year [2]. In a fraction of patients, tumor could also occur in extramedullary sites such as blood, a condition called plasma cell leukemia (PCL) [3]. Despite considerable improvements of patient survival and treatment options over the past few decades, MM remains largely incurable [4]. Thus, identification of critical carcinogenic pathways as well as effective diagnostic and therapeutic targets for this disease remains an urgent medical need.

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 [5, 6]. It has been established that miRNAs might serve as new therapeutic strategies for cancers, as they can act as tumor suppressors or as oncogenes [7]. For example, Yang et al showed that miR-506 was down-regulated in clear cell renal cell carcinoma and inhibited cell growth and metastasis via targeting FLOT1 [8]. Tsukamoto et al suggested that miR-375 was downregulated in gastric carcinomas and regulated cell survival by targeting PDK1 and 14-3-3zeta [9]. Li et al found that miR-150 promoted cervical cancer cell growth and survival by targeting FOXO4 [10]. Those studies suggested that the important roles of miRNAs in the tumor progression.

In the present study, we explored the expression levels of miR-20a in human MM cells and MM cell lines, followed by functional analyses in human MM cell lines. Our findings indicated that miR-20a might function as a tumor oncogene via downregulating SRCIN1 expression, further supporting its development as a potential therapeutic target in MM progression.

## Materials and methods

### *Patient samples*

23 MM and 15 healthy control samples were collected from 2014 to 2015 at Department of Hematology, The Central Hospital of Xinxiang. Plasma cells were isolated from bone marrow samples as described previously [11]. The collection of human tissue samples was approved and supervised by the Ethics Committee of The Central Hospital of Xinxiang. Written informed consent obtained from all patients.

### *Cell culture and transfection*

Human MM cell lines (OPM-2, NCI-H929, and U266) and normal plasma cells (nPCs) were obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 (Gibco) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere. The miR-NC or miR-20a mimics (RiboBio) were inoculated in NCI-H929 using lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. The transfection efficiency of the cells was determined by qRT-PCR.

### *Quantitative real-time PCR*

Total RNA from tissue samples and cell lines was harvested using the miRNA Isolation Kit (Ambion). Expression of mature miRNAs was assayed using Taqman MicroRNA Assay (Applied Biosystems) specific for hsa-miR-20a. Briefly, 10 ng of total RNA was reverse transcribed to cDNA with specific stem-loop real-time (RT) primers. Quantitative real-time PCR was performed by using an Applied Biosystems 7900 Real-time PCR System and a TaqMan Universal PCR Master Mix. All the primers were obtained from the TaqMan miRNA Assays. Small nuclear U6 snRNA (Applied Biosystems) was used as an internal control.

### *Plasmid construction and transfection*

For rescue of the proliferative effect of miR-20a, we used a pcDNA-SRCIN1 open reading frame lacking the entire 3'UTR of endogenous SRCIN1. For luciferase assays, the SRCIN1 3'UTR sequence was inserted into the pGL3 luciferase reporter vector (Promega). Target

sites were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Transfection of plasmids was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

### *MTT assay*

For cell viability assays, transfected NCI-H929 cells were seeded in a 96-well plate at a density of  $1 \times 10^3$ /well. After incubation for 24, 48, 72 or 96 h at 37°C in a humidified incubator, 20  $\mu$ l MTT (5 mg/ml in PBS) was added to each well, and the cells were incubated for a further 4 h. After removal of the medium, 150  $\mu$ l DMSO was added to each well. The absorbance was recorded on a microplate reader at a wavelength of 540 nm.

### *Cell cycle analysis*

At 48 h after transfection, NCI-H929 cells were harvested, washed with PBS and fixed in 70% ethanol at 4°C overnight. After fixation, cells were washed twice with PBS before resuspension in propidium iodide/RNase A solution (5  $\mu$ g/ml propidium iodide and 100 mg/ml RNase A). Cells were incubated with propidium iodide at room temperature in the dark for 1 h. Stained cells were analyzed by flow cytometry for light-scattering properties and for DNA content, using a FACScan flow cytometer (BD Biosciences).

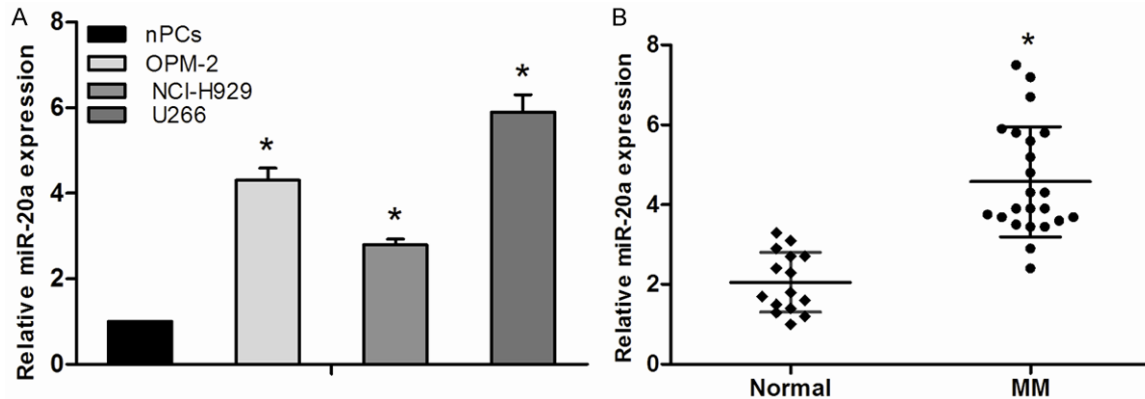
### *Apoptosis assay*

To determine cell apoptosis, the Annexin V-FITC Apoptosis Detection kit (BD Biosciences) and PI was used to assess the apoptotic effect of miR-20a. The transfected NCI-H929 cells were resuspended in 500  $\mu$ l cold Binding Buffer with 1.25  $\mu$ l Annexin V-FITC, and incubated for 15 minutes at room temperature in the dark. Cells were resuspended in 500  $\mu$ l cold Binding Buffer with 10  $\mu$ l PI, incubated for 4 h and analyzed by flow cytometry (BD Bioscience).

### *Luciferase reporter assay*

HEK-293 cells were seeded in a 24-well plate with a density of  $2 \times 10^5$  cells/per well, and co-transfected with Wt 3'UTR or Mut 3'UTR and miR-20a mimics or miR-NC. Cells were collected 48 h after transfection, and firefly and renilla luciferase activities were measured

## MiR-20a target SRCIN1 in MM



**Figure 1.** miR-20a is up-regulated in human multiple myeloma (MM) cell lines and clinical samples. A. Relative expression of miR-20a was examined by qRT-PCR in three MM cell lines (OPM-2, NCI-H929, and U266) and normal plasma cells (nPCs). B. qRT-PCR analysis of miR-20a expression in 23 MM samples and 15 healthy control samples. U6 was used as a control. Data were drawn from three independent experiments, \*P<0.05.

with the Dual-Luciferase Reporter Assay System (Promega).

### Western blot

After 48 h of transfection, the total proteins from the cells were extracted by RIPA lysis buffer with 1 mM proteinase inhibitor PMSF for 15 min on ice. The concentration was determined using the BCA protein assay kit (Santa Cruz). For western blotting, 30  $\mu$ g protein in each well was detached by 10% SDS-PAGE, and then transferred onto PVDF membranes. The membranes were blocked for 1 h in TBST with 5% nonfat milk at room temperature, and incubated with primary antibodies at 4°C overnight. The blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized with enhanced chemiluminescence reagents (Pierce).

### Statistical analysis

All data were presented as mean  $\pm$  SD and analyzed by using SPSS 16.0. Two-tail Student's t test and ANOVA were performed to determine the differences. P<0.05 was considered statistically significant.

## Results

### miR-20a was increased in MM cell lines and patient MM cells

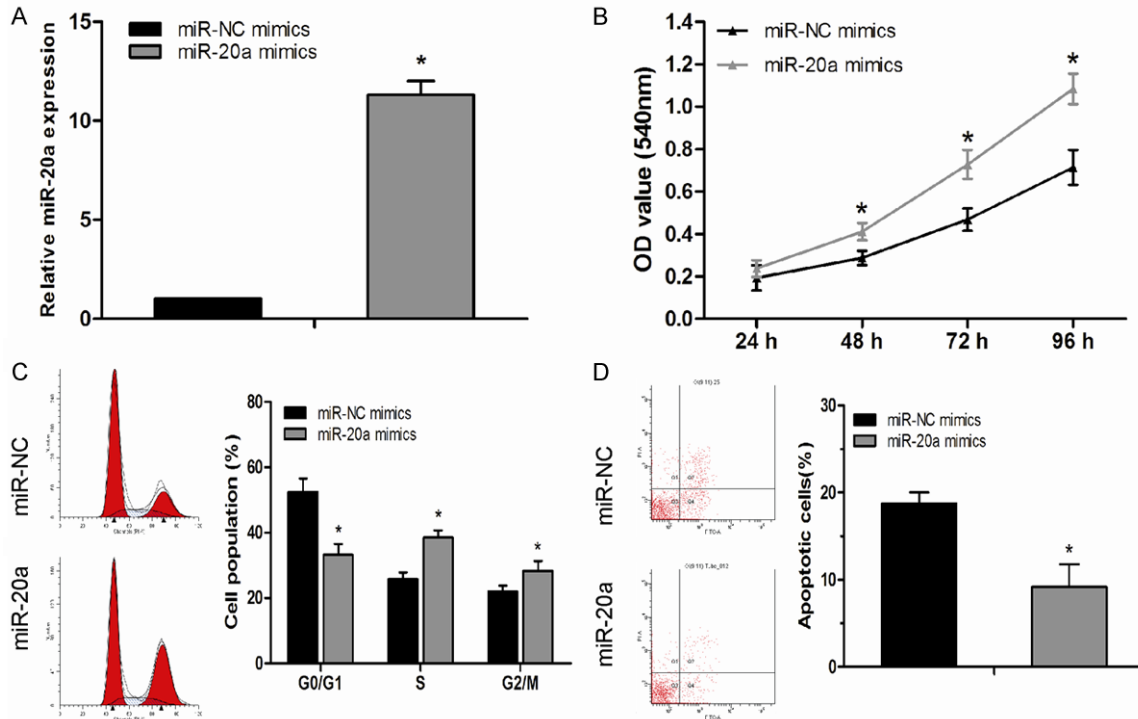
To explore miR-20a expression in MM, we initially examined expression patterns in a series of human mammary tumor cell lines via qRT-

PCR. Our data showed that miR-20a was up-regulated in all three MM cell lines (OPM-2, NCI-H929, and U266) compared with nPCs cells (Figure 1A, P<0.05). Furthermore, we investigated miR-20a expression in patient MM cells and plasma cells from healthy donors, our findings revealed that miR-20a expression was up-regulated to a significant extent in MM samples relative to the healthy donors samples (Figure 1B, P<0.05). Our data suggested that overexpression of miR-20a might play an important role in MM progression.

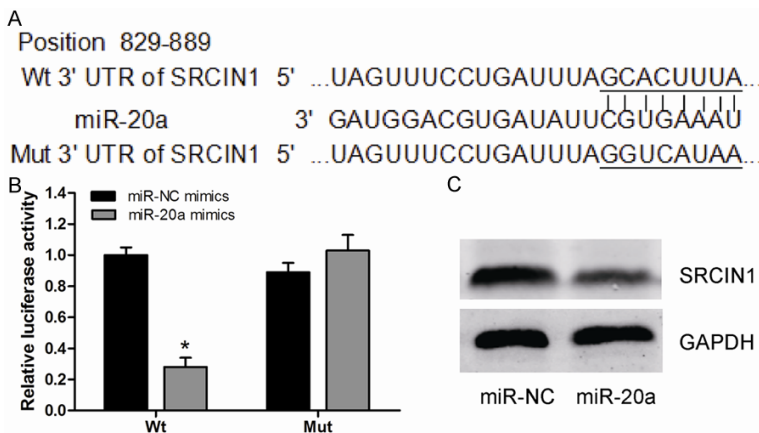
### miR-20a promoted MM cell proliferation

To clarify the role of miR-20a in MM carcinogenesis, the miR-20a mimics and miR-NC mimics were transfected into the NCI-H929 cells using lipofectamine 2000. qRT-PCR showed that the expression of miR-20a was significantly increased after miR-20a mimics transfection (Figure 2A, P<0.05). MTT assay showed that forced overexpression of miR-20a significantly promoted the proliferation of NCI-H929 cells (Figure 2B, P<0.05). This proliferation effect could be partially due to the disruption of cell growth regulation, such as cell cycle regulation and cell apoptosis inhibition. Analysis of cell cycle distribution by flow cytometry showed that miR-20a mimics led to a lower percentage of cells in G0/G1 phase and a higher percentage of cells in S phase (Figure 2C, P<0.05). Cell apoptosis analyzed by flow cytometry showed that miR-20a mimics could noticeably inhibit MM cells apoptosis compared with miR-NC group (Figure 2D, P<0.05). Those data suggested that miR-20a induced promotion of MM cell

## MiR-20a target SRCIN1 in MM



**Figure 2.** Enforced expression of miR-20a promotes MM cell proliferation. (A) qRT-PCR assay of NCI-H929 cells transfected with miR-20a mimics or miR-NC mimics. (B) NCI-H929 cells were transfected with miR-20a mimics or miR-NC mimics, and cell viability was analyzed by MTT assay. (C, D) Flow cytometric analysis of cell cycle (C) and apoptosis (D) of the transfected NCI-H929 cells. U6 was used as a control. Data were drawn from three independent experiments, \*P<0.05.



**Figure 3.** SRCIN1 is a direct target of miR-20a. A. The predicted interactions between miR-20a and its target sites in the SRCIN1 3'UTR were shown. B. Luciferase activity assay shown that miR-20a suppressed Wt SRCIN1 3'UTR luciferase activity, while it had no effect on Mut SRCIN1 3'UTR luciferase activity compared to control in HEK-293 cells. C. The protein expression of SRCIN1 in NCI-H929 cells transfected with miR-20a mimics or miR-NC mimics were analyzed by western blot. GAPDH was used as a control. Data were drawn from three independent experiments, \*P<0.05.

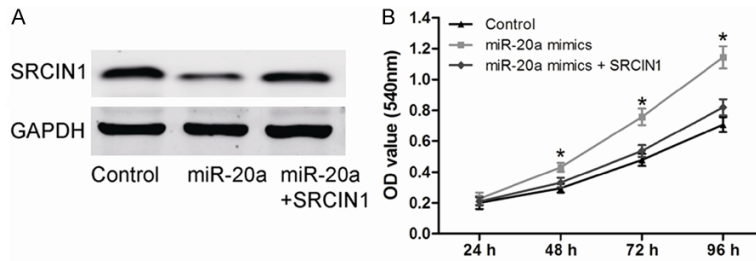
### SRCIN1 was a target of miR-20a

To screen the function target of miR-20a in MM cells, Target Scan 6.2 was used to screen the target gene of miR-20a. SRCIN1 was predicted to be a target of miR-20a (Figure 3A). Luciferase activity assay showed that miR-20a significantly suppressed the luciferase activity of the Wt 3'UTR but not the Mut 3'UTR of SRCIN1 in HEK-293 cells (Figure 3B, P<0.05). In addition, western blot showed that overexpression of miR-20a significantly inhibited SRCIN1 protein level (Figure 3C). Taken together, these results suggested that miR-20a might

proliferation could attributable to cell cycle regulation and cell apoptosis inhibition.

downregulate SRCIN1 expression by targeting a specific site in its 3'UTR.

## MiR-20a target SRCIN1 in MM



**Figure 4.** SRCIN1 overexpression attenuates the promotive effect of miR-20a. A. Expression of SRCIN1 was detected by Western blot in NCI-H929 cells transfected with the vector, miR-20 mimics or miR-20 mimics and SRCIN1. B. NCI-H929 cells were co-transfected with the vector, miR-20 mimics or miR-20 mimics and SRCIN1. MTT assay was used to measure the proliferation. Data were drawn from three independent experiments, \*P<0.05.

### *Ectopic expression of SRCIN1 reversed the effects of miR-20a*

We further evaluated whether reintroduction of SRCIN1 could reverse the effects of miR-20a. NCI-H929 cells were transfected with the vector, miR-20 mimics or miR-20 mimics and SRCIN1 (lacking 3'UTR) were detected by western blot (Figure 4A). As shown in Figure 4B, overexpression of miR-20a significantly promoted cell proliferation whereas reintroduction of SRCIN1 abrogated miR-20a dependent effects (P<0.05). Those data suggested that miR-20a promoted cell proliferation through suppression of SRCIN1 expression.

### Discussion

In the present study, we identified a tumor oncogenic role of miR-20a in MM cells. Our data showed that miR-20a was upregulated in MM cell lines and patient MM cells. Enforced expression of miR-20a promoted MM cell proliferation, regulated cell cycle and inhibited MM cell apoptosis. SRCIN1 was identified as a target of miR-20a in MM cells. Moreover, supplement of SRCIN1 could reverse the tumor oncogenic effects of miR-20a in MM cells.

miRNAs are reported to play essential roles in carcinogenesis and tumor progression [12]. Accumulating evidence revealed the important function of miRNAs in MM development and progression [13]. For example, Hao et al showed that serum high expression of miR-214 and miR-135b could act as novel predictor for myeloma bone disease development and prognosis [14]. Liu et al suggested that miR-186 could inhibit cell proliferation in multiple myeloma by repressing Jagged1 [15]. Liang et al

revealed that miR-301 could promote cell proliferation by directly targeting TIMP2 in multiple myeloma [16]. Peng et al reported that miR-20a has a higher expression in MM and could act as a potential diagnostic biomarker [17]. Our work further expanded the tumor oncogenic role of miR-20a in MM progression.

SRC kinase signaling inhibitor 1 (SRCIN1), also known as p140 Cas-associated protein (p140CAP), is a newly identified

tumor suppressor gene that plays a major role in Src inactivation in tumor cells [18]. For example, Di Stefano et al showed that overexpression of SRCIN1 could inhibit the breast cancer cells spreading, migration and invasion [19]. Recent studies showed that SRCIN1 contributed to cancer progression and was regulated by many miRNAs. For example, Xu et al suggested that miR-374a promoted cell proliferation, migration and invasion by targeting SRCIN1 in gastric cancer [20]. Gao et al found that miR-873 induced lung adenocarcinoma cell proliferation and migration by targeting SRCIN1 [21]. Ye et al indicated that miR-211 promoted non-small cell lung cancer proliferation by targeting SRCIN1 [22]. In our study, we found that SRCIN1 could also be regulated by miR-20a in MM, supporting its oncogenic role in MM.

In conclusion, miR-20a promoted the proliferation of MM cell lines by targeting SRCIN1. Our data suggested that miR-20a play an essential role in regulation of MM cell lines and could serve as a therapeutic target for the treatment of MM. Understanding the precise role played by miR-20a will not only advance our knowledge of MM biology, but also reveal the extensive influence exerted by miR-20a in cancer development.

### Disclosure of conflict of interest

None.

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