

MiR-150 inhibits proliferation of mantle-cell lymphoma cells *via* regulation of MET

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Abstract. – **OBJECTIVE:** The aim of this study was to explore the influences of micro ribonucleic acid (miR)-150 on the proliferation and apoptosis of mantle-cell lymphoma (MCL) cells and to investigate the potential underlying mechanism.

PATIENTS AND METHODS: Differentially expressed miRNAs in MCL tissues were excavated *via* microarray analysis of miRNA expression profiles. Subsequently, the expression of miRNAs were verified by quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR). The influence of miRNA expression on the survival of patients was detected based on clinical data. Besides, the potential targets of miRNAs were determined using Luciferase reporter gene assay combined with qRT-PCR and Western blotting. Primary tumor cells were extracted, and the influences of miR-150 expression on cell proliferation were detected *via* Cell Counting Kit (CCK)-8 assay and 5-ethynyl-2'-deoxyuridine (EdU) staining assay. Finally, Western blotting and flow cytometry were performed to explore the impact of miR-150 on the apoptosis of primary tumor cells.

RESULTS: Microarray analysis of miRNA expression profiles and RT-qPCR verified that the expression levels of hsa-miR-486, hsa-miR-4746, and hsa-miR-3158 rose considerably in MCL tissues, while those of hsa-miR-29b-3p, hsa-miR-150, and hsa-miR-142-5p remarkably declined. According to the results of survival analysis, the survival time was notably prolonged in patients with higher expression levels of miR-150 and miR-486, especially in those with higher expression level of miR-150. Luciferase reporter gene assay and RT-qPCR and Western blotting results demonstrated that miR-150 negatively regulated the expression level of MET. Subsequent CCK-8 assay and EdU staining results revealed that up-regulation of miR-150 significantly suppressed the proliferation of primary MCL cells. Finally, Western blotting and flow

cytometry found that increased expression of MET remarkably facilitated the apoptosis of primary MCL cells.

CONCLUSIONS: MiR-150 inhibits the proliferation and promotes the apoptosis of MCL cells by negatively regulating MET expression.

Key Words:

MiR-150, MET, Mantle-cell lymphoma (MCL), Proliferation, Apoptosis.

Introduction

Mantle-cell lymphoma (MCL) is a subtype of highly aggressive B-cell lymphoma with low morbidity rate. MCL only accounts for 6-8% of total cases of non-Hodgkin's lymphoma¹. Pathologically, it is characterized by chromosome translocation t(11;14)(q13;q32) and overexpression of cyclin D1 (CCND1)². Currently, primary therapies for MCL, including anti-cluster of differentiation 20 (CD20) antibody therapy, CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) and autologous stem-cell transplantation, have remarkably prolonged the survival rate of patients³. Additionally, MCL patients survive longer due to the development of targeted medications and progress in immunotherapy. However, Wu et al⁴ have reported that 57% of patients still suffer from recurrent MCL within 10 years. Generally, MCL persistently recurs, with an average survival of 3-5 years⁵. Therefore, further exploration of the pathological mechanism of MCL is still in dire need to discover novel treatment targets.

Micro ribonucleic acids (miRNAs) are a class of non-coding RNAs that can bind to the 3'-un-

translated region (UTR) of target messenger RNAs (mRNAs), thereby inhibiting mRNA translation or directly degrading mRNAs. Ultimately, this may suppress the post-transcriptional protein expression of target genes⁵. Aberrant expression of miRNAs plays an important role in MCL development. Some miRNAs directly target oncogenes or tumor suppressor genes in MCL, therefore, they are of predictive significance for the prognosis of MCL patients⁶. MiRNAs are implicated in abnormal molecular pathways in MCL, which can be used for predicting the survival rate, prognosis and disease progression of MCL patients^{7,8}. However, aberrant miRNAs in MCL progression have been poorly understood currently.

In the present work, differentially expressed miRNAs in MCL tissues were screened *via* microarray analysis of miRNA expression profiles. The expression of these miRNAs was verified using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Based on clinical data, the influence of miRNA expression on patient survival was analyzed. Direct targets of miRNAs were explored, and the functions of miRNAs were verified *in vitro*. All our findings might help to further deepen the understanding of the pathological mechanism of MCL.

Patients and Methods

General Information

A total of 103 pairs of MCL tissues and para-cancerous tissues were collected from tumor samples surgically resected in our hospital from May 2009 to July 2010. Upon resection, paired tumor tissues and para-cancerous tissues were immediately placed in RNA-fixer reagent (Biotek Corporation, Beijing, China), and preserved in a refrigerator at -80°C before use. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). In this study, factors affecting miRNA expression profiles were excluded based on the following criteria: before operation, no research subjects received medication, namely chemotherapy, radiotherapy and targeted medication. All collected tissue samples were histomorphologically confirmed as MCL tumor tissues. This study was approved by the Ethics Committee of Yantaishan Hospital. Signed written informed consents were obtained from all participants before the study.

Screening of Differentially Expressed MiRNAs Via MiRNA Microarray Analysis

Total RNAs were first extracted from tumor tissues, and quantified using the NanoDrop kit (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of RNAs was assessed with 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). 100 ng of total RNAs were prepared into complementary RNAs (cRNAs) using Affymetrix 3' IVT Express kit (Affymetrix, Santa Clara, CA, USA). Subsequently, they were hybridized on Affymetrix Primeview Human Gene Expression Array at 45°C for 16 h according to the instructions of Affymetrix GeneChip 3' Array. Additionally, the arrays were washed and stained using Affymetrix FS-450 and scanned on Affymetrix GeneChip scanner according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Next, the raw data in CEL files were imported into Partek Genomics Suite Software 6.6, and the probe set was standardized by Robust Multiarray Average. Finally, the significance of differentially expressed genes was determined using one-way analysis of variance, and *p* was corrected with FDR⁸.

Extraction of Primary Tumor Cells

All tumor tissues were collected into 1.5 mL Eppendorf (EP) tubes with medium at 4°C, and cut into pieces. After aspirating the liquid, MCL tissues in EP tubes were transferred into a 15 mL centrifugal tube containing trypsin and trypsinized at 37°C for 3 min. During the process, they were shaken evenly from time to time. The supernatant was then recycled into a neutralization tube and digested with 5 mL of collagenase at 37°C for 5 min, while they were shaken from time to time for mixing evenly, and the neutralization tube was inserted into ice. The resulting supernatant was recycled into another neutralization tube and digested with 4 mL of collagenase at 37°C for 5 min, and the neutralization tube was inserted into ice. The supernatant was harvested and added with 3 mL of collagenase, followed by digestion again at 37°C for 5 min. After that, all the products were collected into a neutralization tube. Some tissues not fully digested were filtered using a 200-mesh screen, ground with the black rubber needle core of a syringe and rinsed by liquid once, followed by centrifugation at 1,000 rpm for 8 min. Finally, the supernatant was removed, and each tube was added with 6 mL of culture medium, followed by beat at the bottom for re-suspending precipitates that were then pi-

petted up and down and inoculated into culture flasks. After 2 h, the medium was replaced and again 24 h later.

Cell Culture

Phosphate buffered saline (PBS), trypsin, fetal bovine serum and Roswell Park Memorial Institute-1640 (RPMI-1640) medium were purchased from Gibco (Rockville, MD, USA). Primary MCL cells were cultured in an incubator with 5% CO₂ at 37°C. When the cells covered the whole culture dish, they were digested by 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid) and sub-cultured.

Western Blotting

Cell lysis buffer was first prepared by mixing an appropriate volume of radioimmunoprecipitation assay buffer and protease inhibitor phenylmethylsulfonyl fluoride evenly at a ratio of 100:1. After trypsinization, the cells were added with lysis buffer and cell lysate was collected into Eppendorf (EP; Hamburg, Germany) tubes, followed by centrifugation in a low-temperature high-speed centrifuge at 4°C and 14,000 rpm for 30 min. The protein supernatant was aspirated, and the proteins were denaturalized *via* heat bath at 95°C for 10 min. Subsequently, prepared protein samples were stored in a refrigerator at -80°C for later use. The concentration of extracted proteins was quantified using bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). Afterwards, gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared, and protein samples were loaded into the wells for electrophoresis under the constant voltage of 80 V for 2.5 h. Next, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by the semi-dry transfer method. Then, the PVDF membranes were immersed in Tris-Buffered Saline with Tween-20 (TBST) buffer containing 5% skim milk powder and shaken slowly on a shaker for blocking. After incubation with primary antibodies diluted using 5% skim milk powder, the membranes were rinsed with TBST for 3 times at 10 min/time. The membranes were then incubated with corresponding secondary antibodies at room temperature for 2 h, followed by rinsing with TBST twice and TBS once at 10 min/time. Immuno-reactive bands were exposed by the enhanced chemiluminescence (ECL) method in a darkroom. The relative protein expression levels of active Caspase-3 (ab76315, Ab-

cam, Cambridge, MA, USA), active Caspase-9 (ab119352, Abcam, Cambridge, MA, USA) and MET (ab119843, Abcam, Cambridge, MA, USA) were finally analyzed using Image-Pro Plus v6 software (Media Cybernetics, Silver Spring, MD, USA).

Luciferase Reporter Gene Assay

The wild-type 3'-UTRs of MET gene were amplified and cloned into psiCHECK-2 Luciferase plasmids (Promega, Madison, WI, USA) to produce a wild-type MET reporter group. Primary MCL cells were cultured in a 24-well plate and co-transfected with miR-150 or miR-control (miR-con) and wild-type plasmids. At 48 h after transfection, the activity of Luciferases was determined using Dual-Luciferase reporter gene assay kit (Promega, Madison, WI, USA).

QRT-PCR Assay

RT and qRT-PCR were conducted to detect the expressions of miRNAs and MET mRNA in cells and tissues. The miRNAs in cells and tissues were reversely transcribed using PrimeScript RT reagent kit, and quantified with TaqMan quantitation kit (Invitrogen, Carlsbad, CA, USA). Briefly, 500 ng of RNA samples were divided into three portions, and 10-fold diluted. 3 µL of total RNAs was taken for RT. PCR amplification was then conducted, and the amplification level of target gene was determined *via* 5% agarose gel electrophoresis. The LabWorks 4.0 image acquisition and analysis software was adopted for quantification and data processing, with U6 as an internal reference. Primers of miR-150 were purchased from ABM (Peterborough, Camb, Canada). To obtain reliable data, the above operations were performed in triplicate in each group of samples. The changes in the relative expression level of target gene were analyzed using the 2^{-ΔΔC_t} method. Primer sequences used in this study were shown in Table I.

Cell Counting Kit (CCK)-8 Assay

After transfection with miR-150 mimic or miR-control (miR-con), primary MCL cells were seeded into 96-well plates at a density of 1×10⁴ cells/well. Six replicate wells were set for each sample. Then, the cells were cultured in an incubator for other 72 h. After discarding the original medium, the cells were incubated with 20 µL of CCK-8 reaction solution (Dojindo Molecular Technologies, Kumamoto, Japan) and 170 µL of cell medium at 37°C in dark for 2 h. Afterwards,

Table I. Primer sequences.

Name	Primer sequence
MiR-150	5'-TACCACTCACAAGTCGGAGGC-3' 5'-CTGCAAGTGCATCATCGTTGTTC-3'
Hsa-miR-486	5'-GAGAGTGATTGAGAGTGGACCAC-3' 5'-CACAAACCCTCTGCACCCAGTTT-3'
Hsa-miR-4764	5'-TGGACCTTCCAGGATGAGGACA-3' 5'-GTTTCATCTCGGAGCCTGTAGTG-3'
Hsa-miR-3158	5'-GTTTCATCTCGGAGCCTGTAGTG-3' 5'-GCCATAGAAGTGTGATGAGAGGGAG-3'
MET	5'-CGGGATCTCATCAGCTTCACAG-3' 5'-TCCTTGTTCGGAGGCAGGTCTA-3'
U6	5'-CGCTTCGGCAGCACATATACTA-3' 5'-CGCTTCACGAATTTGCGTGTCA-3'

the resulting cells were shaken on a micro-vibration device for 3 min. Absorbance at the wavelength of 450 nm was finally measured using a micro-plate reader.

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

Primary MCL cells in the logarithmic growth phase were inoculated into 96-well plates at a density of 1×10^5 cells/well. Normal growing MCL cells were treated with miR-150 mimic or miR-con for 24 h. Subsequently, the cells in each well were incubated with 100 μ L of 50 nM EdU solution (Sigma-Aldrich, St. Louis, MO, USA) diluted by cell medium at 1:1,000 for 2 h. After discarding the medium, the cells were washed with PBS for 2 times at 5 min/time. Next, 100 μ L of cell fixative (namely PBS containing 4% paraformaldehyde) was added in each well, followed by incubation at room temperature for 30 min. Afterwards, 100 μ L of 1 \times Apollo[®] staining solution was added in each well, followed by incubation on a decoloring shaker at room temperature in dark for 30 min. Later, the staining solution was removed, and the resulting cells were added with 100 μ L of penetrant 0.5% Triton X-100-containing PBS and cleansed on a decoloring shaker for 2-3 times at 10 min/time. After discarding the penetrant, the cells were observed under a microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was employed for all statistical analysis. *t*-test was performed to compare the differences among different groups. The calculated data were presented as mean \pm standard deviation

($\bar{x} \pm s$). $p < 0.05$ was considered statistically significant.

Results

Differentially Expressed miRNAs Screened Via Expression Profile Analysis in MCL Patients

According to the analysis results of gene expression profiles, there were notable differences in the expression profiles of miRNAs between MCL tissues and normal lymphatic tissues (Figure 1). A total of 12 miRNAs were considerably different between MCL tissues and para-cancerous tissues in MCL patients (fold change > 1 , $p < 0.05$). Among them, there were 3 considerably upregulated miRNAs, and 9 remarkably downregulated miRNAs.

Differentially Expressed MiRNAs Verified Via qRT-PCR

To verify the reliability of high-throughput screening results, qRT-PCR was performed in 3 upregulated miRNAs and 9 downregulated miRNAs. The results showed that the expression levels of hsa-miR-486, hsa-miR-4746 and hsa-miR-3158 rose considerably ($p < 0.01$), while those of hsa-miR-29b-3p, hsa-miR-150 and hsa-miR-142-5p declined remarkably in MCL tissues compared with para-cancerous tissues ($p < 0.05$, Figure 2).

Influences of Differentially Expressed miRNAs on Patient Survival

To further understand the influences of differentially expressed miRNAs on the prognosis of MCL patients, the survival curves were potted

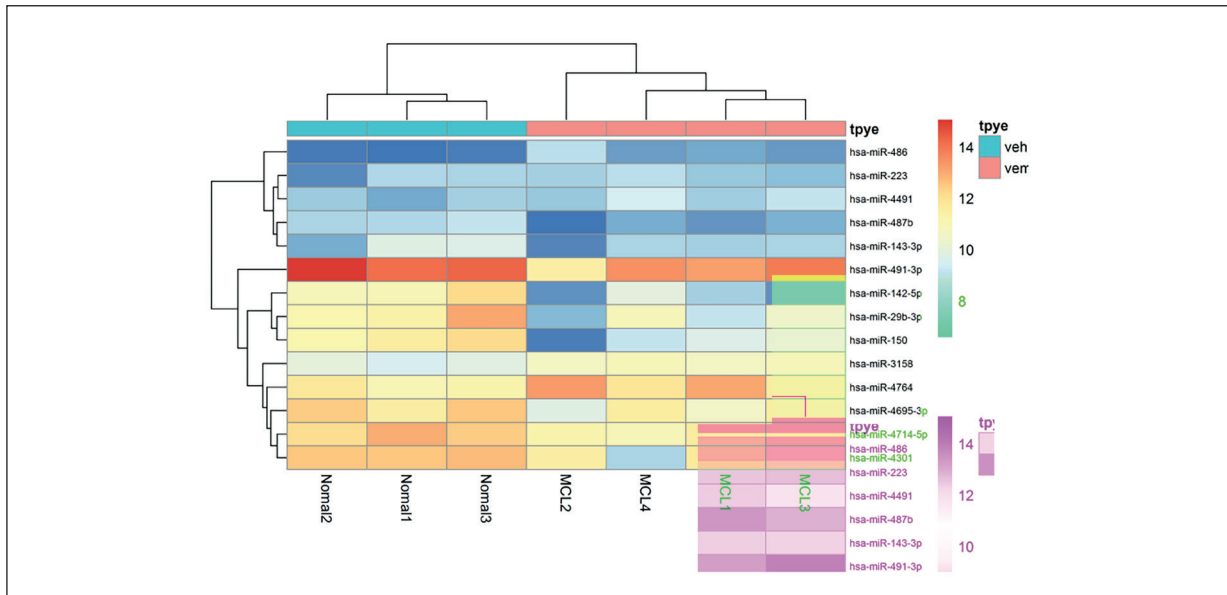


Figure 1. There are significant differences in the expression profiles of miRNAs between MCL tissues and normal lymphatic tissues. Unsupervised cluster heatmap: The expression profiles of the miRNAs in MCL tissues differ substantially from those in normal lymphatic tissues in MCL patients (MCL: tumor tissues, normal: normal tissues, a row: a miRNA, red: a higher expression level, blue: a lower expression level).

based on the differentially expressed miRNAs. It was found that the survival time was notably prolonged in patients with higher expression levels of miR-150 and miR-486, especially in those with higher expression level of miR-150 ($p < 0.05$, Figure 3).

MiR-150 Negatively Regulated MET Expression in MCL

Based on the immunoprecipitation (IP) assay results, MET might be a potential target

of miR-150 in MCL. The expression level of miR-150 was significantly negatively correlated with MET mRNA expression in MCL patients ($p < 0.05$, Figure 4A). To further investigate the influence of miR-150 expression on MET gene in primary MCL cells, the responses of primary MCL cells transfected with wild-type MET plasmids to miR-150 mimic and miR-con were detected *via* Luciferase reporter gene assay. It was found that the fluorescence intensity was considerably weakened in primary MCL cells

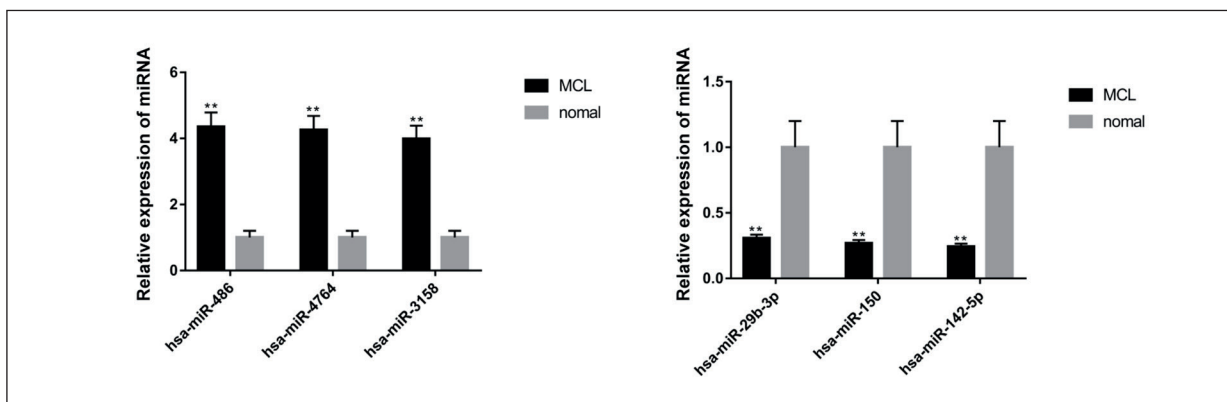


Figure 2. Differences in the expression levels of hsa-miR-486, hsa-miR-4746, hsa-miR-3158, hsa-miR-29b-3p, hsa-miR-150, and hsa-miR-142-5p between MCL tissues and normal tissues detected *via* RT-qPCR. $**p < 0.01$: MCL tissues vs. normal tissues.

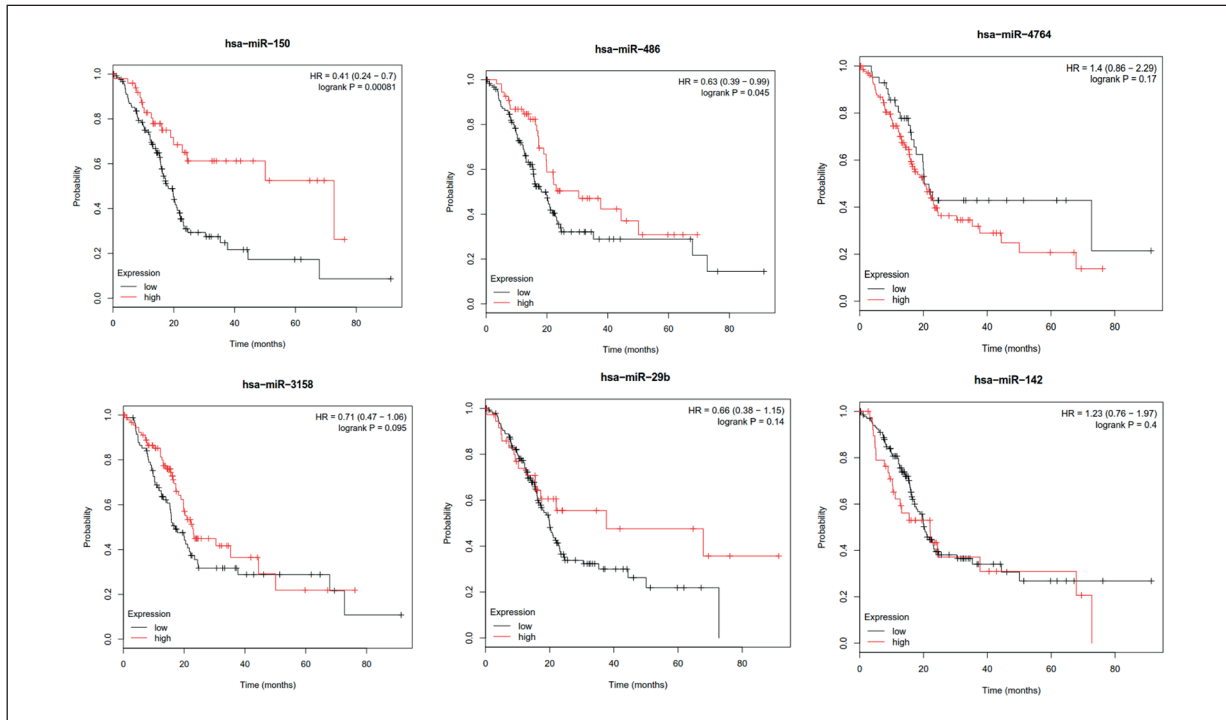


Figure 3. Influences of differentially expressed miRNAs on patient survival. Patients with substantially raised expression levels of miR-150 and miR-486 have a notably longer survival time.

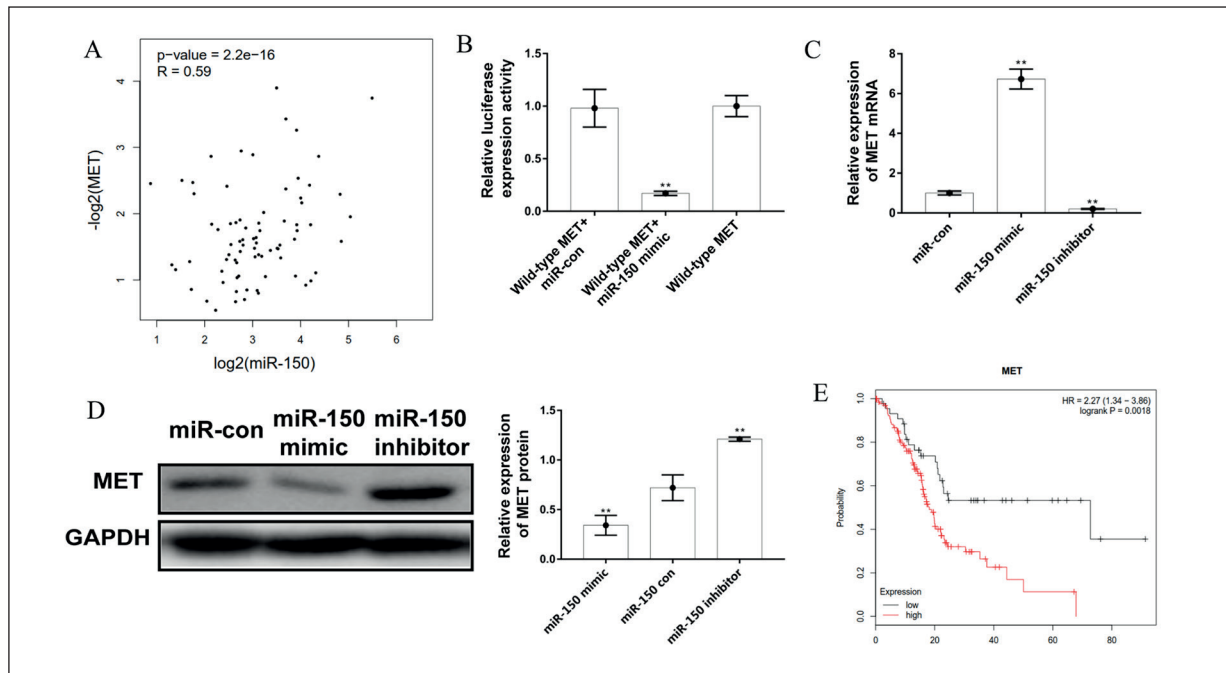


Figure 4. MiR-150 negatively regulates MET expression. **A**, Pearson's correlation coefficient analysis on the correlation between miR-150 expression level and MET mRNA expression level. **B**, Responses of wild-type MET gene in primary cells to miR-150 and miR-con examined using Luciferase reporter gene assay. **C-D**, Influences of transfection with miR-con, miR-150 and miR-150 inhibitor on protein and mRNA expression levels of MET detected *via* Western blotting and qRT-PCR, respectively. **E**, Survival analysis in MCL patients. ****** $p < 0.01$: miR-150 mimic group or miR-150 inhibitor group *vs.* miR-con group.

transfected with miR-150 mimic ($p < 0.05$). However, no significant changes were observed in fluorescence intensity in those transfected with miR-con ($p > 0.05$, Figure 4B). qRT-PCR and Western blotting results further evidenced that transfection with miR-150 markedly decreased the mRNA and protein levels of MET, whereas transfection with miR-150 inhibitor elevated MET gene expression ($p < 0.05$, Figure 4C and 4D). Moreover, the survival analysis results manifested that the survival time was dramatically prolonged in patients with declined expression level of MET ($p < 0.05$, Figure 4E). The above results suggested that miR-150 negatively regulated MET expression in MCL.

MiR-150 Restrained Primary MCL Cell Proliferation

CCK-8 assay demonstrated that the proliferation ability of primary MCL cells declined remarkably in miR-150 mimic group, whereas was enhanced in miR-150 inhibitor group at 24, 48 and 72 h ($p < 0.01$). Similarly, the same results were observed through EdU staining. In other words, miR-150 substantially suppressed the proliferation of primary MCL cells (Figure 5).

MiR-150 Promoted Primary MCL Cell Apoptosis

Flow cytometry was then conducted to detect the impact of miR-150 on the number of apoptotic cells in each group. Compared with miR-con group, the apoptosis rate of cells rose dramatically in miR-150 mimic group, but declined markedly in miR-150 inhibitor group ($p < 0.05$). Consistent with the results of flow cytometry, Western blotting results revealed that miR-150 mimic notably upregulated the protein expression levels of active Caspase-3 and active Caspase-9 ($p < 0.05$, Figure 6A and 6B). The above results implied that miR-150 considerably promoted the apoptosis of primary MCL cells.

Discussion

MCL is a rare subtype of non-Hodgkin's lymphoma. Clinically, it is a significantly invasive lymphoma that still cannot be cured. At present, the molecular mechanisms of the development and progression of MCL remain elusive. Hepatocyte growth factor (HGF) and its receptor MET, also known as c-MET, are essential for embry-

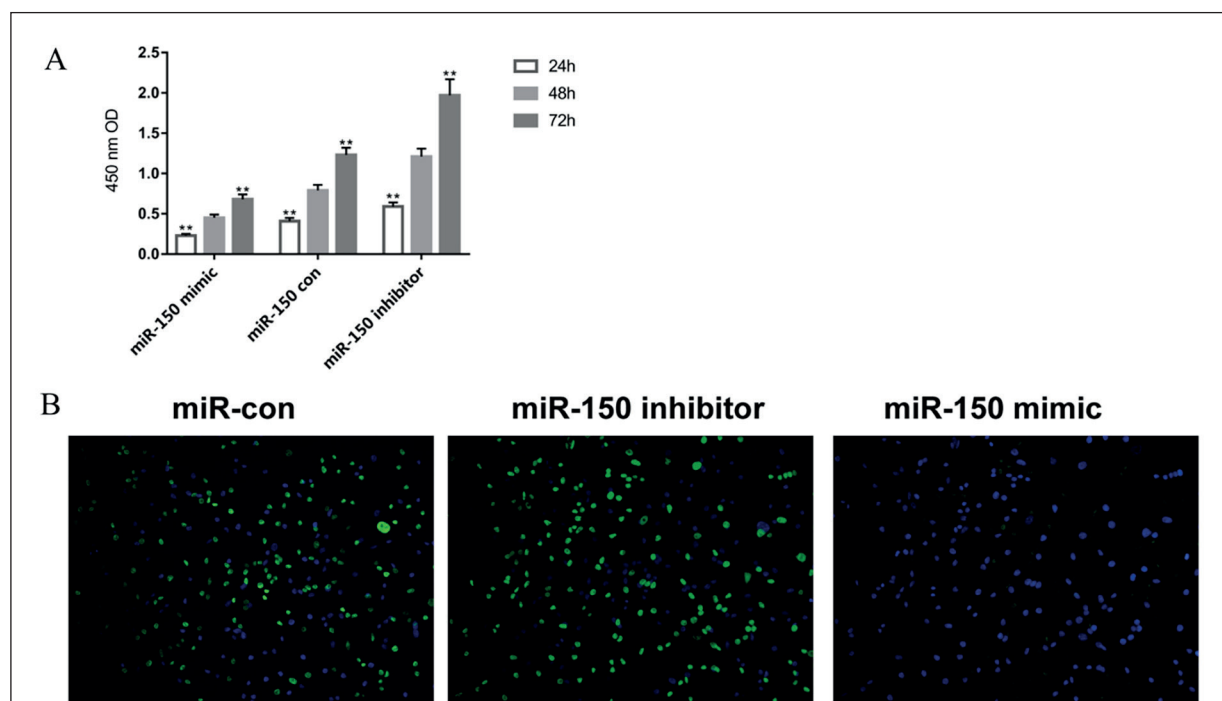


Figure 5. MiR-150 restrains primary MCL cell proliferation. **A**, Influence of miR-150 expression level on MCL cell proliferation detected *via* CCK-8 assay. **B**, Impact of miR-150 expression level on MCL cell proliferation detected *via* EdU staining (magnification: 20 \times). MiR-150 prominently restrains the proliferation of primary MCL cells. $**p < 0.01$: miR-150 mimic group or miR-150 inhibitor group vs. miR-con group.

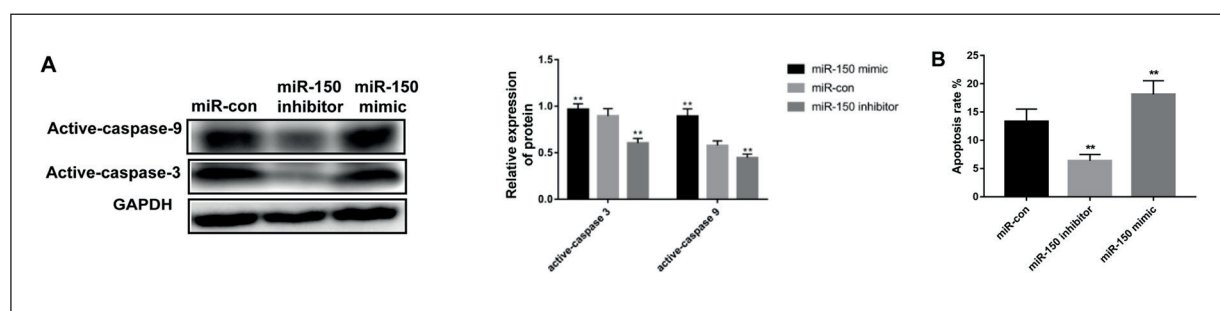


Figure 6. MiR-150 promotes primary MCL cell apoptosis. **A**, Impact of miR-150 expression level on apoptosis of primary MCL cells detected *via* flow cytometry. **B**, Impact of miR-150 expression level on apoptosis of primary MCL cells observed by Western blotting. MiR-150 induces primary MCL cell apoptosis. $**p < 0.01$: miR-150 mimic group or miR-150 inhibitor group vs. miR-con group.

onic development⁹. However, overexpression-induced and mutation-induced abnormal activation of MET contributes to the development and progression of multiple human cancers. Meanwhile, they also tend to be associated with poor clinical outcomes and drug resistance^{10,11}. MET may play a pivotal role in the progression of MCL. The HGF/MET signaling pathway is considered as a treatment target for B-cell lymphoma^{12,13}. In the present study, it was found that the survival of MCL patients with lowly expressed MET was significantly longer. It can be inferred from the above results that MET serves as an oncogene in the development and progression of MCL. Nevertheless, little is known about the molecular mechanism by which MET expression is regulated in MCL.

MiRNAs play vital roles in various tumors since they can epigenetically modulate gene expression. The dysregulation of miRNA expression acts as an important player in the pathological process of MCL. In the present study, microarray analysis of miRNA expression profiles and qRT-PCR verified that hsa-miR-150 was lowly expressed in MCL tissues. Likewise, Zhao et al¹⁴ argued that the expression level of miR-150 declines remarkably in MCL patients, which proves the reliability of this study. MiR-150 is a miRNA with significant correlations with tumor cell proliferation, apoptosis, migration and drug resistance. MiR-150 serves as a tumor suppressor gene. So far, Kim et al¹⁵ have indicated that miR-150 is able to target Notch3 to potentiate the inducing effect of paclitaxel on ovarian cancer cell apoptosis. MiR-150 also inhibits the proliferation of multiple tumor cells by targeting CDK3. However, the biological role of miR-150 in MCL has not been fully elucidated. As con-

firmed by the clinical data analysis results, the survival time was prominently prolonged in patients with higher expression level of miR-150. Luciferase reporter gene assay, qRT-PCR and Western blotting results demonstrated that miR-150 negatively regulated the expression level of MET. Consistent with the findings of this study, MET may act as a direct target for miR-150 according to Xue et al¹⁶ in an IP assay.

The post-transcriptional regulation function of miRNAs is considerably associated with the functions of their target genes. It has been proposed that MET serves as an oncogene in a variety of cancers. Meanwhile, active MET can activate downstream PI3K/Akt and Raf/Ras signaling pathways to induce the proliferation and retard the apoptosis of cells. In this study, CCK-8 assay and EdU staining results revealed that overexpressing miR-150 suppressed the proliferation of primary MCL cells. Besides, flow cytometry results showed that miR-150 overexpression induced the apoptosis of primary MCL cells. The protein expressions of active Caspase-3 and Caspase-9 were detected *via* Western blotting, since both active Caspase-3 and active Caspase-9 play important roles in tumor cell apoptosis. It was found that overexpressing miR-150 substantially downregulated their protein expressions. Therefore, MCL is proved to act as a tumor suppressor gene in MCL. The potential targets of miR-150 include: CCND1¹⁷, Cmyb¹⁸, β -catenin¹⁹, Notch3, MUC4²⁰, and IGF-1R²¹. The results of the present research suggested that MET was a novel target of miR-150. The deficiency of this work was that MET was not overexpressed for exploring whether miR-150 only targeted MET to inhibit the proliferation of MCL tumor cells and induce their apoptosis.

Conclusions

The novelty of this investigation was that miR-150 serves as a tumor suppressor gene in MCL and probably targets MET. Moreover, higher expression level of MET shows an adverse impact on the survival of patients. Thus, both miR-150 and MET may be novel potential targets for MCL.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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