MiR-181a functions as an oncogene by regulating CCND1 in multiple myeloma

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Abstract. MicroRNA-181a (miR-181a) has been demonstrated to be upregulated in patients with multiple myeloma (MM). In several studies, miR-181a has been demonstrated to be significantly overexpressed in MM; however, its potential role in development and progression of MM remains unknown. In the present study, the functions of miR-181a and the potential underlying molecular mechanisms in the pathogenesis of MM were examined. Increased expression of miR-181a was observed in bone marrow samples from patients with MM and the MM RPMI8226 cell line. The role of miR-181a was examined and it was demonstrated that it participated in the proliferation and migration processes of the MM cell line. Furthermore, it was demonstrated that the downregulation of miR-181a inhibited the expression of CCND1, a cell cycle regulatory gene, and caused cell cycle arrest in MM cells. The results of the present study suggested that miR-181a functions as an onco-miRNA in MM, which serves regulatory roles by upregulating expression of CCND1 and may therefore serve as a potential target in patients with MM.

Introduction

Multiple myeloma (MM) is a malignancy of B cells characterized by the aberrant clonal proliferation and expansion of plasma cells producing monoclonal immunoglobulin (Ig) antibodies (1). MM is the second most common hematological malignancy in the world and accounts for ~13% of all hematological malignancies (2). Although the complete remission rate and progression-free survival time has improved as a result of novel treatment strategies and advances in transplantation based therapies, it remains an incurable disease. Despite the central role of genomic changes, epigenetic modifications, including DNA methylation, regulation by non-coding RNAs and histone modifications are crucial in the development and progression of MM (3-5).

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNAs measuring 17-25 nucleotides in length (6). Mature miRNAs regulate a large variety of cellular functions including proliferation, differentiation, angiogenesis, apoptosis, stress response and fat metabolism (7-9).

A number of previous studies have examined the miRNA expression profile in MM, with the aim of identifying novel biomarkers for diagnosis or prognosis prediction, therapeutic targets as well as improving the understanding of the underlying mechanisms regarding miRNA regulation networks (3,10,11). Among the numerous aberrantly expressed miRNA that have been documented in MM, the effects of miR-181a, which is upregulated in MM, have not been extensively examined. miR-181a was the first miRNA reported to serve a regulatory role in B cell differentiation in the bone marrow (BM), and ectopic expression in hematopoietic stem/progenitor cells promoted a substantial increase in the generation of B cells, both *in vitro* and *in vivo* (12). Previous data have revealed that miR-181a is a biomarker of MM and regulates the progression of MM (13,14). In a number of different types of tumor, miR-181a was involved in the regulation of tumor proliferative, migratory and invasive abilities, thereby highlighting its potential as a prognostic factor and therapeutic target (15). In the present study, the expression of miR-181a in patients with MM was measured, and its effect on proliferation and invasion of MM cells was assessed. Additionally, the underlying molecular mechanism of its regulation functions were determined.

Materials and methods

Patient samples. A group of 31 newly diagnosed and previously untreated patients with MM (13 females and 18 males with a median age of 62 years and range 46-77 years) were enrolled in the present study between December 2015 and July 2018 in Ningbo First Hospital. Patients were diagnosed and categorized according to the International Staging System (16). Of the enrolled patients, 5 were classified as stage I, 10 as stage II and 16 as stage III. The class of monoclonal proteins present were: IgG for 13 patients; IgA for 8 patients; and light chain disease

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and others for 10 patients. As a control, 13 healthy individuals (6 females and 7 males; median age, 59 years; age range, 39-74 years) were recruited. Healthy volunteers had not been exposed to any known cytotoxic treatment prior to aspiration of the BM. BM aspirates were collected from patients with MM and normal controls into heparinized syringes in a single aspiration. All BM aspirates were obtained from the posterior iliac crest. Written informed consent was obtained from all patients and all experiments were performed in compliance with the 6th revision of The Declaration of Helsinki (2008) and approved by the Institutional Review Board of Ningbo First Hospital. Briefly, BM samples were diluted in PBS (Sigma-Aldrich; Merck KGaA) and separated using a Ficoll-Paque gradient centrifugation at 1,500 x g for 15 min at 4˚C (GE Healthcare Life Sciences) according to the manufacturer's protocol.

Cell culture. The human MM RPMI8226 cell line was obtained from American Type Culture Collection. Cells were grown in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in 5% CO_2 at 37˚C.

Cell transfection. miR-181a inhibitor and negative control (NC) inhibitor were purchased from Shanghai GenePharma Co., Ltd. RPMI8226 cells in the logarithmic growth stage were seeded into 6-well plates with the density of $1x10^5$ cells/well and transfected with 50 nM either the miR-181a inhibitor or NC inhibitors using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Following transfection for 6 h, the medium was removed from the plate, and serum-free RPMI-1640 was used to wash the cells. Cells were incubated at 37˚C in 5% CO_2 for 24 h for further use. The sequences were as follows: miR-181a inhibitor, 5'-ACUCACCGACAGCGUUGAAUGUU-3'; NC inhibitor, 5'-CAGUACUUUUGUGUAGUACAA-3'.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from freshly isolated RPMI8226 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was reverse transcribed from the mRNA using the One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.). RT-PCR was conducted using One Step TB Green® PrimeScript™ RT-PCR kit (RR066A; Takara Biotechnology Co., Ltd). The PCR conditions were as follows: Initial denaturation at 72˚C for 5 min, denaturation at 95˚C for 10 sec, annealing at 72˚C for 10 sec and final extension at 65˚C for 20 sec for 35 cycles. All primer sequences used in RT-qPCR were purchased from Invitrogen; Thermo Fisher Scientific and the primer sequences were as follows: miR-181a forward, 5'-GCGGTAACATTCAACGCTGTCG-3'; miR-181a reverse, 5'-GTGCAGGGTCCGAGGT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3'; and U6 reverse, 5'-AACGCTTCACGAATTTGCGT-3'. Small nuclear RNA U6 was used as an internal reference. The RT-qPCR was performed on an ABI Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The comparative Cq method ($2^{-\Delta\Delta Cq}$) was used to calculate the relative expression level of each sample (17).

Cell proliferation analysis. The proliferative capacity of the RPMI8226 cells was analyzed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) based on the dehydrogenase activity detection in viable cells. Briefly, cells were seeded into 96-well plates at a density of $1x10^5$ cells per well. After 24 h of culturing, 10 μl CCK-8 solution was added into each well and cells were incubated for 2 h at room temperature. The absorbance at 450 nm was measured using a microplate reader (Model 550; Bio-Rad Laboratories, Inc.).

Cell cycle analysis. RPMI8226 cells in the logarithmic phase were harvested and fixed in ice-cold 70% ethanol on ice for 20 min. The cells were rinsed in PBS, and stained using 20 mg/ml propidium iodide (PI) and 10 U/ml RNase A (Abcam) in a 37˚C water bath for 30 min. Cell cycle distribution was determined using flow cytometry (FACScan; BD Biosciences) and the data were analyzed using ModFit LT™ version 2.0 (Verity Software House, Inc.) to determine the percentage of cells in each phase of the cell cycle.

Wound healing assay. Following transfection, the RPMI8226 cells were collected and seeded in a 48-well plate at a density of $1x10^6$ per well. Upon reaching a confluent monolayer, a line-shaped scratch was created by carefully dragging a 200 μl pipette tip through the cell layer. Cells were washed with PBS 3 times and incubated at room temperature at 5% CO_2 for 24 h. The degree of scratch healing was observed by a light microscope at magnification, x200 and measured and used to determine the cell migratory ability.

Western blot analysis. SDS-PAGE and western blot analysis were performed to measure the protein expression levels of cyclin D1 (CCND1) protein using β-actin as an internal reference. Briefly, RPMI8226 cell lysates were prepared using lysis buffer containing protease inhibitors (Beyotime Institute of Biotechnology). The concentrations of protein were measured using a Bradford protein assay (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. A total of 20 μg protein extracts were resolved using 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). The membrane was blocked using 5% non-fat milk in TBS-Tween solution (Beijing Solarbio Science & Technology Co., Ltd.) for 2 h at room temperature and immunoblotted overnight at 4˚C with a mouse anti-CCND1 monoclonal antibody (cat. no., sc-450; 1:1,000; Santa Cruz Biotechnology, Inc.) or mouse anti-β-actin primary antibody (cat. no., BM0627; 1:1,000; Wuhan Boster Biological Technology, Ltd.). Membranes were washed 3 times in TBS-Tween, and subsequently incubated with a secondary antibody IgG H&L Cy3® (cat. no., ab97035; Abcam) for 1 h at room temperature. Enhanced chemiluminescence was used to visualize the signal and the signal intensity was quantified using VisionWorks LS version 7.0 Analyzing System (Analytik Jena US LLC).

Statistical analysis. Data were analyzed using SPSS version 20.0 (IBM Corp.) to determine statistical significance. All data are expressed as the mean ± standard deviation. Differences between two groups were compared using a Student's t-test.

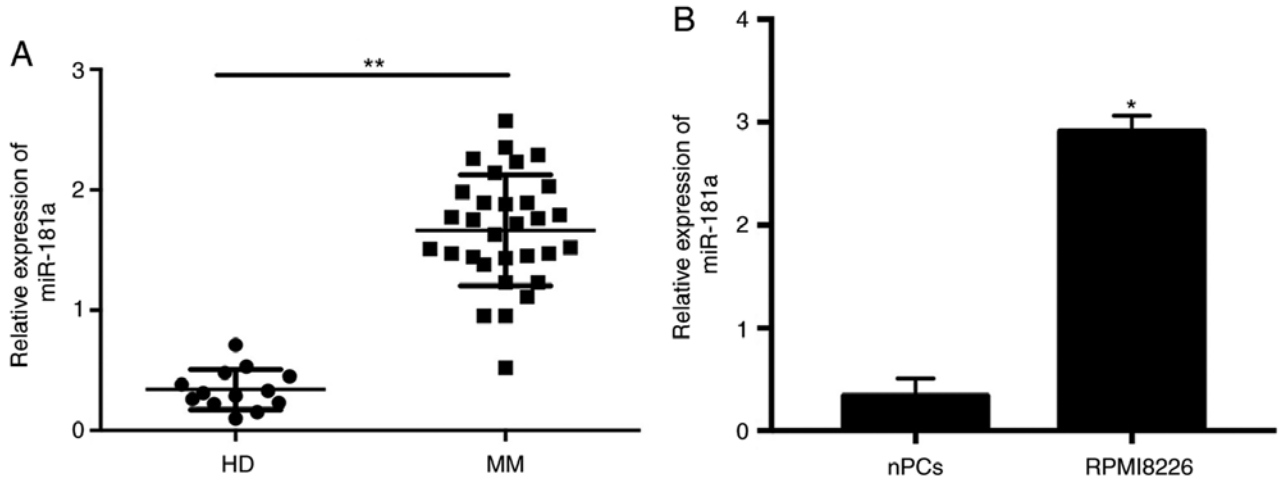

Figure 1. miR-181a is upregulated in MM. (A) Relative expression levels of miR-181a in HD and patients with MM. **P<0.01. (B) Relative expression levels of miR-181a in nPCs and the RPMI8226 cell line. *P<0.05, RPMI8226 vs. nPCs. miR, microRNA; MM, multiple myeloma; HD, healthy donor; nPCs, normal plasma cells.

Table I. Relative expression of miR-181a in patients with multiple myeloma with different clinical characteristics.

Characteristics	Cases (n)	miR-181a level	P-value
Sex			0.743
Male	18	1.64±0.46	
Female	13	1.70±0.48	
Age, years			
<65	16	1.52±0.48	0.080
≥65	15	1.81±0.41	
Stage			0.000[a]
I	5	1.01±0.33	
II	10	1.65±0.30	
III	16	1.88±0.39	
Phenotype			0.859
IgG	13	1.64±0.41	
IgA	8	1.62±0.49	
Other	10	1.73±0.54	
Renal lesion			0.022[a]
Yes	17	1.83±0.38	
No	14	1.46±0.49	

[a]Statistically significant difference. Renal lesion was defined by serum creatinine levels ≥176.8 μmol/l. Data are presented as the mean ± standard deviation. miR, microRNA.

Multiple comparisons were performed using a one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-181a expression is upregulated in patients with MM and an MM cell line. To determine the expression level of miR-181a in patients with MM, RT-qPCR analysis was performed using the MM cells obtained from patients with MM and the normal plasma cells (nPCs) from the healthy controls. In the BM aspirates of the 31 patients with MM and 13 healthy donors, miR-181a expression was significantly increased in the patients with MM patients without treatment compared with the nPCs (Fig. 1A). The expression level of miR-181a was significantly increased in the RPMI8226 cell line compared with the healthy control (Fig. 1B).

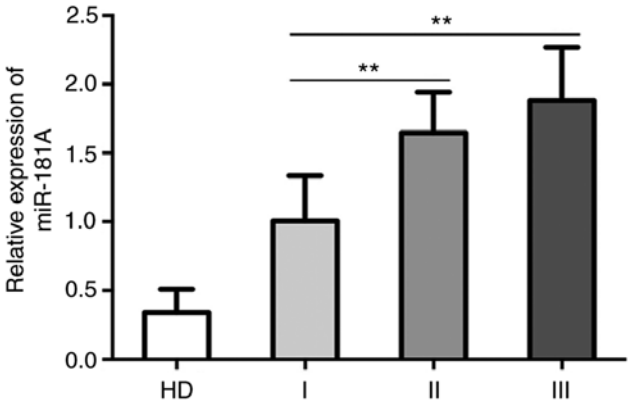

Figure 2. Relative expression levels of miR-181a in different International Staging System stages in patients with multiple myeloma and the HD. **P<0.01, III vs. II, III vs. I. miR, microRNA; HD, healthy donors.

The association between the expression of miR-181a and the clinical features of the patients was subsequently assessed. There was no association between the expression profile of miR-181a and age, sex and cancer isotype; however, expression was increased in the later stages of MM compared with earlier disease stages (Table I). Patients with either stage II (1.65±0.30) and stage III (1.88±0.39) disease presented with significantly increased expression levels of miR-181a compared with patients with stage I MM (1.01±0.33). However, there was no significant difference observed between patients with stage II and III MM (Fig. 2). The expression levels of miR-181a were also increased in patients with MM with renal lesions compared with patients without lesions. These results suggested an oncogenic function for miR-181a in the pathogenesis and progression of MM.

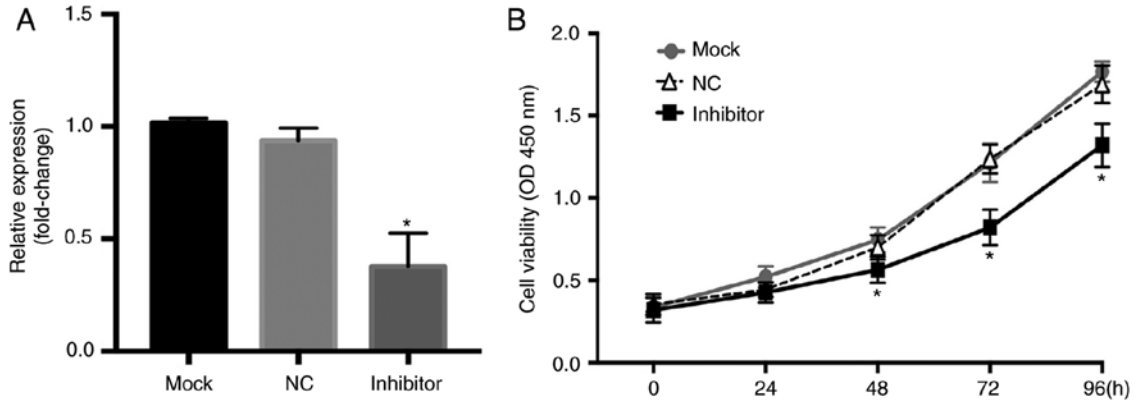

Figure 3. miR-181a promotes proliferation in multiple myeloma cells. (A) Relative mRNA expression levels of miR-181a in RPMI8226 cells following transfection with an miR-181a inhibitor. (B) Proliferation of transfected RPMI8226 cells measured using a Cell Counting Kit-8 assay. *P<0.05 inhibitor vs. NC. miR, microRNA; NC, negative control; OD, optical density.

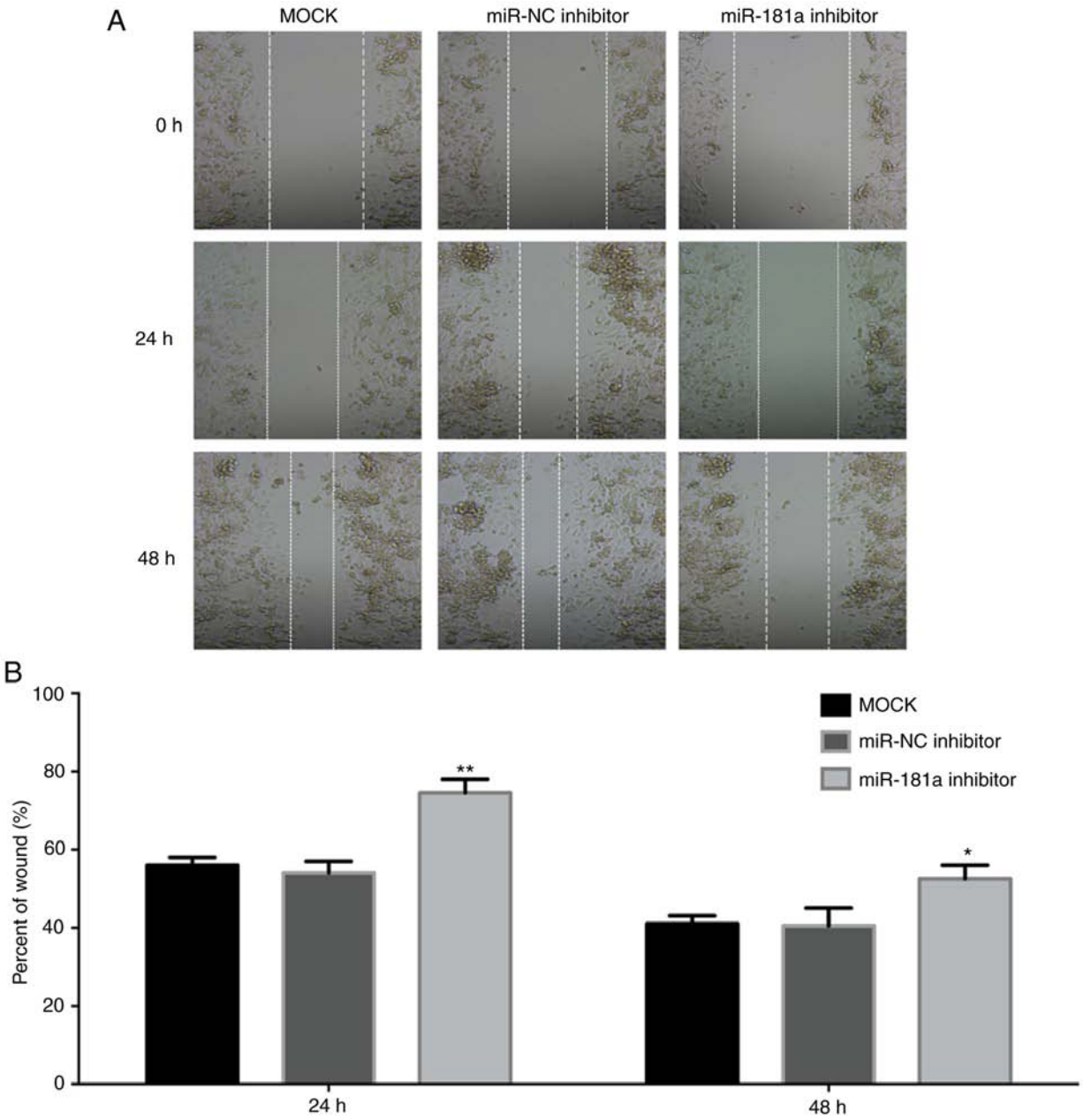

Figure 4. Knockdown of miR-181a inhibits migration in a multiple myeloma cell line. (A) A wound healing assay was performed to determine the effect of miR-181a on migration in RPMI8226 cells. Magnification, x200). (B) Migration ratio presented as a percentage, 24 and 48 h after scratching. *P<0.05 and **P<0.01, miR, microRNA; NC, negative control.

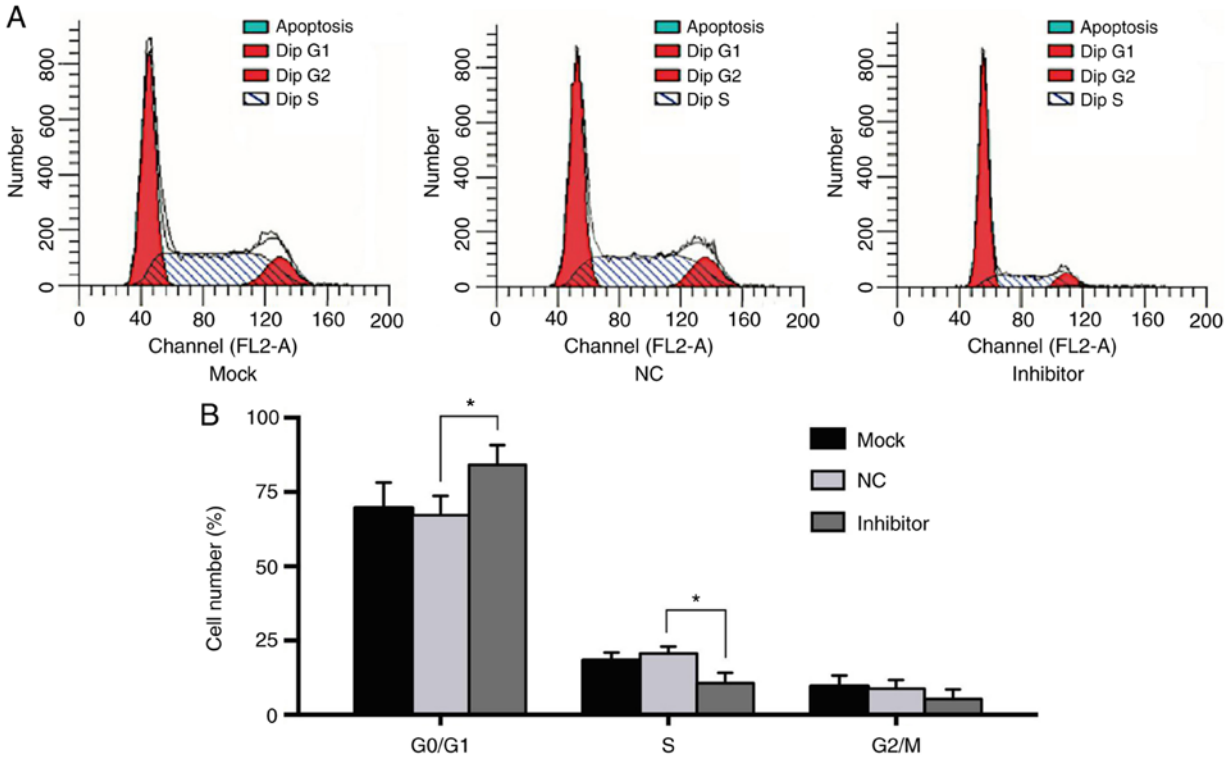

Figure 5. Silencing of microRNA-181a results in arrest at the G0/G1 phase. (A) Cell cycle distributions of three groups of transfected RPMI8226 cells determined using flow cytometry. (B) Proportion of cells in the different phases of the cell cycle. *P<0.05, inhibitor vs. NC. NC, negative control.

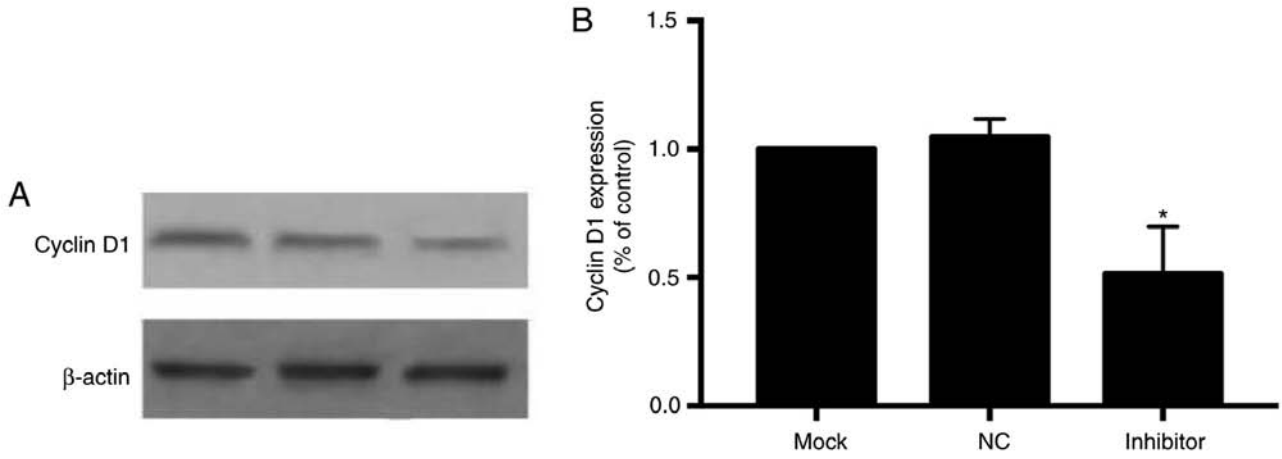

Figure 6. Downregulation of miR-181a decreases the expression of CCND1. (A) Protein expression levels of cyclin D1 in RPMI8226 cells transfected with the miR-181a inhibitor measured by western blot analysis. (B) Quantified protein expression levels of cyclin D1 in cells transfected with the miR-181a inhibitor compared with cells transfected with miR-NC. *P<0.05, inhibitor vs. NC. miR-181a, microRNA; NC, negative control.

Downregulation of miR-181a inhibits the proliferation and migration of MM cells. To determine the effects of miR-181a expression in an MM cell line, miR-181a was inhibited in RPMI8226 cells by transfection with an miR-181a inhibitor (Fig. 3A). By measuring the optical density of the cells, it was demonstrated that the survival rate increased significantly over time in the normal control RPMI8226 cells, and a significant decrease in the viability of cells was observed in the cells transfected with the miR-181a inhibitor compared with the miR-NC group (Fig. 3B). A wound healing assay was used to determine the effect of miR-181a on the mobility of MM cells (Fig. 4). The edge of the wound was neat following scratching. After 24 h incubation, the cells migrated into the wound. Compared with the non-transfected cells and the cells transfected with the NC inhibitor, the number of cells transfected with the miR-181a inhibitor migrating into the wound was significantly decreased (P<0.05). Together, these data suggest that miR-181a serves an important role in promoting proliferation and the migratory ability of MM cells.

Knockdown of miR-181a results in cell cycle arrest in MM cells. To investigate the effect of miR-181a on cell cycle progression in MM cells, RPMI8226 cells were transfected with the miR-181a inhibitor or NC inhibitor, or mock-transfected (Fig. 5). The cytometry results demonstrated that the cells transfected with

the miR-181a-inhibitor exhibited a larger proportion of cells in the G0/G1 phase, and smaller proportion of cells in the S and G2/M phases, suggesting that the inhibition of miR-181a induced G0/G1 cell cycle arrest in RPMI8226 cells. The results suggested that miR-181a contributed to tumor progression of MM by promoting cell cycle progression from G0/G1 to S.

Inhibition of miR-181a suppresses expression of CCND1. To determine the underlying molecular mechanism of action of miR-181a, western blot analysis was performed to determine the expression of the cell cycle regulator CCND1. In cells transfected with the miR-181a inhibitor, CCND1 expression was downregulated compared with the miR-NC group (Fig. 6).

Discussion

miRNAs have been demonstrated to serve a number of essential and diverse physiological and pathological roles in cells (7) and the abnormal expression of numerous miRNAs are implicated in the development and progression of several different types of cancer (18). Furthermore, several miRNAs have been identified as oncogenes or tumor suppressors in the carcinogenesis of MM (3,10,11), highlighting the varying effects of different miRNAs in the development of MM (11,19).

miR-181a was recently determined to be an important noncoding RNA with a wide range of regulatory functions in normal hematopoiesis, organ development and pathological processes of a number of different types of cancer and autoimmune disorders (20-23). In MM, it was one of the most highly upregulated miRNAs in both patient samples and cell lines, and was also demonstrated to exhibit similar expression patterns in peripheral blood and the BM (24). However, its potential role in the pathogenesis of MM remains poorly understood.

In the present study, the expression levels of miR-181a were significantly increased in BM samples from patients recently diagnosed with MM and in the MM RPMI8226 cell line compared with normal plasma cells, which was consistent with the results from previous studies (10,13,25). In the present study, upregulation of miR-181a was associated with disease stage, suggesting its potential contribution to the progression of MM. Aberrant expression of miR-181a has been observed in cases of monoclonal gammopathy of undetermined significance, a probable early pathogenic event of MM (10). Notably, in plasma cell leukemia, which is a rare and aggressive form of MM, characterized by poor prognosis and the presence of malignant plasma cells circulating in the peripheral blood, miR-181a expression levels were considerably increased compared with MM. Additionally, compared with patients who responded to treatment, patients who did not respond to treatment exhibited increased levels of miR-181a (26). Taken together, it may be hypothesized that miR-181a serves a potential role during the entire process of the MM development and progression.

In vitro assays were performed to determine the biological effects of miR-181a in the MM RPMI8226 cell line. Decreasing expression of miR-181a exerted an inhibitory effect on the proliferative and migratory capabilities of the RPMI8226 cells. Furthermore, cell cycle analysis revealed G0/G1 arrest when miR-181a was inhibited in MM cells. These results suggest that miR-181a may function as a novel oncogene in MM and serve a regulatory role in the tumorigenesis of MM.

To identify the regulatory network underlying the effects of miR-181a, the present study focused on CCND1. CCND1 is a cell cycle-regulating protein encoded by the CCND1 gene and is required for the progression through the G1 phase to the S phase in both normal cells and cancerous cells (27,28). CCND1 is activated in MM as an oncogene, and regulates the cell cycle and promotes proliferation (29). The results demonstrated that CCND1 expression was decreased in the miR-181a-inhibited MM cells compared with normal MM cells, and the results consistent with previous studies using co-immunoprecipitation analysis (30-32). The downregulation of CCND1 as a result of miR-181a inhibition in the MM cells may partially explain the effects of miR-181a on the cell cycle distribution results.

However, there are several limitations of the present study. Firstly, only one MM cell line, RPMI8226, was used for the *in vitro* experiments. Secondly, the *in vitro* results were not confirmed *in vivo*.

In conclusion, miR-181a was significantly upregulated in clinical BM samples from patients with MM and an MM cell line compared with the normal controls. Furthermore, the overexpression of miR-181a promoted cell proliferation, migration and cell cycle progression, promoting the development of MM. miR-181a regulation of CCND1 was identified as a potential molecular mechanism underlying the promotion of cell cycle progression, and this interaction was abrogated using an miR-1881a inhibitor. The results of the present study highlight the potential of miR-181a as a novel therapeutic target and the use of synthetic miR-181a inhibitors as novel therapeutic strategies for treating patients with MM.

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Availability of data and materials

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

Authors' contributions

XY, MG, PZ, GO, QM and KX conceived the study. XY, MG and PZ designed the study and performed data analysis. GO, QM and KX conducted the experiments and interpreted the data. XY and MG wrote the manuscript. PZ, GO, QM and KX critically revised the manuscript for important content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and all experiments were performed in compliance with

the Declaration of Helsinki (2008) and approved by the Institutional Review Board of Ningbo First Hospital (approval no. 2015087533).

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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