MicroRNA-324-5p suppresses the migration and invasion of MM cells by inhibiting the SCF$^{\beta\text{-TrCP}}$ E3 ligase

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Received November 4, 2016; Accepted February 20, 2018

DOI: 10.3892/ol.2018.9245

Abstract. Multiple myeloma (MM) is a cytogenetically heterogeneous malignancy of plasma cells in bone marrow. Among the cytogenetic abnormalities of MM, del(17p) is a well-recognized high-risk genetic lesion associated with the late stage and progression of the disease. MicroRNA (miR)-324-5p, located at 17p13.1, was identified to be involved in the dysregulation of a number of types of malignant disease. However, whether miR-324-5p is associated with the development and progression of MM remains unknown. In the present study, the expression status of miR-324-5p in MM, and its effect on the migratory and invasive ability of MM cells were investigated. Using ubiquitination pathway polymerase chain reaction array, the inhibitory effect of miR-324-5p on the ubiquitinated proteins was investigated. It was identified that miR-324-5p levels were decreased in samples from patients with MM and MM cell lines. Increased expression of miR-324-5p by transfection of miR-324-5p mimic suppressed the proliferative, migratory and invasive abilities of MM.1R cells. Furthermore, increased expression of miR-324-5p in MM.1R cells inhibited the ubiquitination pathway and decreased the levels of ubiquitination-associated proteins, particularly the Skp1-Cullin1-F-box β-transducin repeat-containing protein (SCF$^{\beta\text{-TrCP}}$) E3 ligase. In addition, the results of the present study demonstrated that the SCF$^{\beta\text{-TrCP}}$ E3 ligase may contribute to the suppression of MM cell motility by inhibiting the expression of metastasis-associated genes, including metastasis suppressor 1. In conclusion, the results of the present study suggested that miR-324-5p may act as a tumor suppressor by impairing the motility of MM cells by suppressing the ubiquitination pathway.

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Key words: multiple myeloma, microRNA-324-5p, ubiquitination, β-transducin repeat-containing protein, metastasis suppressor 1, migration

Introduction

Multiple myeloma (MM) is a cytogenetically heterogeneous plasma cell dyscrasia, characterized by the abnormal proliferation of malignant plasma cell in bone marrow. Almost all MM evolves from its premalignant stage, monoclonal gammopathy of undetermined significance (MGUS) (between 0.5 and 3% of patients with MGUS annually), and between 2 and 4% of MM may transform into plasma cell leukemia (PCL) (1-3). Del(17p) in MM is a well-recognized high-risk genetic lesion associated with the late stage and progression of the disease (4). Although the incidence of del(17p) in MM is nearly 11%, it increases to 50% in primary PCLs and reaches <75% in secondary PCLs (2,5). A number of critical function genes are located in 17p and del(17p) may result in the progression of MM through abnormalities of those genes.

MicroRNA (miR)-324-5p maps to 17p13.1 and its dysregulation was identified to be involved in a number of types of malignant disease. Ferretti *et al* (6), demonstrated that the decreased expression of miR-324-5p was able to be determined by del(17p) in human medulloblastoma (MB). In certain hematopoietic cell lines, the expression of miR-324-5p was identified to be influenced by copy number status (7). It was also identified that ectopic expression of miR-324-5p may suppress the invasion and metastasis of hepatocellular carcinoma (HCC) cells by counteracting extracellular matrix (ECM) degradation (8). It is hypothesized that the expression and function of miR-324-5p may vary with the chromosomal status of different types of tumor, and be involved in different mechanisms of pathogenesis.

The ubiquitin-proteasome system (UPS) is of great importance in the homeostasis of protein processing in MM cells (9). Proteasome-mediated degradation of proteins is the terminal stage of the UPS. F-box proteins (FBPs) are components of the

SCF (Skp1-Cullin1-F-box protein) type E3 ligase complexes that serve as the substrate recognition subunit which ubiquitinate and target various proteins for degradation by the proteasomes (10,11). To date, ~69 FBPs have been identified in the human genome, including β-transducin repeat-containing protein (β-TrCP, encoded by the BTRC gene). $SCF^{β\text{-}TrCP}$ E3 ligase operates by regulating diverse proteins in multiple signaling pathways, including Gli-1 in the Hedgehog pathway, inhibitor of nuclear factor κB (NF-κB) in the NF-κB pathway and β-catenin in the Wnt pathway (12,13). Mutation of β-TrCP in murine 5TGM1 myeloma cells was identified to significantly decrease the tumor burden of mice bearing this type of transformed cell (14). However, it remains to be elucidated how β-TrCP functions in MM.

Metastasis suppressor 1 (MTSS1), also known as missing in metastasis or basal cell carcinoma-enriched gene 4, is an actin- and membrane-binding protein (15). MTSS1 was identified as a metastasis suppressor in various types of solid tumor, including HCC, breast cancer and bladder cancer (16-18). MTSS1 was identified to be vital for B-cell development, as its knockout may be associated with lymphomagenesis in mice (19). The tumor-suppressive function of MTSS1 has been suggested in acute and chronic myeloid leukemia (20,21). In certain cancer cell lines, MTSS1 was able to be directly ubiquitinated and degraded by β-TrCP (22). The function of MTSS1 in MM, and whether the UPS participates in the regulation of MTSS1, particularly in the later stage of the disease, remains to be investigated.

In the present study, the effect of miR-324-5p on the regulation of the ubiquitination pathway was investigated in MM. The results identified that the expression of miR-324-5p was significantly downregulated in patients with Revised International Stage System (RISS) (23) stage III MM. Overexpression of miR-324-5p in MM cells suppressed the ubiquitination pathway by suppressing the expression of ubiquitination-associated genes, particularly BTRC. By regulating the β-TRCP/MTSS1 axis, miR-324-5p inhibits the motility of MM cells. Together, miR-324-5p as a tumor-suppressive microRNA (miRNA) and β-TRCP as an oncogene in MM may be novel targets for developing therapeutic strategies for the treatment of MM.

Materials and methods

Samples from MM patients and MM cell lines. Primary plasma cells were purified from bone marrow aspirates of 26 patients newly diagnosed with MM and two healthy control (HC) volunteers using cluster of differentiation (CD)138 magnetic beads (Miltenyi Biotec, Inc., Auburn, CA, USA) as described previously (24). Risk stratifications of all patients with MM was defined according to the RISS for MM (serum $β_2$-microglobulin, serum albumin, serum lactate dehydrogenase and chromosomal abnormalities detected using interphase fluorescent *in situ* hybridization) (23). The purity of plasma cells was assessed using flow cytometric analysis and was >95%. CD138+ plasma cells were purified using CD138 magnetic beads (Miltenyi Biotec, Auburn, CA, USA), they were stained with Anti-Igκ Light Chain-FITC (cat. no., 130-093-053, Miltenyi Biotec, Inc.), Anti-Igλ Light Chain-APC (cat. no., 130-093-043, Miltenyi Biotec, Inc.), and CD138-PE (cat. no., 130-081-301, Miltenyi Biotec, Inc.) according the manufacturer's protocol, and analyzed using a flow cytometer. Written informed consent was obtained from all participating patients and HCs. The present study was approved by the Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China).

The human MM cell lines RPMI-8226, U266, MM.1R and MM.1S were purchased from the American Type Culture Collection (Manassas, VA, USA). OPM-2 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). NCI-H929 was a gift from Dr Jian Hou (Second Military Medical University, Shanghai, China). All six cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum at 37˚C in a humidified incubator containing 5% CO_2. MG132 was purchased from Sigma; Merck KGaA (Darmstadt, Germany). MG132, as a proteasome inhibitor, was used to explore its influence on the expression of related genes in MM cell lines. The appropriate concentrations of MG132 for MM cell lines were determined by calculation of their half-maximal inhibitory concentration (IC_{50}) values.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-PCR). Total RNA isolation and the subsequent expression analysis of miRNA and mRNA were performed as described previously (25). The reverse transcription and quantitative amplification for miR-324-5p was performed using a TaqMan® MicroRNA Reverse Transcription kit and TaqMan® Universal Master Mix II (no uracil N-glycosylase) kit respectively (Thermo Fisher Scientific, Inc.). U6 was used as the internal control. The primers of miR-324-5p (cat. no., 000539) and U6 (cat. no., 001973) was purchased from Life Technology (Thermo Fisher Scientific, Inc.). The thermocycling conditions are as follows: Reverse transcription: 16˚C for 30 min, 42˚C for 30 min, 85˚C for 5 min, and held at 4˚C; qPCR: 95˚C for 10 min, and then 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

For mRNAs, cDNA was synthesized using a PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) and used as templates for PCR using a SYBR Green RT-PCR kit (Takara Biotechnology Co., Ltd.). GAPDH was used as the internal control. The primer sequences used were as follows: BTRC, forward, 5'-TGCTCT ATGCCCAGGTCTCT-3', reverse, 5'-AGGGGGTTCGCC ATTATTAC-3'; MTSS1, forward, 5'-TTCCAGACCATCATC AGCGA-3', reverse, 5'-GCTTCAATGCTTCTGTGCCT-3'; GAPDH, forward, 5'-GGTCGGAGTCAACGGATTTG-3, reverse, 5'-GGAAGATGGTGATGGGATTTC-3'. The thermocycling conditions were as follows: Reverse transcription, 37˚C for 15 min, 85˚C for 5 sec, and kept at 4˚C or -20˚C; qPCR, 95˚C for 10 min, and then 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

All PCRs were performed in triplicate using an AB 7500 Fast Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression for each gene was calculated using the $2^{-ΔΔCq}$ method (26).

miRNA transfection. MM cell lines were cultured with fresh medium in a 6-well plate in duplicate ($1x10^6$ cells/well) and transfected with miR-324-5p mimic or its negative control

(mimic-NC). miR-324-5p mimic (5'-CGCAUCCCCUAGGGC AUUGGUGU-3') and mimic-NC (5'-UUUGUACUACACAAA AGUACUG-3') were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Transfection was performed with ribo*FECT*™ CP Transfection kit (Guangzhou RiboBio Co., Ltd.) and the final concentration of oligonucleotides was 50 nM. Variation in miRNA expression was validated using TaqMan RT-PCR, as aforementioned. Following transfection, cells were cultured for between 48 and 96 h, and collected for subsequent experiments.

Proliferation assay. A Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to determine cell proliferation. For the assay, cells were plated in 96-well plates in duplicate ($8x10^3$ cells/well) and transfected with miR-324-5p mimic or mimic-NC. CCK-8 reagent (10 μl/well) was added to the plates at the indicated time points. Following incubation for 4 h, absorbance was determined using a 96-well multiscanner autoreader (BioTek Instruments, Inc., Winooski, VT, USA) at 450 nm.

Ubiquitination pathway PCR array analysis. Total RNAs were extracted from MM.1R cells transfected with miR-324-5p mimic or mimic-NC. cDNAs were synthesized from the RNA templates with a RT^2 First Strand Kit (cat. no. 330401; Qiagen China Co., Ltd., Shanghai, China) and used in an RT^2 SYBR Green Mastermix (cat. no. 330401; Qiagen China Co., Ltd., Shanghai, China) for Ubiquitination RT^2 Profiler PCR Array analysis (cat. no. PAHS-079Z; Qiagen China Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The expression of genes with a fold change >2 ($P<0.05$) was identified.

Western blot analysis. Protein extraction and western blot analysis were performed as described previously (25), with minor alterations, as outlined below. The quantification analysis for western blotting was accomplished with Image Lab (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA). In brief, total proteins from cell lysates were separated by SDS-PAGE (8 or 10% gels) and transferred onto polyvinylidene fluoride (0.45 μm pore size) membranes. The primary antibodies were incubated at 4˚C overnight and the secondary antibodies were incubated at room temperature for 1 h. The membranes were blotted with anti-β-TrCP antibody (dilution, 1:800; cat. no. 4394S; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-MTSS1 antibody (dilution, 1:500; cat. no. 4385S; Cell Signaling Technology, Inc.), anti-matrix metalloproteinase (MMP)9 (dilution, 1:600; cat. no. AM1975b; Abgent Biotech Co., Ltd., Suzhou, China), anti-MMP2 (dilution, 1:1,000; cat. no. AP13693c; Abgent Biotech Co., Ltd.), anti-GAPDH antibody (dilution, 1:4,000; cat. no. A109916A; AntGene Biotechnology Co., Ltd., Wuhan, China) and anti-β-actin antibody (dilution, 1:4,000; cat. no. A107856A; AntGene Biotechnology Co., Ltd.), followed by horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit: Dilution 1:1,500, cat. no. A201708; goat anti-mouse: Dilution 1:2,000, cat. no. A109258A; AntGene Biotechnology Co., Ltd.) and finally visualized using an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc.).

Transwell migration/invasion assays. Transwell assays were performed as described previously (27). The migratory and invasive abilities were determined using 24-well Transwell chambers with 8 μm pore size polycarbonate membranes (Costar, Corning Incorporated, Corning, NY, USA) which were uncoated or coated with Matrigel (BD Biosciences; Franklin Lakes, NJ, USA), respectively. The experiment was performed three times with three wells per group. The results of Transwell assays was visualized under an inverted light microscope (Nikon, Tokyo, Japan) at x400, magnification.

Statistical analysis. All data were analyzed using SPSS software (version 17; SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 5.0; GraphPad Software, San Diego, CA, USA). Results are presented as the mean ± SD were analyzed using the Mann-Whitney U test or analysis of variance and Bonferroni's correction. $P<0.05$ or $P<0.0167$ was considered to indicate a statistically significant difference. The correlation analyses between the expression of miR-324-5p and other genes, or between MG132 concentration and gene expressions were accomplished using Pearson's correlation coefficient.

Results

Expression of miR-324-5p in patients with MM and MM cell lines is downregulated and negatively associated with disease stage. First, the expression levels of miR-324-5p were determined and it was identified that miR-324-5p in plasma cells from bone marrow aspirates of patients newly diagnosed with MM was downregulated during the disease progression, and its expression in patients with stage III MM ($P<0.05$) was significantly decreased compared with in patients with stage I MM (Fig. 1A). No significant difference was identified between patients with stage I MM and patients with stage II MM. Furthermore, miR-324-5p expression was decreased in all six MM cell lines investigated compared with HCs (Fig. 1B). The miR-324-5p expression level in MM.1R cells was the lowest.

Effect of miR-324-5p on the ubiquitination pathway. The effect of miR-324-5p overexpression on the ubiquitination pathway was investigated. Compared with mimic-NC-transfected cells, increased expression of miR-324-5p in miR-324-5p mimic-transfected MM.1R cells was confirmed using RT-qPCR ($P=0.0011$; Fig. 1C). The expression levels of ubiquitinated proteins in MM.1R cells were slightly decreased by the overexpression of miR-324-5p (Fig. 1D). Among the 89 genes investigated using the Ubiquitination Pathway PCR Array, five genes (BTRC, DAZ-interacting protein 3, HECT, C2 and WW domain-containing E3 ubiquitin protein ligase 2, ubiquitin protein ligase E3 component N-recognin 2 and von Hippel-Lindau protein) were downregulated and only one (CBL) was upregulated (Table I). Specifically, BTRC was most downregulated with a 2.42-fold decrease.

Effect of miR-324-5p on the β-TrCP/MTSS1 axis in MM cells. BTRC encodes β-TrCP and is located at 10q24.32. The metastasis-associated gene MTSS1 has been identified as the target of β-TrCP (22). In the present study, the expression of BTRC

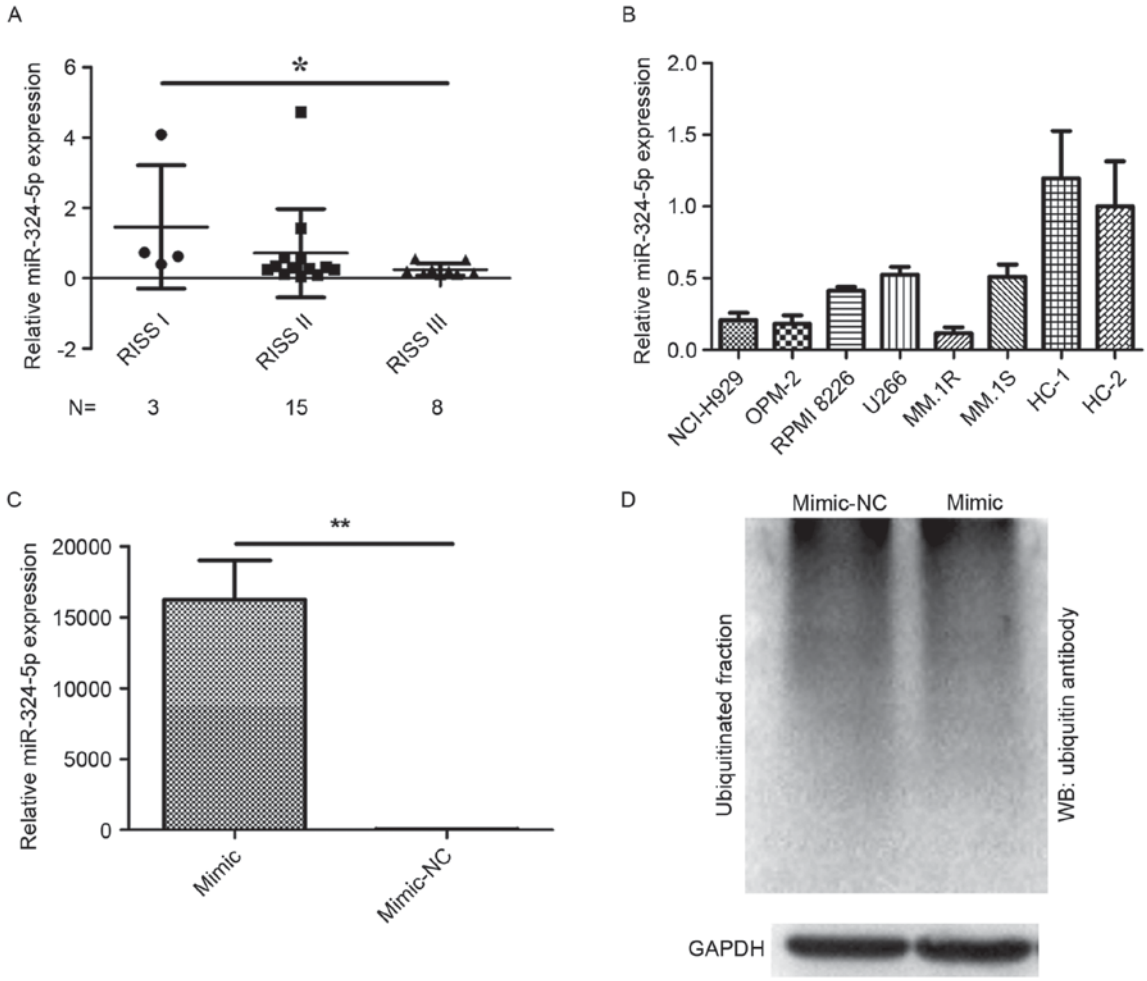

Figure 1. Expression status of miR-324-5p in patients with MM and MM cell lines, and its effect on the levels of ubiquitinated proteins. (A) Using the reverse transcription-quantitative polymerase chain reaction, the expression levels of miR-324-5p were determined in patients with MM. Results between different RISS groups were compared using the Mann-Whitney U test with Bonferroni's correction. A significant difference was identified between patients with stage III and I MM. *P<0.05. (B) Expression of miR-324-5p in the six MM cell lines was decreased compared with in HCs. (C) MM.1R cells were transfected with miR-324-5p mimic and the mimic negative control and cultured for a further 48 h. A significant difference in miR-324-5p expression was identified between MM.1R cells transfected with mimic and the cells transfected with mimic-NC. **P<0.01. (D) Ubiquitinated proteins in MM.1R cells transfected with mimic or mimic-NC were extracted for WB analysis. The level of ubiquitinated proteins was slightly decreased by overexpression of miR-324-5p. MM, multiple myeloma; miR, microRNA; RISS, Revised International Stage System; HC, healthy control; WB, western blot; NC, negative control.

in the majority of the MM cell lines was increased compared with that in HCs (Fig. 2A and B). However, MTSS1 expression in the MM cell lines was low or undetectable (Fig. 2A and B). Correlation analysis identified that the expression levels of miR-324-5p and BTRC mRNA were negatively correlated (Pearson correlation coefficient=-0.374). In addition, the expression of BTRC and MTSS1, at either the mRNA or the protein level, was negatively correlated (Pearson correlation coefficient=-0.538 and -0.678, respectively). Furthermore, the expression levels of BTRC and MTSS1 were investigated in MM cell lines in which the expression of miR-324-5p was upregulated. The results identified that BTRC was down-regulated significantly by overexpressed miR-324-5p in MM.1R (P=0.0153) and NCI-H929 (P=0.0111) cells (Fig. 2C). However, the expression of MTSS1 in MM.1R (P=0.0026) and NCI-H929 (P=0.0386) cells was significantly upregulated with incresed expression of miR-324-5p (Fig. 2D). The same trend was confirmed at the protein level in MM.1R and H929 cell lines with the upregulation of miR-324-5p expression (Fig. 2E and F).

Effect of MG132 on the β-TrCP/MTSS1 axis in MM cells. Furthermore, for the purpose of identifying the regulation of β-TrCP on MTSS1, the proteasome inhibitor MG132 was used to restrain the expression of β-TrCP and the expression levels of MTSS1 were determined. According to IC_{50} analysis, final concentrations of 0.1, 0.15 and 0.20 μM were used for the treatment of MM.1R cells, and final concentrations of 5, 25 and 50 μM were used for the treatment of NCI-H929 cells. For MM.1R cells, the expression of β-TrCP was decreased in a concentration-dependent manner (Fig. 3A). Conversely, the expression of MTSS1 was gradually increased with the increase in MG132 concentration. Similar results were observed in NCI-H929 cells (Fig. 3B). The correlation analysis revealed that the expression of β-TrCP and MTSS1 was negatively correlated in MM.1R (Pearson correlation coefficient=-0.603) and NCI-H929 (Pearson correlation coefficient=-0.538) cells following treatment of MG132.

miR-324-5p suppresses the proliferation and motility of MM cells. The proliferation of MM.1R cells transfected with

Table I. Results of ubiquitination pathway array.

	Gene	Variation (A/B)
Downregulation	BTRC	2.42
	DZIP3	2.29
	HECW2	2.20
	UBR2	2.15
	VHL	2.05
Upregulation	CBL	2.89

BTRC, β-transducin repeat-containing protein; DZIP3, DAZ-interacting protein 3; HECW2, HECT, C2 and WW domain-containing E3 ubiquitin protein ligase 2; UBR2, ubiquitin protein ligase E3 component N-recognin 2; VHL, von Hippel-Lindau protein.

miR-324-5p mimic was significantly suppressed compared with MM.1R cells transfected with mimic-NC after 48 h (Fig. 4A).

MTSS1 has been verified as a metastasis suppressor in a number of tumors (17,18). Therefore, it was investigated whether miR-324-5p was able to affect the motility of MM cells. MM.1R cells were transfected with miR-324-5p mimic or mimic-NC, cultured for 48 h, and subsequently collected for a Transwell assay. The results identified that overexpressed miR-324-5p in MM.1R cells significantly restricted cell migratory ability ($P<0.01$; Fig. 4B); images are presented in Fig. 4C. Furthermore, the invasive ability of MM.1R was inhibited (Fig. 4D). Additionally, the expression of MMP2 and MMP9 in MM cells transfected with miR-324-5p mimic was decreased compared with the cells transfected with mimic-NC (Fig. 2E and F). In summary, overexpressed miR-324-5p suppressed the proliferation and motility of MM cells.

Discussion

The results of the present study revealed that the expression levels of miR-324-5p in patients with stage III MM were significantly decreased compared with that in patients with stage I MM. In addition, miR-324-5p levels in MM cell lines were also downregulated compared with $CD138^+$ cells from HCs. We hypothesize that miR-324-5p may serve a vital function in MM. It was identified that overexpressed miR-324-5p was able to suppress ubiquitination pathway activity and decreased the levels of ubiquitinated proteins in MM cells. Using ubiquitination pathway PCR arrays, certain genes whose expression was downregulated by ectopic miR-324-5p were identified and BTRC (encoding β-TrCP) was the most significantly decreased gene. Subsequently, the expression status of β-TrCP and the functions of miR-324-5p were examined in MM cell lines. Results suggested that the expression of β-TrCP in MM cell lines was upregulated, whereas ectopic miR-324-5p was able to reverse this. Furthermore, the motility of MM cells was repressed by miR-324-5p overexpression.

miR-324-5p, located at 17p13.1, has been identified to be associated with various types of cancer. In HCC, miR-324-5p was downregulated and associated with the invasion of HCC cells (8). The downregulation of miR-324-5p in MB resulted from del(17p) (6). Del(17p) is considered one of the secondary cytogenetic abnormalities which occurs with progression of MM and predicts an adverse prognosis irrespective of the treatment context of patients with MM (28-30). The results of the present study indicated that miR-324-5p in patients with stage III MM was significantly downregulated and the levels of miR-324-5p in MM cell lines were decreased compared with in HCs. Therefore, we hypothesized that decreased expression of miR-324-5p may be associated with the progression of MM.

The UPS serves an important function in cellular protein homeostasis and dysfunction of the system is important in pathogenesis, including tumorigenesis, particularly of MM (31,32). According to the results of the present study, increased expression of miR-324-5p was able to repress the ubiquitination pathway by inhibiting the expression of β-TrCP in MM. β-TrCP is a vital member of the F-box proteins in UPS and a key component of the $SCF^{β\text{-}TrCP}$ E3 ligase complex (12,33). In triple-negative breast cancer, β-TrCP was decreased by phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor and its knockdown decreased the proliferation of tumor cells (34). Compared with the mutated β-TrCP, the wild-type β-TrCP in the murine 5TGM1 myeloma cell was able to promote the myeloma cell proliferation and increase the tumor burden in the mice which were inoculated with 5TGM1 cells (14). The expression level of β-TrCP in all six MM cell lines screened in the present study was significantly increased compared with in HCs. Ectopic miR-324-5p significantly suppressed β-TrCP expression in MM cells. In addition, increased expression of miR-324-5p markedly suppressed the proliferation of MM.1R cells. Collectively, these results indicate that β-TrCP may act as an oncogene in MM and be repressed by miR-324-5p.

Zhong *et al* (22), demonstrated that β-TrCP regulated the stability of MTSS1 and mediated its degradation by the 26S proteasome. In the present study, it was identified that the expression of MTSS1 was increased when β-TrCP was suppressed by overexpression of miR-324-5p or by MG132 treatment. Furthermore, there was a negative correlation between β-TrCP and MTSS1 in MM cell lines. MTSS1 has been identified to be associated with migration and invasion of various types of cancer. The overexpression of MTSS1 may inhibit the proliferation, colony formation, migration and invasion of glioblastoma cells (35). In the aggressive tumor cholangiocarcinoma, loss of MTSS1 was associated with lymph nodal metastasis and overexpressed MTSS1 was able to decrease cell migration (36). In the present study, it was identified that the overexpressed miR-324-5p significantly inhibited the migratory and invasive ability of MM cells. This may be partly attributed to the decreased expression of MMP-2 and MMP-9. Therefore, increased expression of miR-324-5p may inhibit the migratory and invasive ability of MM cells, and dysregulation of the β-TrCP/MTSS1 axis may be the underlying molecular mechanism.

Taken together, the results of the present study indicate that miR-324-5p may be a tumor suppressor in MM and associated with the progression of this disease. It may suppress the ubiquitination pathway and inhibit the migration/invasion of MM cells by targeting the β-TrCP/MTSS1 axis. The results of the present study emphasized the potential therapeutic function of miR-324-5p for patients with MM, particularly those in disease progression.

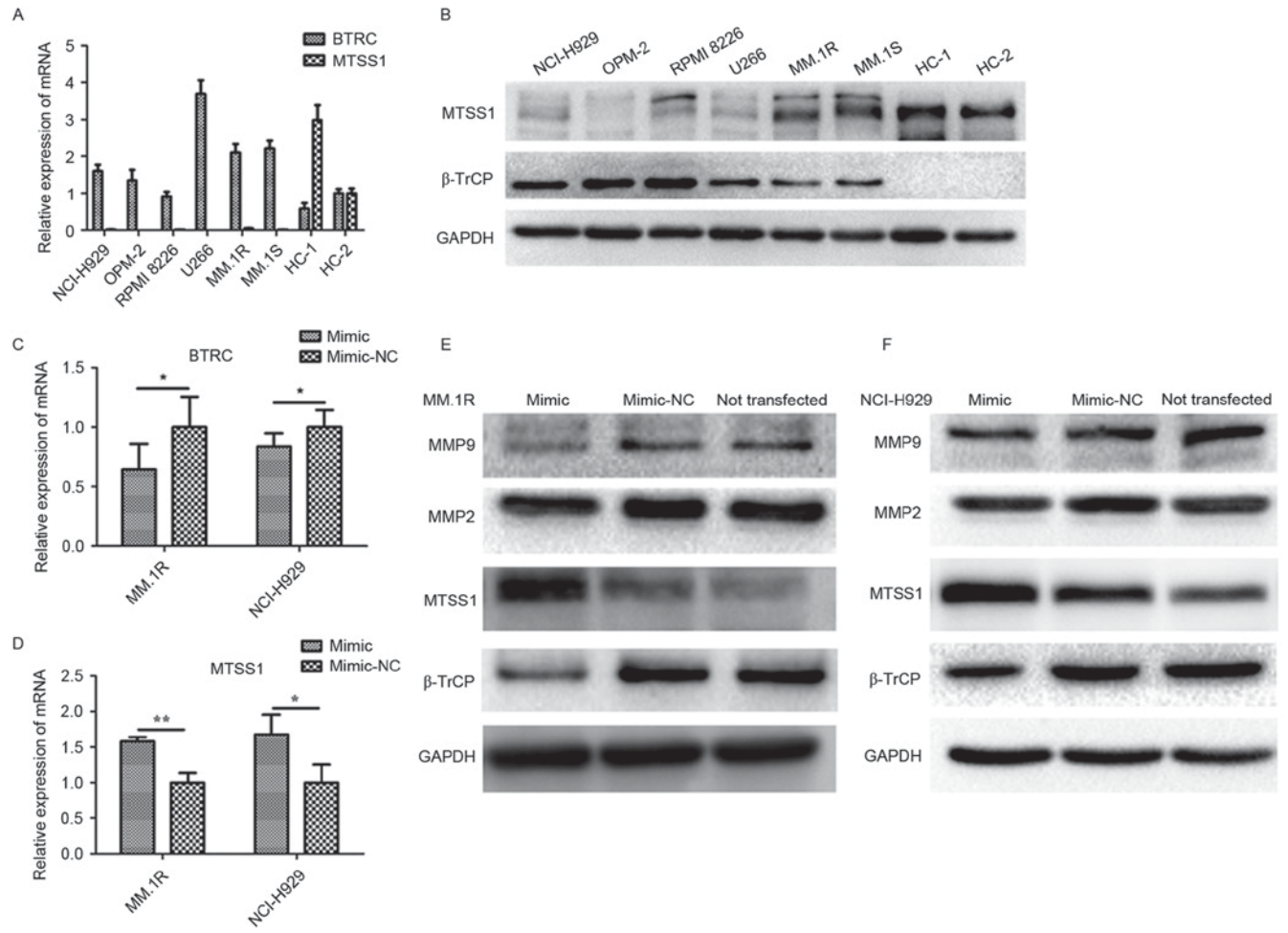

Figure 2. Expression of BTRC and certain metastasis-associated genes, and their variation when miR-324-5p was upregulated. (A) The mRNA expression levels of BTRC and MTSS1 in MM cell lines and HCs were determined using RT-qPCR. The expression of BTRC was negatively correlated with the expression of MTSS1 (Pearson correlation coefficient=-0.538). (B) Protein levels of β-TrCP and MTSS1 in MM cell lines were determined using western blot analysis. As well as the mRNAs, β-TrCP and MTSS1 proteins were also negatively correlated (Pearson correlation coefficient=-0.678). MM.1R or NCI-H929 cells inoculated in 6-well plates were transfected with miR-324-5p mimic, mimic-NC or not transfected. Using RT-qPCR, mRNA expression of (C) BTRC in MM.1R (P=0.0153) and NCI-H929 (P=0.0111) cells, and (D) MTSS1 in MM.1R (P=0.0026) and NCI-H929 (P=0.0386) were significantly decreased by overexpression of miR-324-5p. Variations in the level of the proteins β-TrCP, MTSS1, MMP2 and MMP9 were determined using WB analysis in (E) MM.1R and (F) NCI-H929 cells. β-TrCP was markedly suppressed by the miR-324-5p mimic, but MTSS1 was markedly increased. Levels of the other metastasis-associated proteins, MMP2 and MMP9, were decreased. *P<0.05, **P<0.01. BTRC/β-TrCP, β-transducin repeat-containing protein; miR, microRNA; MM, multiple myeloma; MTSS1, metastasis suppressor 1; HC, healthy control; NC, negative control; WB, western blot; MMP, matrix metalloproteinase.

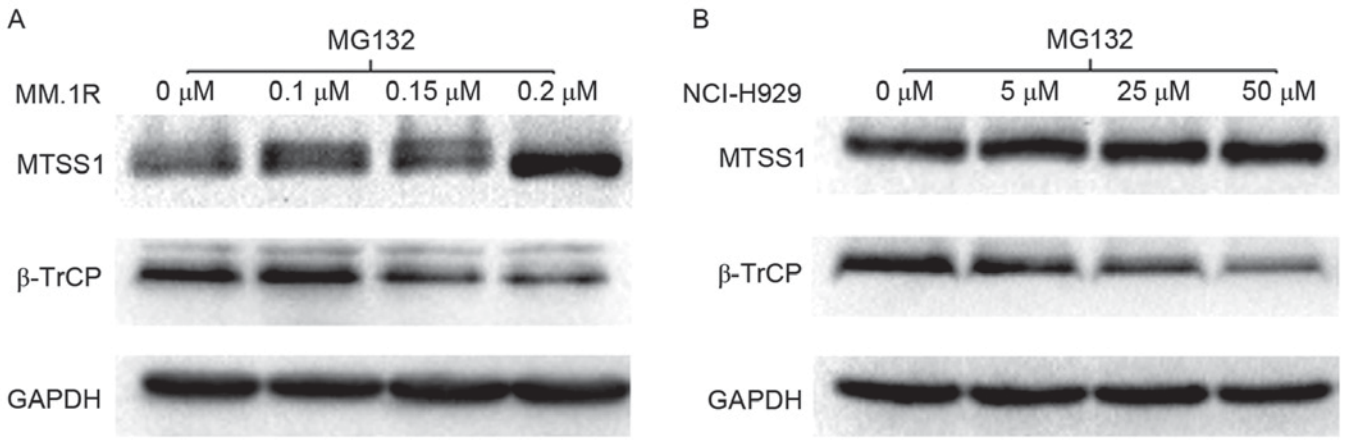

Figure 3. Effect of MG132 on the protein levels of β-TrCP and MTSS1. (A) In MM.1R cells, β-TrCP was decreased by MG132 in a concentration-dependent manner. Furthermore, the expression of MTSS1 was increased over the same concentrations. A negatively correlation was identified (Pearson correlation coefficient=-0.603). (B) For NCI-H929 cells, as in MM.1R cells, there was an inverse correlation between β-TrCP and MTSS1 expression (Pearson correlation coefficient=-0.538). β-TrCP, β-transducin repeat-containing protein; MTSS1, metastasis suppressor 1.

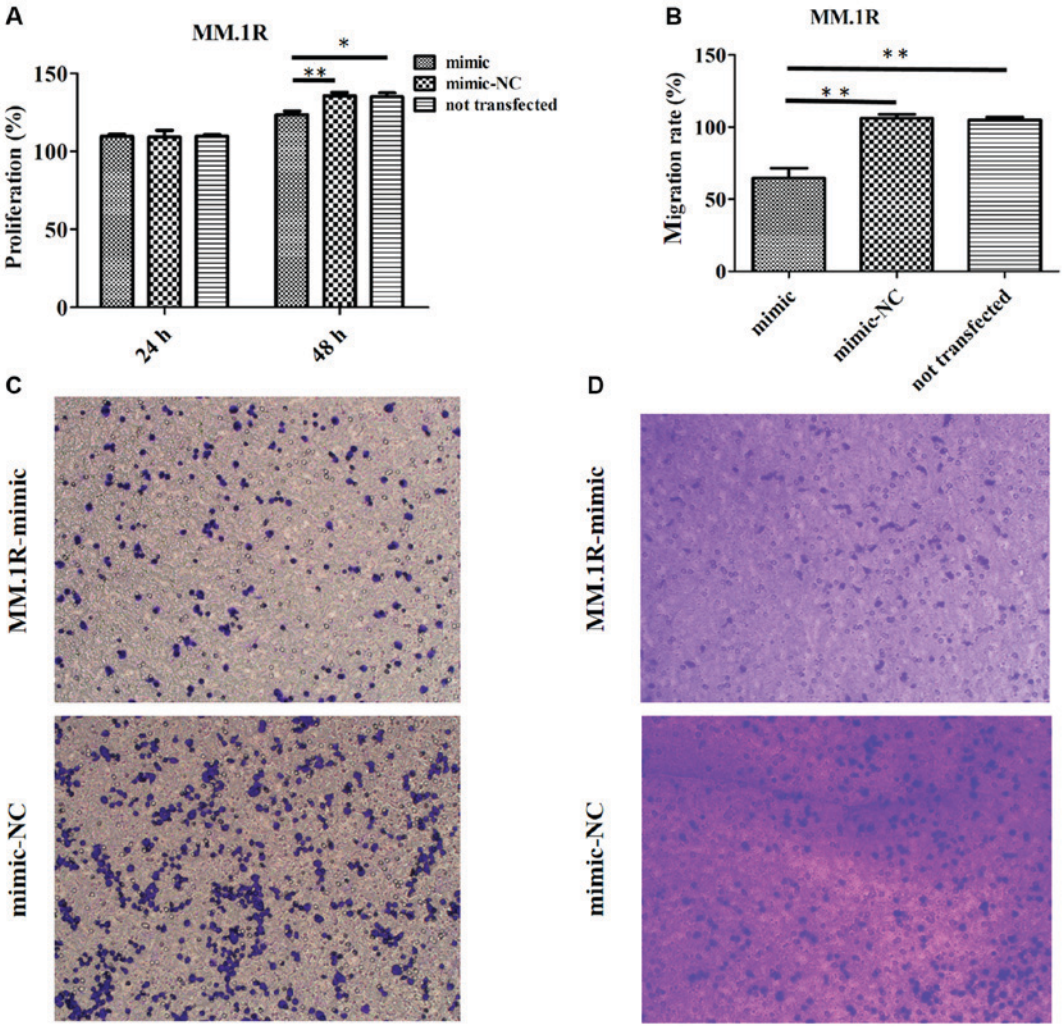

Figure 4. Proliferation and motility of MM.1R cells. (A) Proliferation was assessed using CCK-8 assays. Following transfection with miR-324-5p mimic, mimic-NC or not transfected, the proliferation of MM.1R cells was significantly suppressed at 48 h. (B) The motility of MM.1R cells were analyzed using a 24-well Transwell chamber and the number of MM.1R cells that had migrated into the lower chamber was determined using a CCK-8 assay. There was a significant difference between MM.1R cells transfected with miR-324-5p mimic and those transfected with mimic-NC. Results were analyzed using analysis of variance and Bonferroni's correction. $^{*}P<0.05$, $^{**}P<0.01$. (C) The difference in the migratory ability of MM.1R cells resulting from overexpressed miR-324-5p was visualized using an inverted microscope at x400, magnification. (D) Following coating of the upper Transwell chamber with Matrigel, the transfected MM.1R cells were added and 48 h later the invasive ability was analyzed. The invasive ability was similarly suppressed. CCK-8, Cell Counting Kit-8; miR, microRNA; NC, negative control.

Acknowledgements

Not applicable.

Funding

The present study was funded by the National Natural Science Foundation of China (grant no. 81272625).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LZ, DL, CS and YH contributed to study conception and design. LZ, DL, YS, CS and YH were responsible for development of methodology. LZ, DL, BT, AX, HH, JX and JD were responsible for acquisition of data. LZ, DL, BT, CS and YH performed data analysis and interpretation. LZ, DL, LT, CS and YH wrote, reviewed and revised the manuscript. DL, CS and YH provided study supervision. LT, CS, and YH approved the version to be published.

Ethics approval and consent to participate

Written informed consent was obtained from all participating patients and healthy controls. The present study was approved by the Institutional Review Board of Huazhong University of Science and Technology (Wuhan, China).

Patient consent for publication

All the participating patients and healthy volunteers consented to the publication of the present study.

Competing interests

The authors declare that they have no competing interests.

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