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MicroRNA-506 inhibits the proliferation and invasion of mantle cell lymphoma cells by targeting B7–H3

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ABSTRACT

Background: Aberrant expression of B7 homologue 3 (B7–H3) has been observed in various malignancies. Our previous study demonstrated that knocking down of B7–H3 inhibited cell proliferation, invasion and enhanced the therapeutic efficacy of chemotherapy in mantle cell lymphoma (MCL). However, the mechanism regulating of B7–H3 expression remains unknown. Here, we present a new regulatory microRNA of B7–H3, miR-506, that directly targets B7–H3 and may play an inhibitory role in MCL progression.

Methods: The expression of miR-506 and B7–H3 was investigated by real-time quantitative PCR (RT-qPCR). B7–H3 was confirmed to be a novel direct target gene of miR-506 by a dual-luciferase assay and western blot analysis. MiR-506 overexpression in the Maver and Z138 MCL cell lines was established using lentiviral transduction. Cell counting kit-8, flow cytometry and Transwell assays were used to detect changes in cell proliferation, cycle distribution, migration and invasion, respectively.

Results: The RT-qPCR results showed that miR-506 was expressed at a low level, while B7–H3 was overexpressed in MCL patients and cell lines. By using a bioinformatics analysis combined with a dual-luciferase assay, we determined that miR-506 could target the 3'-untranslated region (3′-UTR) of B7–H3 mRNA. Moreover, miR-506 had a negative regulatory effect on B7–H3 expression according to the western blotting and RT-qPCR results. In terms of function, increased expression of miR-506 led to reduced MCL cell proliferation, invasion and migration, caused cell cycle arrested at G0/G1 phase, similar to the effects of B7–H3 knockdown. Furthermore, we measured the expression of invasion-related proteins by western blotting and found that miR-506 could reduce MMP-2 and MMP-9 expression in MCL cells. Rescue experiments suggested that the restoration of B7–H3 expression in MCL cells reversed the inhibition of proliferation and invasion induced by miRNA-506 overexpression.

Conclusions: Our findings suggest that miR-506 functions as a tumor suppressor miRNA and plays a significant role in inhibiting human MCL cell proliferation and metastasis by suppressing B7–H3 expression.

1. Introduction

Mantle cell lymphoma (MCL) is a relatively uncommon subtype of B cell non-Hodgkin's lymphoma (NHL), accounting for 6%–8% of NHL cases [1]. Patients with an aggressive clinical course present with a continuous relapse pattern and a median survival time of only 3–5 years [2]. Recently, specifically targeted efficacious agents, immunotherapy agents, and cellular therapies have dramatically improved MCL outcomes because of an improved understanding of the molecular pathogenesis of MCL [3]. Nevertheless, the clinical behavior of MCL is usually aggressive. There is an urgent need for the identification of potential mechanisms contributing to MCL and the development of novel therapeutic strategies.

B7 homologue 3 (B7–H3), located on chromosome 15q24.1, is a novel member of the B7 immunoregulatory family that is ubiquitously expressed in a wide spectrum of tissues [4]. An increasing number of studies have shown that B7–H3 may promote cancer progression and subsequently a poor prognosis in several solid human cancers, including, lung cancer [5], hepatocellular carcinoma [6], pancreatic cancer [7], and gastric cancer [8], as well as hematologic malignancies [9]. Our previous study showed that

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knocking down of B7−H3 inhibits the proliferation and invasion of MCL cell lines Maver and Z138, and silencing B7−H3 enhances the therapeutic efficacy of chemotherapeutic drugs [10]. Our findings suggest that B7−H3 might act as a carcinogenic factor in MCL, but the regulatory mechanism of B7−H3 expression in MCL remains unclear.

MicroRNAs (miRNAs) are a group of small noncoding single-stranded RNAs of ~22 nucleotides that are involved in the post-transcriptional regulation of gene expression through binding to the 3′-untranslated region (UTR) of target gene messenger RNA (mRNA) [11]. MiRNAs have been found to be important in tumorigenesis and are abnormally expressed in many malignancies [12]. miR-506 can serve as a tumor suppressor gene or oncogene in different types of cancer via the regulation of molecules involved in physiological processes [13]. Moreover, several studies have reported that miRNAs can regulate B7−H3 expression in breast cancer, osteosarcoma and renal cell carcinoma [14–16]. However, the expression pattern of miR-506 in MCL is not well known. Whether miR-506 is involved in the posttranscriptional regulation of B7−H3 expression has not been studied.

In this study, we demonstrated the decreased expression of miR-506 and the high expression of B7−H3 in MCL patients and cell lines. Furthermore, we predicted and confirmed that B7−H3 is a target of miR-506 using a bioinformatics analysis and a dual-luciferase assay, respectively. Functional studies further demonstrated that miR-506 exerts its biological function by directly targeting B7−H3. Our study provides a better understanding of MCL pathogenesis and novel findings for targeted therapeutic tools for MCL.

2. Materials and methods

2.1. Patient samples and MCL cell lines

Bone marrow mononuclear cells (BMNCs) were isolated from 12 de novo MCL patients with bone marrow involvement using Ficoll-Paque Plus (GE, USA). All the patients were diagnosed with MCL according to the classification criteria of lymphoid hematopoietic neoplasms issued by the WHO. The characteristics of patients are summarized in Table 1. The BMNCs samples collected from 9 healthy donors were used as controls. This study was approved by the Ethics Committee of Peking University Third Hospital. Written informed consent was obtained from all participants.

The human MCL cell lines Maver and Z138 were obtained from American Type Culture Collection. They were cultured in RPMI 1640 medium, Iscove's modified Dulbecco's Medium (IMDM), respectively; all media were supplemented with 10% FBS. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors after written informed consent was obtained. All cells were cultured in a humidified chamber with 5% CO_2 at 37 °C.

Table 1
Patient characteristics.

Parameters	No. of cases	%
Gender		
Male	9	75
Female	3	25
Age		
≥60 years	8	66.7
<60 years	4	33.3
Stage		
I-II	0	0
III-IV	12	100
WBC		
$>10 \times 10^9$/L	4	33.3
$\leq 10 \times 10^9$/L	8	66.7
LDH		
Normal	5	41.7
Elevated	7	58.3

WBC, white blood cells; LDH, lactate dehydrogenase.

2.2. RNA extraction and real-time quantitative PCR

Total RNA was extracted from cells with Trizol reagent (Invitrogen, USA) and reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen, China) following the manufacturers' instructions. The relative gene expression level of B7−H3 was determined by real-time quantitative PCR (RT-qPCR) using a SuperReal PreMix Color kit (Tiangen, China). The expression of miR-506 was detected by RT-qPCR using a miRcute Plus miRNA qPCR Detection Kit (Tiangen, China) with the manufacturer-provided miScript universal reverse primer. The relative expression levels of B7−H3 and miR-506 were calculated by the comparative $2^{-\Delta\Delta Ct}$ method using β-actin and U6 small nuclear RNA levels for normalization.

2.3. Western blotting

Total protein was extracted with RIPA lysis buffer containing a protease inhibitor. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Then, the membranes were blocked in TBST containing 5% BSA for 1 h and incubated overnight at 4 °C with the primary antibodies, including anti-B7-H3, anti-MMP-2, anti-MMP-9, and anti-β-actin antibodies (Abcam, USA). The membranes were washed 3 times for 10 min each time with TBST, incubated with the appropriate anti-rabbit secondary antibodies for 1 h at room temperature and washed again. Fluorescent bands were visualized with an Odyssey infrared imaging system.

2.4. Transfection and lentivirus infection

Stably infected Maver and Z138 cell lines overexpressing miR-506 were generated by lentiviral transduction. The recombinant lentiviruses used to overexpress miR-506 were purchased from Vigene Biosciences (Vigene Bio, China). Maver and Z138 cells were infected with recombinant lentivirus in the presence of 8 μg/mL Polybrene (Sigma-Aldrich, USA). Antibiotic-resistant clones were isolated and maintained in medium containing 200 μg/ml puromycin (Sigma-Aldrich, USA). MiR-506 overexpression was confirmed by RT-qPCR. A recombinant lentiviral vector containing a random sequence was used as the negative control.

To generate the B7−H3 overexpression plasmid, a pcDNA3.1 vector was used. The B7−H3-overexpressing pcDNA3.1-B7-H3 vector and empty pcDNA3.1 vector were used to transfect into stable miR-506-overexpressing Maver and Z138 cells. Cells were suspended in OPTI-MEM® I and diluted to a cell density of 3×10^6 cells/100 μl pcDNA3.1-B7-H3 or empty vector were mixed with diluted cells and electroporated by a Super Electroporator NEPA21 Type II (NEPAGENE, Japan).

2.5. Dual-luciferase assay

Both the B7−H3 3'-UTR wild-type sequence (wt 3′-UTR) and the B7−H3 3'-UTR mutant sequence (mut 3′-UTR) containing the predicted miRNA-506 binding sites were amplified by PCR and cloned into the pMIR-GLO dual luciferase reporter vector (Promega, USA). HEK293T cells were cotransfected with B7−H3 wt or mut 3′-UTR plasmids and either the miR-506 mimics or miR-NC using Lipofectamine 2000 (Invitrogen, USA). After a 48 h transfection,

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HEK293T cell lysates were harvested by direct lysis. Luciferase activity was detected using a Dual-Glo luciferase assay system (E1960, Promega) in a GloMax 96 Microplate Luminometer. Renilla luciferase was used for normalization. The miR-506 mimics and negative controls were purchased from RiboBio (RiboBio, China). Assays were performed three times.

2.6. Cell proliferation assays

Cell proliferation was determined using a Cell Counting Kit-8 assay (CCK-8; Dojindo, Japan) in accordance with the manufacturer's instructions. Cells in the logarithmic growth phase (1×10^4 cells/mL per well) were grown in 96-well cell culture plates in medium containing 10% FBS in an incubator with 5% CO_2 at 37 °C for 0, 24, 48, and 72 h. At each time point, 10 μl of CCK-8 solution was added to each well, and the plates were then incubated for an additional 4 h. The absorbance in each well was measured at a wavelength of 450 nm with a microplate reader.

2.7. Cell cycle assays

The cell cycle distribution was evaluated using a flow cytometric analysis. A total of 1×10^6 transduced and control cells were collected, washed twice with PBS, and then fixed in cold 70% ethanol overnight at 4 °C. After that, the collected cells were stained with an RNase A/PI solution (KeyGEN, China) in the dark for 30 min at room temperature and then analyzed using flow cytometry. The data were analyzed using the ModFit program.

2.8. Cell migration and Transwell invasion assays

Transwell chamber (8.0 μm PC, Corning-Costar, Corning, USA) inserts uncoated or coated with Matrigel (BD Biosciences, USA) were used for cell migration and invasion assays, respectively. A total of 5×10^5 (migration) or 2×10^5 (invasion) cells in 100 μl of serum-free RPMI 1640 medium or IMDM were seeded into the upper chamber, while 600 μl of complete medium supplemented with 10% FBS was added to the lower chamber. After incubation at 37 °C for 24 h, the migrated cells in the lower chamber were collected and counted. The migration rate was calculated according to the following formula: migration rate = (cell count in the lower chamber/total number of cells on the top of the Transwell chamber) × 100%. The invasive cells were fixed with methyl alcohol for 30 min and stained with 0.1% crystal violet. The number of migrated cells in five random fields was counted under an inverted microscope (Nikon, Japan).

2.9. Statistical analysis

Statistical analysis was performed using the Statistical Program for Social Science (SPSS) software 19.0 package. Data are presented as the mean ± standard deviation of at least three independent experiments. The differences between two groups were calculated with Student's t-test. Differences in miR-506 and B7−H3 expression between MCL patients and control specimens were analyzed by the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-506 and B7−H3 were inversely expressed in MCL specimens and cell lines

To determine the roles of miR-506 and B7−H3 in MCL carcinogenesis, RT-qPCR was performed to detect the expression levels of miR-506 and B7−H3 in BMNCs from patients with MCL and controls. The results showed that B7−H3 was overexpressed and miR-506 was underexpressed in MCL patients compared to controls (Fig. 1A and C). The MCL cell lines (Maver and Z138) and PBMCs from healthy donors were used in this study. The expression of B7−H3 in MCL cell lines was significantly higher than that in normal PBMCs, whereas the expression of miR-506 in MCL cell lines was markedly lower than that in normal PBMCs (Fig. 1B and D).

3.2. The establishment of stable miR-506 overexpression cell lines

In Maver and Z138 cells, the silencing of B7−H3 leads to cell proliferation inhibition, cell cycle arrest and metastasis suppression. However, the role of miR-506 in MCL cells is unclear. We used lentivirus-mediated gene transduction to stably increase miR-506 expression in Maver and Z138 cells. Recombinant lentivirus-infected cells composed the miR-506 (miR-506/Maver; miR-506/Z138) and miR-NC (miR-NC/Maver; miR-NC/Z138) groups. These groups of each cell line were used for the following assays. RT-qPCR confirmed that the miR-506 mRNA expression level was significantly increased in the miR-506 group compared with the miR-NC group after transfection (Fig. 1E and F).

3.3. MiR-506 downregulated B7−H3 expression by binding to the 3′-UTR of B7−H3 mRNA

A bioinformatics analysis by TargetScan (http://www.targetscan.org/) predicted that the 3′-UTR of B7−H3 has potential miR-506 binding sites (Fig. 2A). To verify whether miR-506 could directly interact with the B7−H3 3′-UTR, B7−H3 wt/mut 3′-UTRs were cloned into the pMIR-GLO vector (Fig. 2B). These recombinant vectors were cotransfected with the miR-506 mimics or miR-NC into HEK293T cells, and a dual-luciferase assay was performed. We found that miR-506 mimics reduced the luciferase activity of the B7−H3 wt 3′-UTR construct. In contrast, miR-NC did not affect the luciferase activity of either B7−H3 wt or mut 3′-UTR construct. No significant difference was observed in the luciferase activity of the B7−H3 mut 3′-UTR with the miR-506 mimics transfection (Fig. 2C). In addition, the mRNA and protein expression levels of B7−H3 were analyzed by RT-qPCR and western blotting after the transfection of Maver and Z138 cells with the miR-506-overexpressing lentiviruses. As shown in Fig. 2D and E, B7−H3 expression was remarkably downregulated after the upregulation of miR-506 expression. Collectively, these data indicated that miR-506 directly binds to the 3′-UTR of B7−H3 mRNA and downregulates the expression of B7−H3.

3.4. MiR-506 inhibited MCL cell proliferation

A CCK-8 assay was performed to define the function of miR-506 in MCL cell proliferation, and the growth curves are shown in Fig. 3A. After 48 h and 72 h of incubation, the OD values of the miR-506/Maver and miR-506/Z138 cells were significantly lower than those of the corresponding miR-NC cells. Similarly, knockdown of B7−H3 by siRNA in MCL cells also resulted in reduced cell proliferation.

3.5. MiR-506 induced MCL cell cycle arrest in the G0/G1 phase

To further investigate the role of miR-506 overexpression in MCL progression, cell cycle features of miR-506-overexpressing cells were detected by flow cytometry. As shown in Fig. 3B and C, ectopic expression of miR-506 arrested cells in the G0/G1 phase and decreased the S phase population of miR-506/Maver and miR-506/Z138 cells when compared to that of the corresponding miR-

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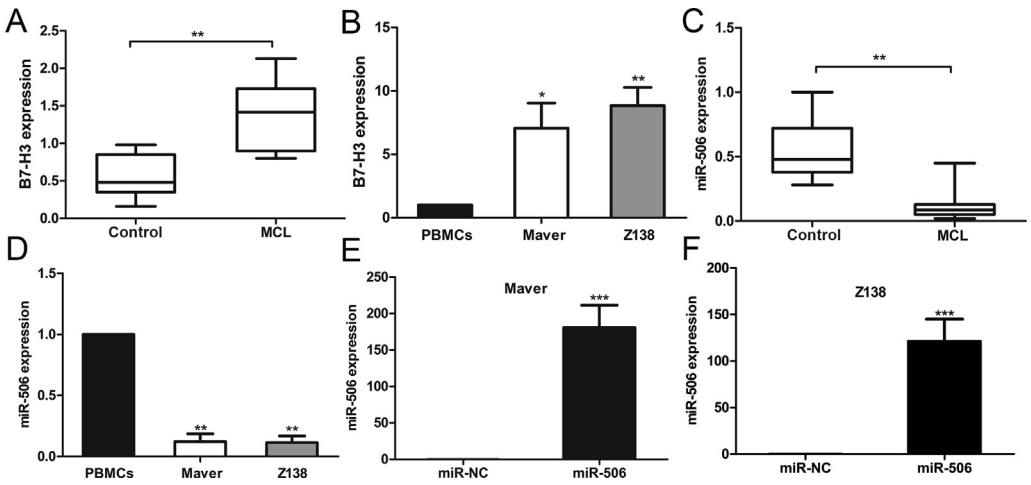

Fig. 1. miR-506 and B7−H3 expression in human MCL patients and cell lines. The expression level of B7−H3 in MCL patients (A) and cell lines (B). Analysis of miR-506 expression in MCL patients (C) and cell lines (D). The stable expression of miR-506 in Maver (E) and Z138 (F) cell lines was evaluated using RT-qPCR. The data were obtained from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

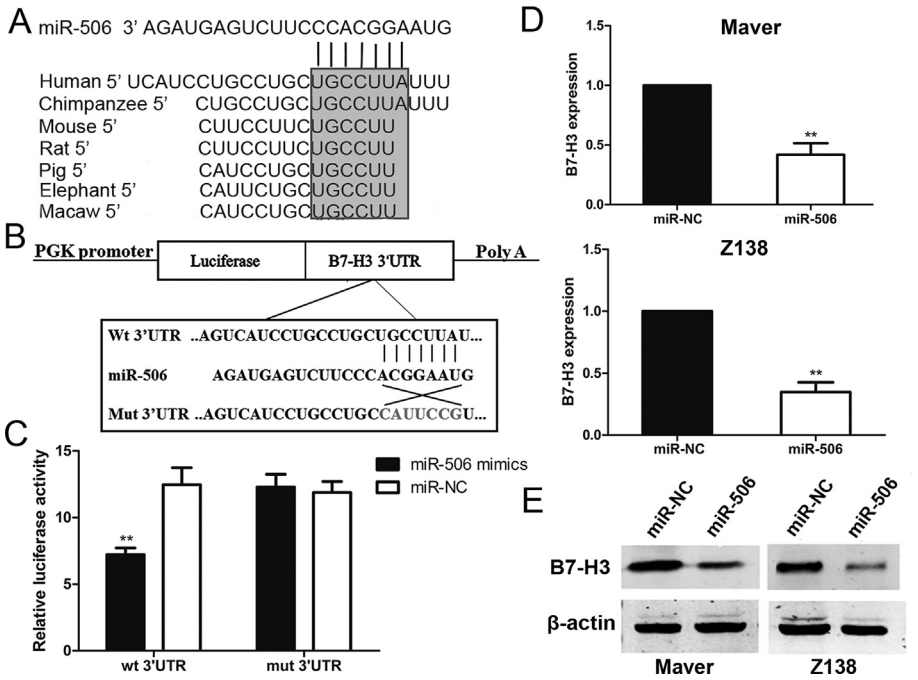

Figure 2. miR-506 downregulated the expression of B7−H3 by directly targeting the B7−H3 3′-UTR. (A) Schematic representation of the miR-506 binding site in the 3′-UTR of B7−H3 and the interspecies conservation of seed sequences. (B) Schematic diagram showing the cloning strategy of the miR-506 targeting sequence (named wt B7−H3-3′-UTR) and the mutated targeting sequence (named mut B7−H3-3′-UTR) into the pMIR-GLO luciferase vector. (C) HEK293T cells were cotransfected with the miR-506 mimics or the negative control and the B7−H3 wt/mut 3'-UTR reporter plasmid. B7−H3 mRNA (D) and protein (E) levels in the Maver and Z138 cells after miR-506 overexpression. The data were obtained from at least three independent experiments. $^{**}P < 0.01$.

NC cells. The percentage of cells in the G0/G1 phase was 49.98% in the miR-506/Maver cells, 40.57% in the miR-NC/Maver cells, 48.02% in the miR-506/Z138 cells and 37.66% in the miR-NC/Z138 cells. These results indicated that overexpression of miR-506 possibly induced MCL cell cycle arrest in the G0/G1 phase to inhibit cell proliferation. Consistent with this result, B7−H3 knockdown also arrested the MCL cell cycle in the G0/G1 phase.

3.6. MiR-506 expression repressed MCL cell invasion and migration

We further investigated the migration and invasion abilities in each group by Transwell assays. The results revealed that the migration rates of the miR-506/Maver and miR-506/Z138 cells were significantly lower than those of the corresponding miR-NC cells after a 24 h incubation (Fig. 3D). Furthermore, compared with miR-NC cells, miR-506/Maver and miR-506/Z138 cells showed an obvious inhibition of their invasive capacities (Fig. 3E). Knockdown of B7−H3 in Maver and Z138 cells obtained similar results. Accompanying the cell metastatic change, a set of protein markers related to migration and invasion were also changed. MMP-2 and MMP-9 are considered key migratory and invasive regulators in tumor cells. Here, we found that miR-506 reduced the protein expression of MMP-2/MMP-9 (Fig. 3F). Thus, these findings illustrated that miR-506 inhibited migratory and invasive abilities in

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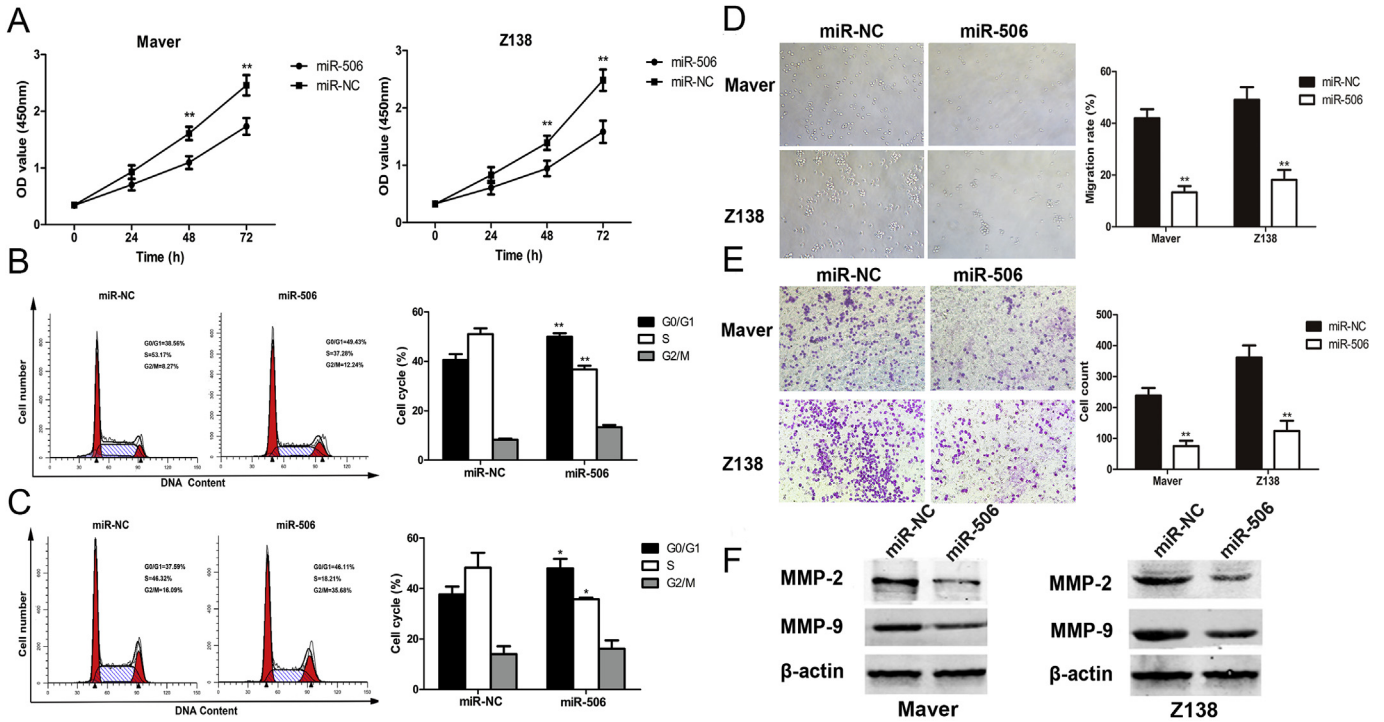

Figure 3. miR-506 overexpression inhibited MCL cell proliferation, migration, invasion and caused cell cycle arrest. (A) The effects of miR-506 on cell viability were measured by CCK-8 assay in Maver and Z138 cells. (B, C) Flow cytometry was performed to detect the effect of miR-506 on the cell cycle distribution of Maver and Z138 cells. (D, E) The effects of miR-506 on invasion and migration were determined by Transwell assays of Maver and Z138 cells. (F) The expression of the invasion-related proteins MMP-2 and MMP-9 was measured by western blotting. The data were obtained from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Maver and Z138 cells by downregulating MMP-2/MMP-9 levels.

3.7. Overexpression of B7−H3 could rescue the suppression of miR-506

To elucidate whether B7−H3 was a functional target of miR-506, we performed rescue experiments by restoring B7−H3 expression in stable miR-506-overexpressing Maver and Z138 cells. Western blotting showed that the decreased level of B7−H3 caused by miR-506 overexpression was restored in Maver and Z138 cells after transfection with pcDNA3.1-B7-H3 (Fig. 4A). A CCK-8 assay indicated that B7−H3 overexpression could significantly reverse the growth inhibition mediated by miR-506 (Fig. 4B and C). The migration and invasion arrays also showed that the effects of miR-506 overexpression on the migration and invasion of Maver and Z138 cells were partially abrogated by re-overexpressing B7−H3 (Fig. 4D and E). These results indicated that miR-506 performs tumor-suppressing roles in MCL cells by targeting and inhibiting B7−H3.

4. Discussion

MCL is a rare subtype of B-NHL with clinically aggressive behavior that is still considered an incurable type of lymphoma [17]. The fundamental molecular mechanisms of MCL oncogenesis and progression remain poorly understood to date. B7−H3, also known as CD276, is frequently overexpressed in various human cancers and may play a crucial role in disease progression and poor patient outcome [18]. We previously found that knockdown of B7−H3 suppressed the proliferation and invasion of the MCL cell lines [10]. In this study, we showed that B7−H3 expression was significantly higher in MCL patients and cell lines. These results indicate that B7−H3 functions as an oncogene in the development and progression of MCL. However, relatively little is known about the molecular mechanisms that regulate B7−H3 expression in MCL.

MiRNAs are of great importance in major biological processes due to their regulation of target gene expression [19,20]. Dysregulated miRNA expression is involved in the pathogenesis of MCL [21−23]. MiR-506 is one of the miRNAs closely associated with proliferation and metastasis in cancer [13]. Mounting studies indicate that the tumor suppressor function of miR-506 is due to the suppression of cell proliferation, induction of cell cycle arrest, and enhancement of apoptosis and chemosensitivity in cancer cells, such as ovarian cancer [24], pancreatic cancer [25], and glioblastoma [26]. However, the biological roles of miR-506 in MCL are still unknown. In the current study, MCL patients and cell lines showed decreased expression of miR-506. According to the results of the bioinformatic analysis, the B7−H3 3′-UTR includes potential binding sites that perfectly match miR-506. In addition, a luciferase reporter assay showed that miR-506 binds directly to the 3′-UTR of B7−H3. We also revealed that miR-506 significantly reduced B7−H3 mRNA and protein expression in Maver and Z138 cells, further indicating that B7−H3 is a miR-506 target gene.

The posttranscriptional regulatory functions of microRNAs are closely related to their target genes. Our prior study revealed that B7−H3 exerted an oncogenic function in MCL processes. Correspondingly, the effects of miR-506 on biological behavior were investigated in MCL cells. Here, we found that miR-506 overexpression significantly suppressed MCL cell proliferation, migration and invasion. Since MMP-2 and MMP-9 are important for invasion and metastasis in malignancy [27,28], we measured the expression of these two proteins, and our western blotting results showed that miR-506 significantly suppressed the expression levels of MMP-2 and MMP-9. These lines of evidence imply that

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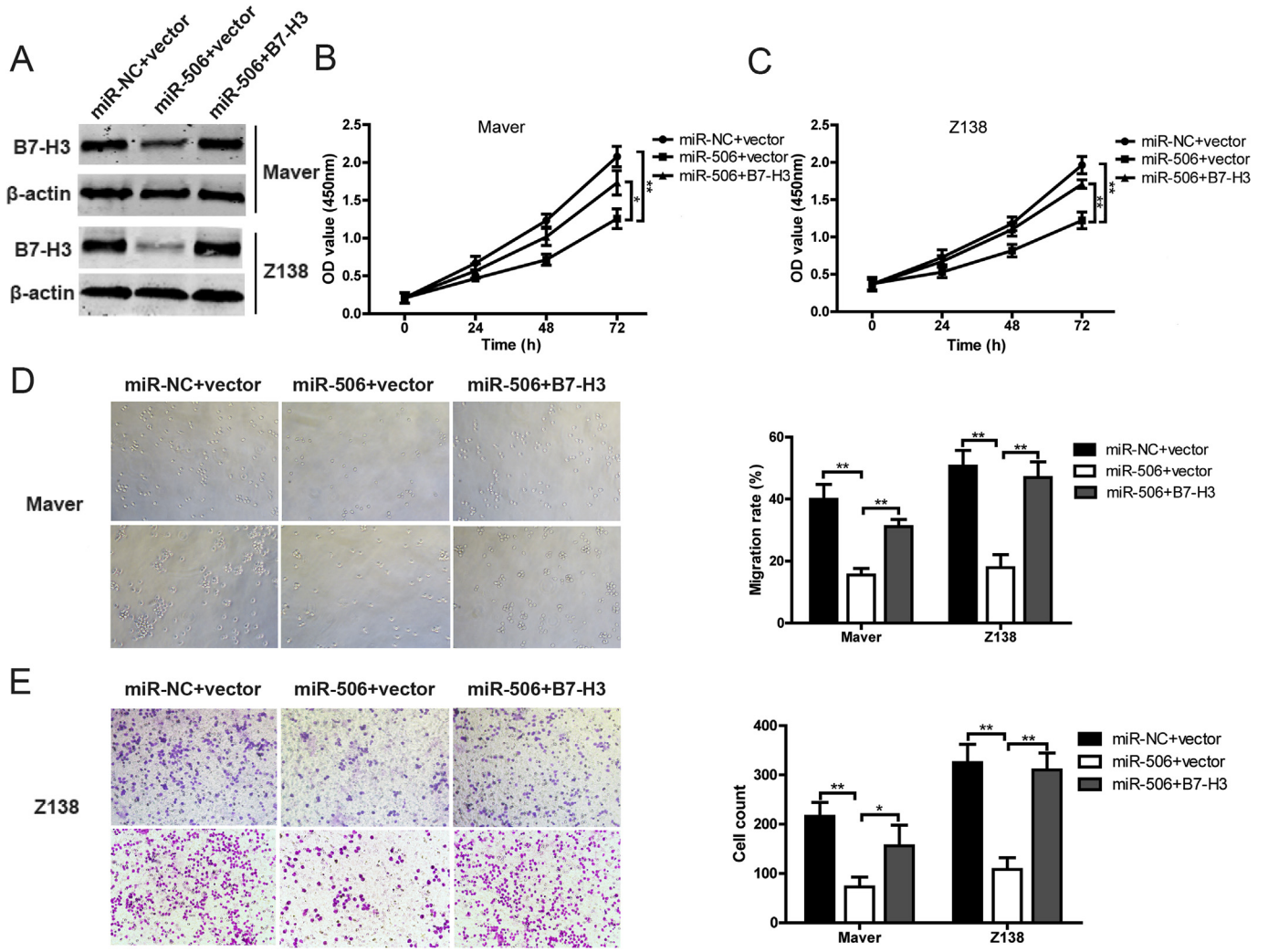

Fig. 4. Restoration of B7−H3 expression rescued the inhibitory effect of miR-506 on MCL cell proliferation and invasion. (A) The expression levels of B7−H3 in the miR-506-overexpressing Maver and Z138 cells with different transfections were determined by western blotting. The effects of overexpressing B7−H3 in the miR-506-overexpressing Maver and Z138 cells on cell proliferation (B), migration (C) and invasion (D) were measured. The data were obtained from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

miR-506 acts as a tumor suppressor miRNA in MCL and its upregulation may be a useful therapeutic strategy for inhibiting MCL progression. Currently, several reports have found that miR-506 suppresses the development and progression of cancers by targeting proteins such as CREB1 [29], RAB3D [30], NEK6 [31], MDR1/P-gp [32] and ROCK1 [33]. In this study, B7−H3 was identified as a novel target of miR-506 in MCL. To clarify whether the suppressive effect of miR-506 was mediated by repression of B7−H3, a rescue experiment was performed. We found that restoration of B7−H3 rescued the inhibitory effects of miR-506 on MCL cells. Our study provided powerful evidence that miR-506 exerts tumor suppressive functions through directly downregulating B7−H3 in MCL.

In summary, we observed that miR-506 serves as a potential tumor suppressor gene for MCL development by directly targeting B7−H3. Our study provides a potential mechanism for B7−H3 dysregulation and contribution to MCL progression. These findings open the possibility that miR-506 and B7−H3 could be potential targets for the treatment of MCL in the future.

Conflicts of interest

The authors declare no conflicts of interest.

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Transparency document

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