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MiR-135b-5p inhibits the progression of malignant melanoma cells by targeting RBX1

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Abstract. – OBJECTIVE: The aim of this study was to investigate the potential effects of microRNA-135b-5p (miR-135b) on the development of malignant melanoma (MM) and the relevant mechanism.

PATIENTS AND METHODS: The expression level of miR-135b in MM tissues and cells was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Online prediction software and luciferase reporter assays were used to predict and verify the possible target of miR-135b, respectively. Furthermore, the effects of the miR-135b on MM A375 cells were determined by Western blotting, MTT, and transwell assays.

RESULTS: MiR-135b was significantly down-regulated in MM. RING-box protein 1 (RBX1) was verified as a direct target of miR-135b. Subsequent experiments showed that down-regulation of RBX1 resulted from miR-135b up-regulation could significantly inhibit the proliferation, invasion, and migration abilities of MM cells.

CONCLUSIONS: MiR-135b inhibited the progression of MM by targeting RBX1. Our findings revealed that miR-135b/RBX1 might be a potential therapeutic target for the treatment of MM.

Key Words:
MicroRNA-135b-5p (miR-135b), Malignant melanoma (MM), RING-box protein 1 (RBX1).

Introduction

Malignant melanoma (MM) is a highly malignant skin tumor. It is characterized by strong invasion, proneness to early metastasis, high mortality rate, as well as resistance to routine chemotherapy or radiotherapy. Currently, MM only accounts for less than 5% of all skin tumors. However, its mortality rate accounts for 65% of the total, with 50000 deaths every year[1]. Previous studies have found that the 5-year survival rate of MM patients after local excision is more than 90%. However, the rate of metastatic patients is lower than 5-10%, with a mean survival time of only 6-9 months[2]. Therefore, understanding the molecular mechanism of MM is of great importance for its early diagnosis and treatment.

RNA interference (RNAi) refers to gene silencing induced by double-stranded RNA in molecular biology. Its possible mechanism is to inhibit gene expression by blocking the transcription or translation of specific genes. RNAi is an accurate, efficient, and stable method of gene suppression, superior to anti-transcription[3]. MicroRNA is a kind of small, single-stranded, non-coding RNA with 22-25 nucleotides in length. MicroRNA regulates mRNA stability and expression by binding to the 3′ untranslated region (3′UTR) of target mRNAs[4]. Studies[5-9] have demonstrated that dysfunction of miRNAs affects the occurrence of a variety of tumors, including MM. This is possibly achieved by regulating the transcription and translation of oncogenes and tumor suppressor genes. Therefore, the roles of miRNAs in tumors have become a research hotspot in recent years. However, more reports[9] are still needed to further determine the significance of specific miRNAs and to explore efficient miRNA-targeted therapies to increase the survival rate of MM patients.

MicroRNA-135b-5p (miR-135b) has been found to play an important role in multiple life activities. For example, miR-135b inhibits the production of lipopolysaccharide (LPS)-induced reactive oxygen species (ROS), the activation of nuclear factor-κB (NF-κB), and the mRNA expression of tumor necrosis factor-α (TNF-α) in human macrophages by influencing adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) signaling[10]. In a cerebral ischemia/reperfusion model, miR-135b can effectively protect hypoxic neurons through the down-regulation of glycogen synthase kinase-3β (GSK-3β) and the promotion of Nrf2/ARE signaling pathway-mediated antioxidant responses[11]. However, in an animal model of

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heart ischemia/reperfusion, miR-135b improves myocardial damage, playing a negative role[12]. In the field of cancer research, miR-135b acts as a double-edged factor. It promotes the development of pancreatic cancer[13] and gastric cancer[14]. However, miR-135b can inhibit the proliferation of tumor cells in osteosarcoma[15]. Therefore, the aim of this work was to explore the expression and function of miR-135b in MM and to investigate its possible underlying mechanism.

Patients and Methods

MM Clinical Samples and Cell Lines

MM tissues and normal tissues were obtained from patients who received treatment in Shengli Oilfield Central Hospital. All collected tissue samples were confirmed by pathological examination. Liquid nitrogen was used to freeze the tissue samples. The Declaration of Helsinki should be mentioned and respected. This research was approved by the Ethics Committee of Shengli Oilfield Central Hospital. Informed consents were obtained from all participants before the study.

MM cell line (A375) and normal human epidermal melanocyte line (PEM) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C. The culture medium was replaced once every other day. Cell passage was performed when 80% of cell fusion. When the density of cells reached 70-80%, cell transfection was conducted according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then, the cells were routinely cultured under 5% CO_2 at 37°C for subsequent experiments.

Luciferase Reporter Gene Assay

Bioinformatics was used to predict the base complementary relationship between miR-135b and the 3′UTR of RBX1. The mutant-type vector of RBX1 3'UTR (3'UTR MUT) was constructed through point mutation. Subsequently, miR-135b mimics, its control miR-NC and RBX1 3'UTR wild-type (WT), as well as mutant-type vectors (MUT) were co-transfected into A375 cells. After 48 h, the cells were collected. Finally, firefly luciferase and *Renilla* luciferase activity were detected according to the instructions of the dual luciferase reporter assay kit (Promega, Madison, WI, USA).

Cell Transfection

MiR-135b mimics, NC-mimics, and LV-RBX1 were designed, synthesized, and transfected into A375 cells by using Lipofectamine 2000. All cells were divided into three groups, including NC group (negative control), miR-135b group (A375 cells transfected with miR-135b mimics), and miR-135b+RBX1 (A375 cells transfected with miR-135b mimics and LV-RBX1).

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Analysis

Gene expression in cells was detected *via* Real Time fluorescence quantitative Polymerase Chain Reaction (qRT-PCR). Total RNA in cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to measure the expression level of miR-135b and RBX1 expression normalized to U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. The QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: RBX1, F: 5'-CgACAGGACgCTCCTTACgCCTC-3', R: 5'-CAGGGATGTGTCCGTAGGAA-3'; miR-135b, F: 5'-AGTGTACCACACTCGAACTCTG-3', R: 5'-GTCCGTCGTGGAGAATTGCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

After different treatment, A375 cells were lysed with protein lysis buffer and the total protein samples were collected. After quantification, the same amount of protein sample was separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto membranes. After sealing with 5% skim milk powder for 1 h, the membranes were incubated with primary antibodies of RBX1 and GAPDH at room temperature for 1 h. After washing, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 1 h, followed by washing again. Color development was performed with chemiluminescence solution. Finally, gray values were analyzed using the software.

Cell Proliferation

A375 cells in logarithmic phase were collected and digested with 0.25% trypsin. After centrifugation, the cells were inoculated into 96-well plates at a density of 5×10^3 cells per well. Then the cells were cultured in an incubator with 5% CO_2 at 37°C. 6 repeated wells were set in each group. After 24 h, 10 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, followed by incubation for 3-4 h. Next, 150 μL of dimethyl sulfoxide (DMSO) was added to each well and shaken for 5-10 min to dissolve crystals. Optical density at 450 nm (OD_{450}) was detected using a microplate reader. Finally, MTT graph was plotted.

Cell Invasion and Migration

After transfection, the cells were digested with 0.25% trypsin and prepared into single-cell suspension with serum-free Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA). The concentration of cells was counted using a cell counter. After dilution, 400 μL of cell suspension (about 1×10^5 cells) were added into the upper transwell chamber (pore size: 8 μm). Meanwhile, 500 μL of DMEM containing 15% FBS was added into the lower chamber. Then, the cells were cultured for 24 h. Subsequently, cells on the upper surface of upper transwell chambers were wiped with cotton swabs and washed with phosphate-buffered saline (PBS) twice. Next, the cells were fixed with absolute methanol and stained with 0.4% crystal violet dye at room temperature for 2 h, followed by photography under a microscope. Three fields (upper, middle and lower) were randomly selected for each sample, with 3 replicates in each group. For cell invasion, the steps were the same as those of migration assay, except that the upper surface of upper transwell chambers was coated with Matrigel to simulate the extracellular matrix.

Statistical Analysis

Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided and $p<0.05$ was considered significant. Prism 6.02 software (La Jolla, CA, USA) was applied for all the statistical analysis.

Results

MiR-135b Expression Was Significantly Down-regulated in Both MM Tissues and Cells

QRT-PCR was used to detect the expression of miR-135b in 27 MM tissues and normal skin tissues. Results showed that the expression of miR-135b in MM tissues decreased significantly when compared with normal skin tissues (Figure 1A). Additionally, we detected the expression of miR-135b in two different cell lines [MM cell line (A375) and normal human epidermal melanocyte line (PEM)] as well. As shown in Figure 1B, the expression of miR-135b in A375 cells was significantly lower than that of PEM cells. All the above results demonstrated that miR-135b acted as a tumor suppressor gene in MM.

RBX1 Was a Direct Target of MiR-135b in MM Cells

Online prediction software provides many advantages for target gene detection of microRNAs.

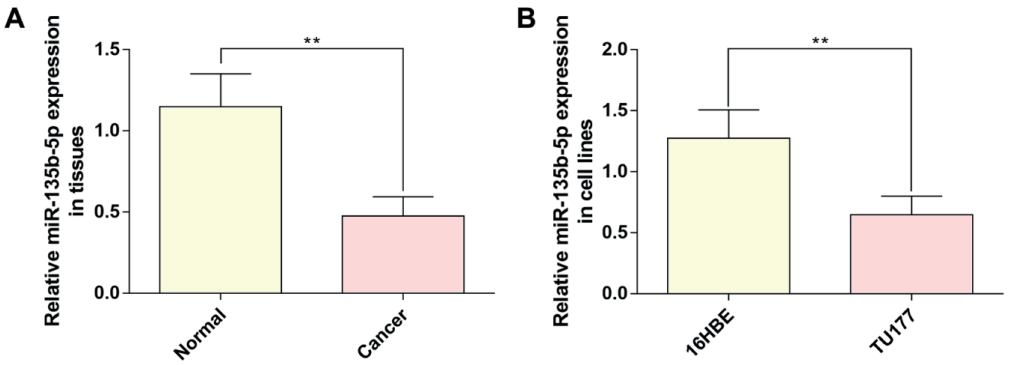

Figure 1. The expression of miR-135b in MM tissues and cells. **A,** Difference in the expression of miR-135b in MM tissues and normal skin tissues. (**$p<0.01$). **B,** Expression of miR-135b in cell lines. (**$p<0.01$).

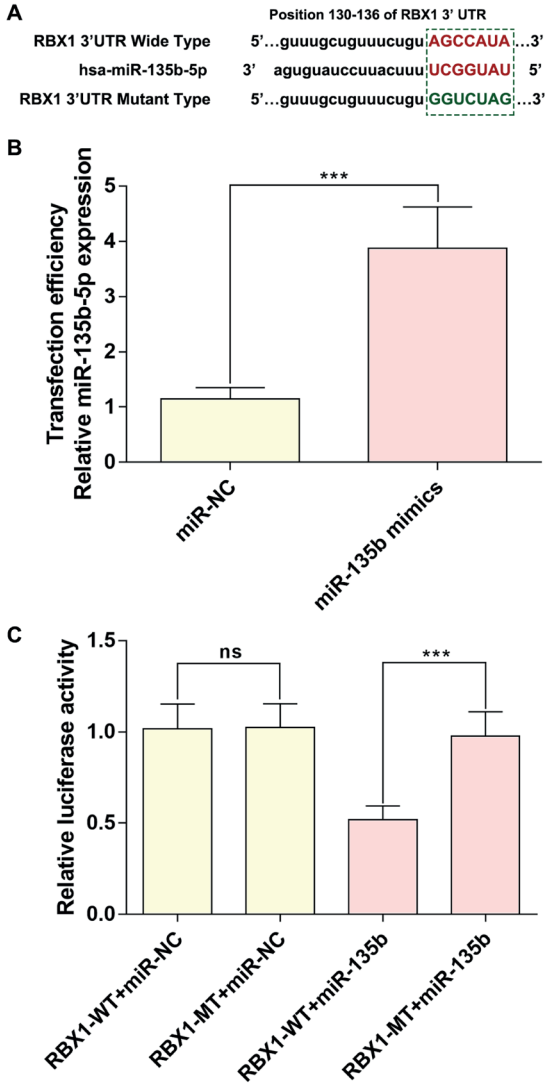

Figure 2. RBX1 was a direct and functional target of miR-135b. **A,** Diagram of putative miR-135b binding sites of RBX1. **B,** Transfection efficiency detected by qRT-PCR. (***p<0.001). **C,** Relative activities of luciferase reporters. (ns p>0.05, ***p<0.001).

In this study, three common websites (including TargetScan, miRDB, and microRNA) were used to search for the potential target genes of miR-135b. Finally, RBX1, an oncogene that could promote the malignant process of tumor cells, was screened out (Figure 2A).

To further explore the relationship between miR-135b and RBX1, we first established over-expressed miR-135b in A375 cells by miR-135b mimics transfection (Figure 2B). Subsequently, luciferase reporter vectors containing wild or mutant-type seed sequences of RBX1 3'-UTR (RBX1-WT and RBX1-MT) were established. Luciferase reporter gene assay indicated that miR-135b mimics transfection could significantly inhibit the luciferase activity of RBX1-WT. However, no significant changes were observed in the luciferase activity of RBX1-MT (Figure 2C). These results revealed the direct regulatory effect of miR-135b on RBX1.

MiR-135b Decreased the Expression Level of RBX1

From the perspective of RBX1 expression level, Western blotting assay was performed. The protein expression of RBX1 was found significantly reduced in A375 cells with overexpression of miR-135b when compared with control group. These results indicated the posttranscriptional regulation of miR-135b on RBX1 (Figure 3A).

MiR-135b Suppressed the Proliferation of MM Cells

The MTT assay was used to evaluate the changes in cell proliferation ability after different treatments. As shown in Figure 3B, the up-regulation of miR-135b could significantly reduce the proliferation of A375 cells. On the fourth day, the OD value of A375 cells in miR-135b group was significantly different from that of control group. However, there was no significant difference between the co-transfection group and control group.

MiR-135b Inhibited the Invasion and Migration of MM Cells

As shown in Figure 4, the invasion and migration of cells were detected by transwell assay. By photographing and counting the number of cells on the filtration membrane, it was found that the invasion and migration of A375 cells were significantly inhibited after transfection of miR-135b mimics. On the contrary, when we artificially up-regulated the expression of RBX1 in A375 cells with miR-135b overexpression, the migration and invasion of A375 cells returned to the same level as the control group. This evidence suggested that RBX1 was a functional target of miR-135b affecting the ability of A375 cells.

Discussion

MM is a highly malignant tumor derived from melanocytes. Early-stage MM is mainly treated by excision. However, the therapeutic effect is

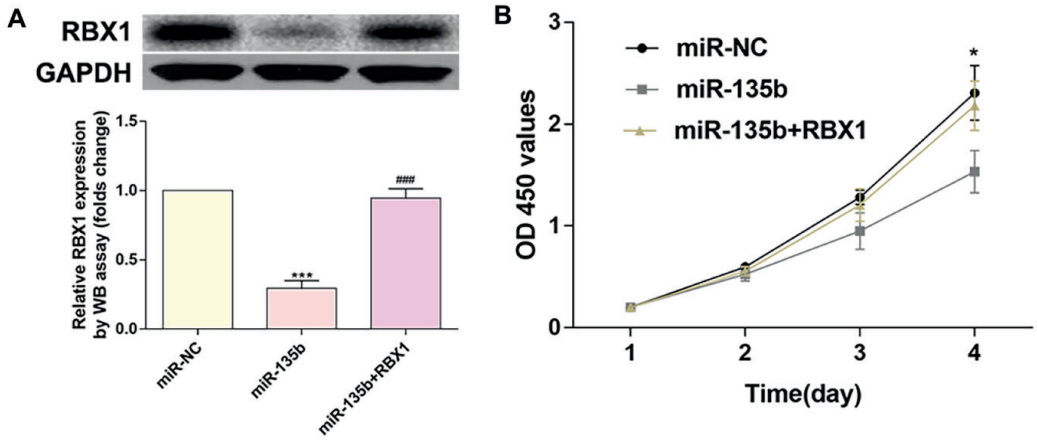

Figure 3. A, MiR-135b decreased the protein expression level of RBX1 detected by Western blot (***p<0.001 *vs.* miR-NC group; ###p<0.001 *vs.* miR-135b group). **B,** MiR-135b decreased the proliferation of MM cells. Cell proliferation detected by MTT assay (*p<0.05).

far from satisfactory for metastatic MM, which is extremely easy to relapse. In recent years, significant efficacy has been achieved in the molecular targeted therapy in the clinical treatment of MM. However, they have little effect on patients with advanced MM. Therefore, exploring the molecular mechanism of MM has become a new direction for MM treatment[16]. Calin et al[17] have confirmed that miRNA can regulate the development of tumors. Meanwhile, they can also specifically control the transcription and translation of target genes through base pairing, thereby regulating the expression of target genes. Moreover, miRNA is involved in regulating various biological behaviors of cells, whose expression level is closely related to MM development[18-21]. In the current study, we revealed that miR-135b was significantly lowly expressed in both MM tissues and cells.

After the clarification of differentially expressed miRNAs, further understanding of regulated targets has become another emphasis of studies on miRNAs. It has been manifested that miRNAs are able to regulate at least 1/3 of human genes. The same miRNA can regulate various genes, meanwhile, the same gene can be regulated by different miRNAs coordinately[4,22]. In this way, a complex regulatory network is formed by miRNAs and genes. With the development of genetic testing, the emergence of online prediction software has greatly helped the detection of miRNA targets[23-25]. In this work, RBX1 was screened out as a possible target gene for miR-135b in MM.

RBX1 is a kind of conserved protein that plays an important role in human embryogenesis[26,27]. Abnormal expression of RBX1 may lead to dysfunction of SCF ligase, eventually causing embryo damage[28]. Previously, Wei and Sun[29] have demonstrated that RBX1 not only plays an important regulatory role in various cellular physiological activities, but also shows a certain correlation with the occurrence and development of tumors. Xing et al[30] on non-small cell lung cancer (NSCLC) has indicated that high RBX1 expression is correlated with poor overall survival, suggesting that high RBX1 expression is an unfavorable prognostic factor for NSCLC patients. Likewise, Wang et al[31] has found that overexpression of RBX1 contributes to the progression and poor prognosis of non-muscle-invasive bladder transitional cell carcinoma (NMIBC). Migita et al[32] have revealed that RBX1 is identified as an independent prognostic factor for gastric cancer. Furthermore, silencing of RBX1 gene significantly inhibits the proliferation of gastric cancer cells *in vitro*. Our results suggested that protein expression of RBX1 was significantly down-regulated after up-regulating miR-135b expression in A375 cells. Changes in RBX1 affected many characteristics of tumor cells, including inhibition of the proliferation, migration, and invasion of A375 cells.

Conclusions

We revealed that miR-135b expression was significantly down-regulated in both MM tissues

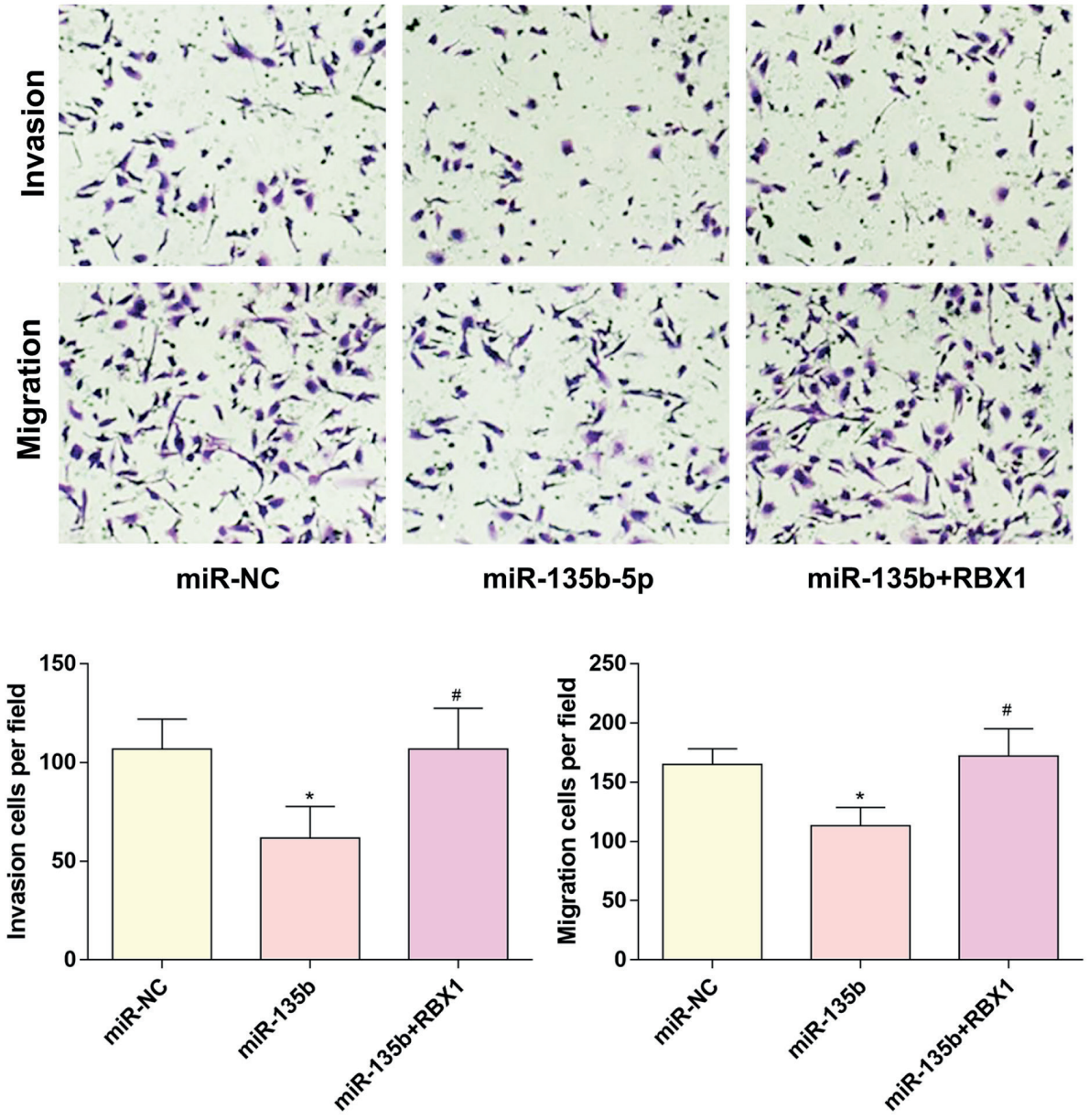

Figure 4. MiR-135b/RBX1 axis inhibited the invasion and migration of MM cells. Cell invasion and migration detected by transwell assay (magnification 40×). All data were presented as means ± standard deviations. (*p<0.05 *vs.* miR-NC group; #p<0.05 *vs.* miR-135b group).

and cell. By targeting RBX1, miR-135b significantly inhibited the malignant behaviors of MM cells. In addition, our findings might provide a new molecular therapeutic target for the MM treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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