Original Article

The microRNA miR-19a-3p suppresses cell growth, migration, and invasion in multiple myeloma via the Wnt/β-catenin pathway

Zhengxiao Wei¹, Wang Wang², Qingfeng Li¹, Linfang Du², Xuemei He³

¹Department of Clinical Laboratory, Public Health Clinical Medical Center of Chengdu, Chengdu, China; ²College of Life Sciences, Sichuan University, Chengdu, China; ³Medical Experimental Center, Affiliated Hospital of Southwest Medical University, Luzhou, China

Contributions: (I) Conception and design: Z Wei, L Du, X He; (II) Administrative support: Q Li, L Du, X He; (III) Provision of study materials or patients: Z Wei, W Wang, L Du, X He; (IV) Collection and assembly of data: Z Wei, W Wang; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Background: MicroRNAs have been suggested as potential regulators in the development of multiple myeloma (MM) through affecting the expression of their target genes. This study aimed to investigate the effects of miR-19a-3p in MM, and its underlying mechanisms in regulating cell proliferation and invasion.

Methods: Bone marrow samples from 25 MM patients and 12 healthy donors were collected and miR-19a-3p and Wnt1 mRNA expression was assessed. The effects of miR-19a-3p on cell proliferation, migration, and invasion in U226 and RPMI-8226 MM cells were evaluated by miR-19a-3p overexpression. Luciferase assays were performed to explore the potential target genes. Knock down or overexpression of Wnt1 was used to explore the effects of miR-19a-3p on cell growth, migration, and invasion.

Results: The expression of miR-19a-3p was downregulated in MM and cell lines, while Wnt1 mRNA levels were increased. Overexpression of miR-19a-3p inhibited cell proliferation, migration, and invasion in U226 and RPMI-8226 cells. Additionally, western blot assays revealed that miR-19a-3p could suppress Wnt1, β-catenin, cyclin D1, and c-Myc expression. Knockdown of Wnt1 also inhibited cell growth, migration, and invasion. Moreover, luciferase reporter assay revealed direct binding between Wnt1 and miR-19a-3p. Wnt1 overexpression partially reversed the suppressive effects of miR-19a-3p on cell proliferation, migration, and invasion in U266 cells.

Conclusions: The expression of miR-19a-3p was downregulated in MM patients and MM cell lines. Overexpression of miR-19a-3p inhibited proliferation, migration, and invasion by targeting Wnt1 via the Wnt/β-catenin signaling pathway.

Keywords: miR-19a-3p; multiple myeloma (MM); proliferation; invasion; migration; Wnt1/β-catenin pathway

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Introduction

Multiple myeloma (MM) is the second most common hematological malignancy (1,2) characterized by the uncontrolled growth of monoclonal plasma cells in the bone marrow, resulting in hypercalcemia, bone disease, renal failure, anemia and other complications (3,4). The latest treatments for MM include immunomodulatory drugs (IMiD), second-generation proteasome inhibitors (PI), anti-CD38 monoclonal antibodies (MoAb), and chimeric antigen receptor T cell (CAR T cell) therapy (5-8). Although these therapies significantly improve the rate of survival in MM patients, issues of relapse and refractory disease still require urgent attention (9). Therefore, it is necessary to explore the pathogenesis and diagnostic markers of MM to provide new directions for the treatment of MM.
MicroRNAs (miRNAs) are small non-coding RNA with a length of 19–25 bases that regulate gene expression by degrading mRNA or inhibiting its translation (10). In MM, a variety of miRNAs (such as miR-21 and miR-221) are abnormally expressed or dysfunctional. In addition, the miRNAs in the tumor microenvironment regulate MM cell functions through gene/protein targets, signaling molecules, and pathways leading to the transfer of MM cells (11-13). It has been reported that the regulation of miRNAs (such as miR-15a, miR-16, and miR-34) in MM cells could weaken their functional interaction with the bone marrow microenvironment and produce significant anti-tumor activity (10,14). Other studies have demonstrated that miR-19a-3p was abnormally expressed in MM, and promoted cell proliferation and inhibited cell apoptosis by degrading the F-box only protein 32 (FBXO32) mRNA (15). Therefore, miRNAs may be potential therapeutic targets for MM.

**Methods**

**Patient sample collection**

Bone marrow samples from 25 patients with MM and 12 patients with non-hematological diseases were collected in the Affiliated Hospital of Southwest Medical University from May 2017 to May 2018. The collected samples were stored at −80 °C. This study was approved by the Research Ethics Committee of the Affiliated Hospital of Southwest Medical University, and all patients provided signed informed consent. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

**Cell culture and transfection**

The human MM cell lines U266 and RPMI-8226 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone; Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37 °C with 5% CO₂ atmosphere.

The small interfering RNA of Wnt1 (si-Wnt1), the miR-19a-3p mimic, and the pcDNA3.1-Wnt1 vector, as well as their corresponding negative controls (NC) were obtained from Shanghai GenePharma Co., Ltd., (Shanghai, China) and transfected into U266 and RPMI-8226 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer’s protocol. The sequences of miR-19a-3p mimic and si-Wnt1 were listed in Table 1. Cell samples were then harvested for further research.

**Cell counting kit-8 (CCK-8) assay**

Cells at the logarithmic growth phase were harvested and digested with trypsin to prepare a cell suspension. The cells were seeded into 96-well plates at a cell density of 2×10⁴ cells/
well, and the wells at the edge of the plate were filled with sterile phosphate buffered saline (PBS). After cell culture for 0, 24, 36, 48, and 60 hours, 10 μL of the CCK-8 reagent was added to each well and cultured for a further 4 hours. A microplate reader was used to measure absorption at 450 nm.

**Flow cytometry analysis**

U226 cells were transfected with miR-19a-3p mimic and mimic NC and cultured for 24 hours. After trypsin digestion, cell suspension at a cell density of 1×10^6 cells/mL was made by 1x Banding Buffer dilution. Then cell suspension was added 5 μL Annexin V-FITC and 10 μL PI, and incubate for 15 min at room temperature in the dark. Finally, flow cytometry was used to analyze apoptosis of each sample group.

**Wound healing analysis**

The transfected cells were seeded into 24-well cell culture plates at a density of 5×10^4 cells/well. After 24 hours, the cell confluence reached 70–80%. A sterile 10 μL pipette tip was used to gently draw a straight line through the monolayer of cultured cells. After scratching, each well was gently washed with PBS to remove the exfoliated cells. Cells were cultured for a further 48 hours and wound closure was examined under light microscopy.

**Transwell invasion assay**

A 24-well transwell chamber (Corning Inc., Corning, NY, USA) was pre-coated with Matrigel (BD Biosciences, USA). The transfected cells were resuspended with serum-free Dulbecco's Modified Eagle Medium (DMEM) and then seeded into the upper chamber at a density of 5×10^4 cells/well. DMEM containing with 10% FBS was added into the lower chamber. After 48 hours at 37 °C, the cells on the lower surface were fixed with 4% methanol for 10 minutes and stained with 0.1% crystal violet for 5 minutes. Invasive cells were observed in six randomly selected fields of view under a light microscope.

**Quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

Total RNA was extracted from clinical samples and transfected cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. To obtain the cDNA from miRNA, the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme Biotech Co., Ltd) was used to reverse transcribe miR-19a-3p according to the product instructions. For mRNA, template RNA was reverse transcribed to obtain cDNA using Hsircipt® II Q RT SuperMix (Vazyme Biotech Co., Ltd) following the manufacturer's protocols. RT-qPCR was performed using AceQ® qPCR SYBR Green Master Mix (Vazyme Biotech Co., Ltd). The following qPCR program was used: pre-denaturation at 95 °C for 10 minutes; 40 cycles of 95 °C for 10 seconds, 55 °C for 10 seconds, 72 °C for 20 seconds. The relative miR-19a-3p and Wnt1 mRNA expression levels were calculated using the 2^-ΔΔCt method. The primer sequences used in this study are shown in Table 2.

**Western blot analysis**

Total protein was isolated from the MM tissues and cells using RIPA lysis buffer (Thermo Scientific, Waltham, MA, USA). The Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) was used to measure protein concentration. Equal amounts of protein (40 μg) for each sample were separated by 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) at a constant voltage (120 V) and then transferred onto polyvinylidene fluoride (PVDF) membranes at a constant current (200 mA). After blocking with 5% skimmed milk at room temperature for 1 hour, the membranes were incubated with primary antibodies (Wnt1 #ab15251 dilution 1:1,000, Abcam.}

### Table 2 The sequence of primers used in this study

| Name       | Sequence (5’-3’)         |
|------------|--------------------------|
| miR-19a-3p | Sense: GCGTGTGCAAAATCTATGCAA; antisense: AGTGCAAGGTCCGAGGTATT |
| Wnt1       | Sense: CTCATGTGATCTATGCCCCG; antisense: AGGTGATACAACTCGTTTCAGT |
| U6         | Sense: CTGGCTTCGGCAGGACACA; antisense: AACGCTTCAGATTTGCGT |
| GAPDH      | Sense: AGGAGGCTGGGGCTCATTG; antisense: AGGGGCCATCCACAGTCTTC |
β-catenin #8480, dilution 1:1,000; Cyclin D1 #55506, dilution 1:1,000; c-Myc #5605, dilution 1:1,000; β-actin #4970, dilution 1:1,000, Cell Signaling Technology, Inc.) overnight at 4 ℃. Membranes were washed with tris buffered saline tween (TBST) and incubated with secondary antibodies (anti-rabbit IgG, HRP-linked antibody #7074, dilution 1:2,000, Cell Signaling Technology, Inc.) for 1 hour at room temperature and visualized using enhanced chemiluminescence.

**Dual-luciferase reporter assay**

The online miRNA target predication databases miRDB and TargetScan 7.2 predicted that the target gene of miR-19a-3p was Wnt1. The wild-type (WT) and mutant-type (Mut) of the 3′UTR of Wnt1 was cloned and amplified by PCR, and the psi-CHECK2™ plasmids containing the wild-type and mutant-type fragments of the 3′-UTR of Wnt1 were constructed. Cells were cotransfected with the miR-19a-3p mimic, the corresponding negative control (NC) mimic, or the Wnt1 WT, or Mut luciferase reporter vector using Lipofectamine 2000 reagent according to the instruction manual. After transfection for 48 hours, the Dual-Luciferase Reporter Assay kit (Promega Corporation) was used to detect Firefly and Renilla luciferase activities following the manufacturer's protocol.

**Statistical analysis**

GraphPad Prism 6.0 software (GraphPad, Inc.) was used to conduct data analysis. Data were expressed as the means ± standard deviation (SD). Student's t-test and one-way of variance analysis (ANOVA) was used for comparisons between multiple groups. A P value <0.05 was considered statistically significant.

**Results**

**The expression of miR-19a-3p was decreased, while the expression of Wnt1 was upregulated in MM tissues and cell lines**

To investigate the expression of miR-19a-3p and Wnt1 in MM samples and cells, RT-qPCR and western blot analysis were performed. The expression of miR-19a-3p was significantly reduced in the bone marrow samples from patients with MM compared to samples from healthy donors (*Figure 1A*). The expression of Wnt1 mRNA was increased in MM patients (*Figure 1B*). Western blot detection demonstrated that the expression of the Wnt1 protein was consistent with the mRNA changes (*Figure 1C*). In addition, the expression of miR-19a-3p and Wnt1 was studied in two human MM cell lines (U226 and RPMI-8226). The results showed that miR-19a-3p was down-regulated in both U226 and RPMI-8226 cells compared to healthy donor cells (*Figure 1D*). At the same time, Wnt1 mRNA and protein levels increased significantly in both U226 and RPMI-8226 cells (*Figure 1E,F*).

**Overexpression of miR-19a-3p suppressed cell proliferation in MM cell lines**

The miR-19a-3p overexpression model was established in U226 and RPMI-8226 cells, and RT-qPCR was used to detect the transfection efficiency of the miR-19a-3p mimic. As illustrated in *Figure 2A*, the level of miR-19a-3p expression was greatly increased in U226 and RPMI-8226 cells after transfection with the miR-19a-3p mimic compared to the NC mimic. The CCK-8 assay was performed to assess the effects of miR-19a-3p overexpression on cell proliferation. The ability of U226 and RPMI-8226 cells to proliferate was inhibited following transfection with the miR-19a-3p mimic compared to the NC mimic. The CCK-8 assay was performed to assess the effect of miR-19a-3p on the apoptosis of U226 cells. The result discovered that miR-19a-3p mimic promoted cell apoptosis on U226 cells compared to mimic NC group (*Figure 2D,E*).

**Overexpression of miR-19a-3p inhibited cell migration and invasion in MM cell lines**

To explore the effects of miR-19a-3p on cell migration and invasion, wound healing analysis and transwell invasion experiments were performed on U226 and RPMI-8226 cells. The ability of U226 cells to migrate decreased following transfection with the miR-19a-3p mimic, compared with the NC mimic (*Figure 3A*). Quantitative analysis of wound closure further verified that overexpression of miR-19a-3p suppressed U226 cell migration (*Figure 3B*). Cell migration ability in RPMI-8226 cells was similarly greatly inhibited following transfection with the miR-19a-3p mimic (*Figure 3C,D*). In addition, miR-19a-3p overexpression significantly depressed the ability of both U226 and RPMI-8226 cells to invade into the Matrigel as shown by the transwell experiment (*Figure 3E*). Quantitative assessment of the number of invasive cells also demonstrated that the invasion capability of U226 and RPMI-8226 cells were blocked by
miR-19a-3p overexpression (Figure 3F). The above data suggested that miR-19a-3p overexpression inhibited the cell migration and invasion ability of MM cells.

**Wnt1 was a direct target of miR-19a-3p**

To further explore the mechanisms by which miR-19a-3p affects the progression of MM, the Wnt1/β-catenin pathway was analyzed using U226 and RPMI-8226 cells. The results demonstrated that overexpression of miR-19a-3p significantly suppressed Wnt1 mRNA expression in both U226 and RPMI-8226 cells (Figure 4A). As presented in Figure 4B, Western blot analysis detected the proteins involved in the Wnt1/β-catenin pathway. Wnt1, β-catenin, cyclin D1, and c-Myc were all significantly inhibited in the miR-19a-3p mimic group compared to the NC mimic group in both U226 and RPMI-8226 cells. Furthermore, analyses using the Targetscan and miRBD databases predicted that miR-19a-3p and Wnt1 have complementary base pairing sequences (Figure 4C). In addition, the interaction between miR-19a-3p and Wnt1 was verified by dual luciferase reporter analysis. The results demonstrated that miR-19a-3p targeted and negatively regulated Wnt1 mRNA expression in both U226 and RPMI-8226 cells (Figure 4D,E). These data indicated that miR-19a-3p targeted Wnt1 and affected the activation of the Wnt1/β-catenin pathway.

**Silencing of Wnt1 suppressed cell proliferation, migration, and invasion in U266 cells**

To determine whether silencing of Wnt1 had the same effect as miR-19a-3p overexpression, siRNA was used. The silencing efficiency of si-Wnt1 was assessed by RT-qPCR and Western blot analysis. The results demonstrated that the expression of Wnt1 mRNA and protein was significantly suppressed after transfection with si-Wnt1 (Figure 5A,B). Knockdown of Wnt1 greatly inhibited U226 cell proliferation compared to the si-NC (Figure 5C). The
wound closure speed was also attenuated by silencing Wnt1 in U226 cells (Figure 5D). Moreover, the number of invasive cells was similarly reduced by silencing Wnt1 in U226 cells (Figure 5E). The above results indicated that silencing Wnt1 had the same effect as miR-19a-3p overexpression on the proliferation, migration, and invasion of U226 cells.

Wnt1 overexpression partially reversed the suppressive effects of miR-19a-3p on cell proliferation, migration, and invasion in U226 cells

Wnt1 is one of the key proteins that affect the proliferation, migration, and invasion of MM cells. Therefore, the effects of Wnt1 overexpression on the miR-19a-3p-mediated suppression of cell proliferation, migration, and invasion were assessed in U266 cells. The overexpression efficiency of Wnt1 was examined by RT-qPCR, and the results revealed that the levels of Wnt1 mRNA increased significantly after transfection of the pcDNA3.1-Wnt1 plasmid (Figure 6A). Western blot analysis was performed to explore the effects of Wnt1 overexpression on the Wnt1/β-catenin pathway. As shown in Figure 6B, the levels of Wnt1, β-catenin, cyclin D1, and c-Myc in cells transfected with the miR-19a-3p mimic and pcDNA3.1-Wnt1...
Figure 3 Overexpression of miR-19a-3p inhibited cell migration and invasion in multiple myeloma cells. U226 and RPMI-8226 cells were transfected with a miR-19a-3p mimic or a NC mimic. (A,C) The effect of miR-19a-3p overexpression on the migratory ability of U226 and RPMI-8226 cells was evaluated by the wound healing assay (magnification, 100×). (B,D) The wound closure rate was calculated with Image J software. (E) The effect of miR-19a-3p overexpression on the ability of U226 and RPMI-8226 cells to invade was assessed by the transwell invasion assay. The cells in the lower layer of the chamber were fixed with 4% paraformaldehyde for 20 minutes and stained with crystal violet solution for 15 minutes, and finally photographed under a microscope (magnification, 400×). (F) The number of invading cells was counted. **P<0.01. NC, negative control.

(mimic+pcDNA3.1-Wnt1) were significantly higher than that in cells transfected with the miR-19a-3p mimic and pcDNA3.1 (mimic+pcDNA3.1) (Figure 6B). In addition, the proliferation, migration, and invasion capabilities of U226 cells were analyzed. The CCK-8 assay results revealed that overexpression of Wnt1 significantly improved the viability of U226 cells overexpressing miR-19a-3p (Figure 6C). Wound healing analysis also demonstrated that the cell migration ability was significantly enhanced in the mimic+pcDNA3.1-Wnt1 group compared to the mimic+pcDNA3.1 group (Figure 6D). Transwell invasion analysis suggested that overexpression of Wnt1 reversed the inhibitory effects of miR-19a-3p on cell invasion (Figure 6E). These data indicated that Wnt1 overexpression partially reversed the suppressive effects of miR-19a-3p on cell proliferation, migration, and invasion in U266 cells.
Discussion

MM is one of the most common hematological malignancies, accounting for about 1% of all cancer cases (22). Currently, there is no drug or treatment that can completely cure MM. Resistance and relapse are the biggest obstacles hindering the development of successful MM treatments (8). Therefore, it is crucial to investigate the potential pathogenic mechanisms of the occurrence and development of MM, especially in terms of proliferation and apoptosis in order to develop new treatment strategies (1). In recent years, the use of non-coding RNAs (including miRNAs, long non-coding RNAs, circular RNAs, and siRNA) has become increasingly popular for studying the mechanisms and treatments of MM (23,24). This present study demonstrated that miR-19a-3p was significantly down-regulated in MM patients and cell lines. This result was consistent with the changes in miR-19a-3p reported in other cancers (15,25).

Furthermore, the mechanisms of miR-19a-3p were explored in MM cells. Our results revealed that miR-19a-3p inhibited cell proliferation, migration, and invasion in U226 and RPMI-8226 cells. This agreed with previous reports showing that overexpression of miRNA-19a-3p significantly reduced the cell proliferation, migration, and invasion ability of HCT116 human colon cancer cells (26).

To investigate the mechanisms of miRNA-19a-3p-mediated inhibition of cell proliferation, migration, and invasion, bioinformatics software was used to predict the target genes of miRNA-19a-3p. The results identified that miRNA-19a-3p binds to the 3’-UTR of Wnt1. Moreover, the dual luciferase reporter assay confirmed that microRNA-19a-3p
negatively regulated the expression of Wnt1. Subsequently, Wnt1 knockdown and overexpression experiments demonstrated that miRNA-19a-3p inhibited cell proliferation, migration, and invasion by targeting Wnt1.

The Wnt/β-catenin pathway plays an important role in embryogenesis, cell growth, and proliferation (27). Over-activation of the Wnt signaling pathway is inextricably linked to cancer initiation and progression (28). Wnt binds to the receptor and initiates a signal cascade to activate β-catenin which promotes the expression of oncogenes such as cyclin D1 and c-Myc (29). In this study, miR-19a-3p significantly inhibited the expression of Wnt1, β-catenin, cyclin D1, and c-Myc. When Wnt1 was knocked down, the expression of β-catenin, cyclin D1, and c-Myc was also suppressed. These data suggested that Wnt1 played a vital role in the miR-19a-3p-mediated regulation of cell proliferation, migration, and invasion.

**Conclusions**

In summary, miR-19a-3p was down-regulated in MM tissues and cell lines and was negatively correlated with...
Wnt1 expression. Moreover, miR-19a-3p overexpression in U226 and RPMI-8226 cells inhibited cell growth, invasion, and migration by targeting Wnt1. The data in this report indicated that miR-19a-3p may be a potential target for the treatment of MM.

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Figure 6 Wnt1 overexpression partially reversed the suppressive effects of miR-19a-3p on cell proliferation, migration, and invasion in U266 cells. U266 cells were transfected with the pcDNA3.1 or pcDNA3.1-Wnt1 vector. (A) The mRNA levels of Wnt1 were measured to detect the transfection efficiency using RT-qPCR. (B) Western blot analysis was performed to determine Wnt1, β-catenin, cyclin D1, and c-Myc protein levels following Wnt1 overexpression. β-actin served as internal reference. (C) The CCK-8 assay was used to assess cell viability. (D) Cell migration ability was evaluated by wound healing experiments (magnification, 100×). (E) Cell invasion ability was assessed using the transwell invasion assay. The cells in the lower layer of the chamber were fixed with 4% paraformaldehyde for 20 minutes and stained with crystal violet solution for 15 minutes, and finally photographed under a microscope (magnification, 400×). **P<0.01. RT-qPCR, quantitative reverse transcription polymerase chain reaction; CCK-8, cell counting kit-8.

Footnote

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Conflicts of Interest: All authors have completed the ICMJE
uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-3490). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Research Ethics Committee of the Affiliated Hospital of Southwest Medical University, and all patients provided signed informed consent. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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