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Effects of miR-32 targeting PTEN on proliferation and apoptosis of myeloma cells

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Abstract. – OBJECTIVE: To explore the effects of micro ribonucleic acid (miR)-32 on the proliferation and apoptosis of myeloma cells, and to verify whether it exerts its function by targeting phosphatase and tensin homolog deleted on chromosome ten (PTEN).

PATIENTS AND METHODS: The differentially expressed miRNAs were screened in healthy people and myeloma patients. The myeloma U266 cells transfected with negative control (NC) were used as control group, those transfected with miR-32 inhibitor as transfection group, and those transfected with miR-32 inhibitor and treated with PTEN inhibitor SF1670 as the transfection + inhibitor group. Then, the cell proliferation and apoptosis in each group were detected using the 5-Ethynyl-2'-deoxyuridine (EdU) kit and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, respectively. Finally, the expressions of apoptosis-related proteins B-cell lymphoma-2 (Bcl-2), Bcl-2 homologous antagonist/killer (Bak), caspase-9, and survivin were detected.

RESULTS: The expressions of some miRNAs and genes in myeloma patients were significantly different from those in healthy people. In myeloma patients, miR-32, miR-126, miR-123, and miR-183 were significantly highly expressed, while miR-5, miR-76, and miR-50 were remarkably lowly expressed. After myeloma U266 cells were transfected with the miR-32 inhibitor, the expression of miR-32 markedly declined. In addition, the mRNA expression of PTEN in myeloma cells rose after transfection with the miR-32 inhibitor, and declined after addition of the PTEN inhibitor SF1670, which were consistent with the results of Western blotting. Besides, the proliferation ability of myeloma cells was evidently weakened after transfection with the miR-32 inhibitor, while it was restored to a certain extent after addition of the PTEN inhibitor SF1670. Moreover, the number of apoptotic myeloma cells was remarkably larger after transfection with the miR-32 inhibitor, while it was remarkably smaller after addition of the PTEN inhibitor SF1670. The expressions of pro-apoptotic proteins Bak and caspase-9 in myeloma cells were significantly increased after transfection with the miR-32 inhibitor ($p<0.05$), and significantly decreased after addition of the PTEN inhibitor SF1670, while the expressions of anti-apoptotic proteins Bcl-2 and survivin were opposite to those of Bak and caspase-9.

CONCLUSIONS: MiR-32 targeting PTEN will have certain effects on the proliferation and apoptosis of myeloma cells.

Key Words:
MiR-32, PTEN, Myeloma.

Introduction

Myeloma is a kind of malignant tumor originating from the bone marrow, and it frequently arises in the hematopoietic system, dominated by multiple myeloma[1,2]. Myeloma is a plasma cell-related malignant tumor, and plasma cells mainly secrete antibodies and exert immune function, so the immune system of myeloma patients is often in a state of collapse[3]. In addition to external environmental factors, the occurrence of myeloma may be related to multiple microenvironment and cell signal transduction in patients, such as MCL1 gene expression[4] and abnormal expression of Hedgehog pathway[5]. Therefore, studying the specific pathogenesis of myeloma is of great help for its early diagnosis and treatment.

Micro ribonucleic acids (miRNAs) are a kind of conserved non-coding small RNAs, which, by binding to the 3'-untranslated region (UTR) of target mRNA, regulate the post-transcriptional gene expression in the occurrence and progression of diseases, and they have become research hotspots in recent years in China and foreign countries[6]. Dysregulated miRNAs play key roles in many biological processes in myeloma patients[7]. Furthermore, the changes in content of miRNAs in myeloma patients can be used as observation indexes for early diagnosis and progression of disease. MiR-21

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can affect the myeloma cell functions through STAT3, which may be a potential therapeutic target[8]. The content of peripheral circulating miR-203 can serve as a marker for early diagnosis of myeloma[9]. Hypoxia-induced miR-210 can promote the growth of myeloma through DIMT1-IRF4[10]. However, the expression of miR-32 in myeloma patients and its effects on cell functions have not been reported yet.

In this paper, therefore, the differentially expressed miRNAs were obtained by sequencing in healthy people and myeloma patients. Then, with the myeloma U266 cells as the vector, the miR-32 inhibitor was used to interfere in the miR-32 expression, and the expression of phosphatase and tensin homolog deleted on chromosome ten (PTEN) was inhibited using SF1670, so as to prove that miR-32 regulates the proliferation and apoptosis of myeloma cells through PTEN.

Patients and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA), fetal bovine serum (FBS; Gibco, Rockville, MD, USA), trypsin, phosphate-buffered saline (PBS) and PTEN inhibitor SF1670 (Selleck, Houston, TX, USA). MiR-32 inhibitor, miR-32, PTEN, Bcl-2 homologous antagonist/killer (Bak), caspase-9, Bcl-2, survivin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA primers (Sangon, Shanghai, China), PTEN, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibodies (Abcam, Cambridge, MA, USA). 5-Ethynyl-2'-deoxyuridine (EdU) assay kit (RIBOBIO, Guangzhou, China) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit (Beyotime, Shanghai, China).

Screening of Differentially Expressed MiRNAs in Myeloma Patients

The myeloid tissues were taken from 13 healthy people and 16 myeloma patients to extract the total RNA. The total RNA was sent to Sangon (Shanghai, China) for sequencing, and the miRNA and gene expressions were detected according to the standard quality control procedures. After the expression profile was obtained, the differentially expressed miRNAs in myeloma patients were analyzed. This study was approved by the Ethics Committee of First Affiliated Hospital of Jinzhou Medical University.

Transfection of MiR-32 Inhibitor and Inhibition of SF1670 on PTEN Expression With

The myeloma U266 cells in logarithmic growth phase were taken and paved onto the plate at 1 d before transfection. At the time of transfection, the old medium was replaced with serum-free OPTI-MEM. 5 μL of miR-32 inhibitor and 5 μL of Lipo 2000 were added into 245 μL of OPTI-MEM, mixed evenly after 5 min, placed for 20 min, and added with the cell culture fluid. After 24 h, the PTEN inhibitor SF1670 was added (5 μL/well), followed by observation of cell growth.

The myeloma U266 cells transfected with negative control (NC) were used as control group, those transfected with miR-32 inhibitor as transfection group, and those transfected with miR-32 inhibitor and treated with PTEN inhibitor SF1670 as transfection + inhibitor group.

MRNA Detection

The mRNA expressions of miR-32, PTEN, Bak, caspase-9, Bcl-2, and survivin were determined using real-time fluorescence quantitative polymerase chain reaction (qPCR), with GAPDH as an internal reference. The total RNA was extracted in each group using TRIzol method (Invitrogen, Carlsbad, CA, USA). Then, 1 μL of RNA samples was diluted 50-fold, the optical density (OD) value was determined, and the RNA samples with the OD_{260}/OD_{280} ratio >1.8 were used for subsequent experiments. After that, the RNA was reversely transcribed into cDNA and stored at –20°C. The gene primers were designed using Primer Premier 5.0 and synthesized by Sangon. The primer sequences and Tm values are shown in Table I. The total system of PCR was 25 μL, including 1 μL of forward primers, 1 μL of reverse primers, 0.5 μL of cDNA template, 12.5 μL of SYBR premix Taq, and 10 μL of dH_2O. The PCR conditions are as follows: 95°C for 2 min (95°C for 35 s, 58°C for 40 s, and 72°C for 35 s) × 40 cycles.

Western Blotting

The protein expression of PTEN was detected using Western blotting, with GAPDH as the internal reference. The myeloma cells in each group were lysed on ice using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) added into 1% protease inhibitor phenylmethylsulfonyl fluoride (PMSF) for 30 min, vortexed for 3 times midway, centri-

Table I. Primer sequences in qPCR.

Gene		Primer sequences (5′→3′)
miR-32	Forward	TGGATTCGACTTAGACTTGACCT
	Reverse	GGTGGGTTATGGTCTTCAAAAGG
PTEN	Forward	TTTGAAGACCATAACCCACCAC
	Reverse	ATTACACCAGTTCGTCCCTTTC
Caspase-9	Forward	AGGGACGAACTGGTGTAATGA
	Reverse	CTGGTCCTTACTTCCCCATAGAA
Bcl-2	Forward	TGGATTCGACTTAGACTTGACCT
	Reverse	GCGGTGTCATAATGTCTCTCAG
Survivin	Forward	TGGATTCGACTTAGACTTGACCT
	Reverse	TGGCGGTGTCATAATGTCTCT
Bak	Forward	CCTTTTGAAGACCATAACCCACC
	Reverse	GAATTGCTGCAACATGATTGTCA
GAPDH	Forward	CAATCATGTTGCAGCAATTCACT
	Reverse	CCCCATAAAAATCTAGGGCCTCT
U6	Forward	CTCGCTTCGGCAGCACATAT
	Reverse	TTGCGTGTCATCCTTGCG

fuged at 14,000 rpm for 15 min, and added with loading buffer, followed by boiling water bath and storage at –20°C. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with 5% skim milk for 1 h, and incubated with antibodies at 4°C overnight. After washing on the next day, the protein was incubated again with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h and washed, followed by exposure using an imager.

EdU Analysis

The myeloma cells in logarithmic growth phase in each group were inoculated into a 96-well plate (8,000-10,000 cells/well), cultured overnight, labeled with EdU reagent diluted using the cell culture fluid. Thereafter, the cells were washed with PBS for 3 times and fixed with 4% paraformaldehyde for 2-4 h, followed by staining with Apollo dye and DNA dye. Finally, the cells were photographed under a fluorescence microscope.

TUNEL Assay

The myeloma cells in each group were inoculated, fixed with 4% paraformaldehyde for 24 h, and detected using the TUNEL apoptosis assay kit in strict accordance with the instructions. After the color developer was added, the TUNEL-positive cells were obtained under the excitation light of 488 nm, counted, and photographed in 5-10 fields.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The *t*-test and analysis of variance (ANOVA) were performed for the comparison of intergroup difference. $p<0.05$ suggested the statistically significant difference.

Results

Differentially Expressed MiRNAs in Healthy People and Myeloma Patients

As shown in Figures 1 and 2, the expressions of some miRNAs and genes in myeloma patients

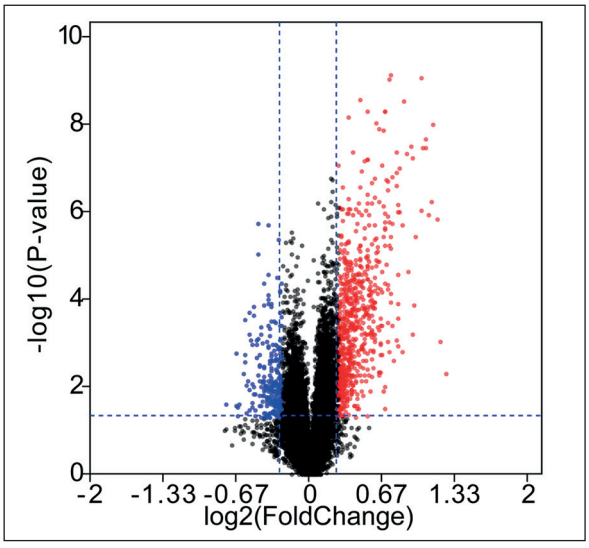

Figure 1. Volcano map of differentially expressed genes and miRNAs in healthy people and myeloma patients.

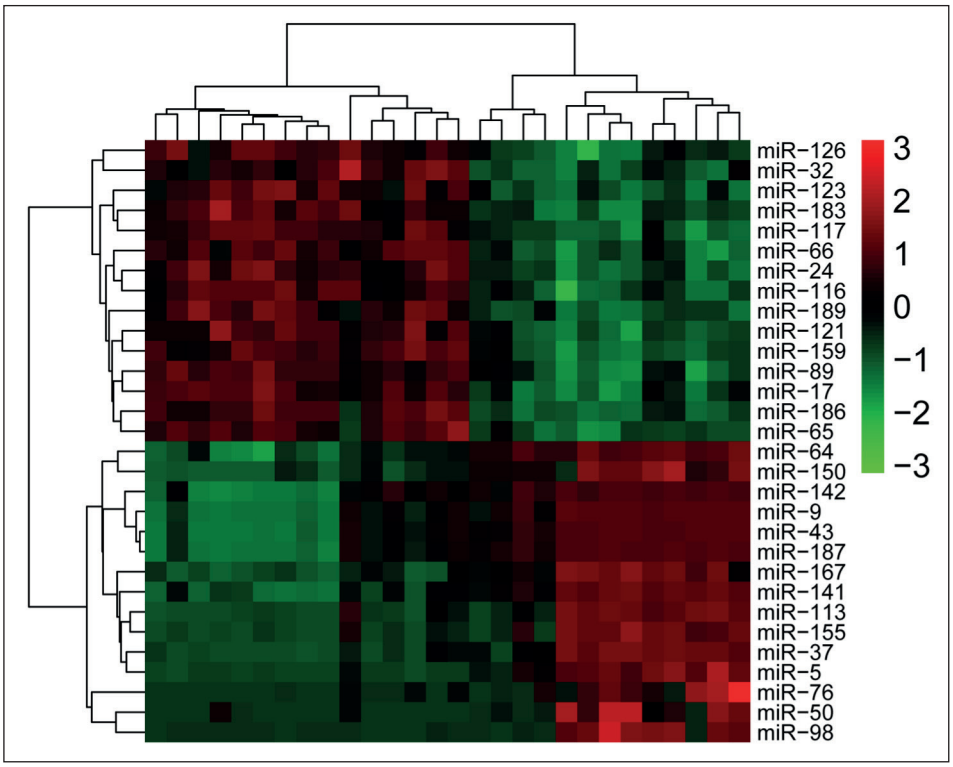

Figure 2. Heat map of differentially expressed miRNAs in healthy people and myeloma patients.

were significantly different from those in healthy people. In myeloma patients, miR-32, miR-126, miR-123, and miR-183 were significantly highly expressed, while miR-5, miR-76, and miR-50 were remarkably lowly expressed.

Expression of MiR-32 in Control Group and Transfection Group

As shown in Figure 3, after myeloma U266 cells were transfected with the miR-32 inhibitor, the expression of miR-32 markedly declined.

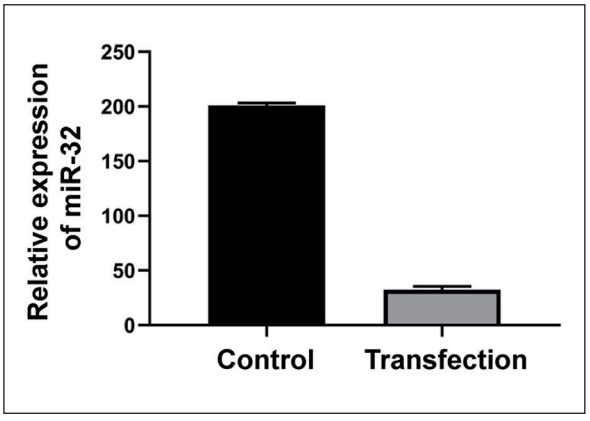

Figure 3. Expression of miR-32 in myeloma cells after transfection with miR-32 inhibitor.

Expression of PTEN in Control Group, Transfection Group, and Transfection + Inhibitor Group

According to the miRNA target gene database analysis (TargetScan) and literature reports, the targeted regulatory gene of miR-32 was predicted as PTEN, so the influence of the change of miR-32 expression on PTEN expression was studied. The mRNA expression of PTEN in myeloma cells rose after transfection with the miR-32 inhibitor, and declined after addition of the PTEN inhibitor SF1670, which were consistent with the results of Western blotting (Figures 4 and 5).

EdU Cell Proliferation Assay in Control Group, Transfection Group, and Transfection + Inhibitor Group

The proliferation ability of myeloma cells was evidently weakened after transfection with the miR-32 inhibitor, while it was restored to a certain extent after addition of the PTEN inhibitor SF1670 (Figure 6).

Apoptosis in Control Group, Transfection Group, and Transfection + Inhibitor Group

The number of apoptotic myeloma cells was remarkably larger after transfection with the miR-

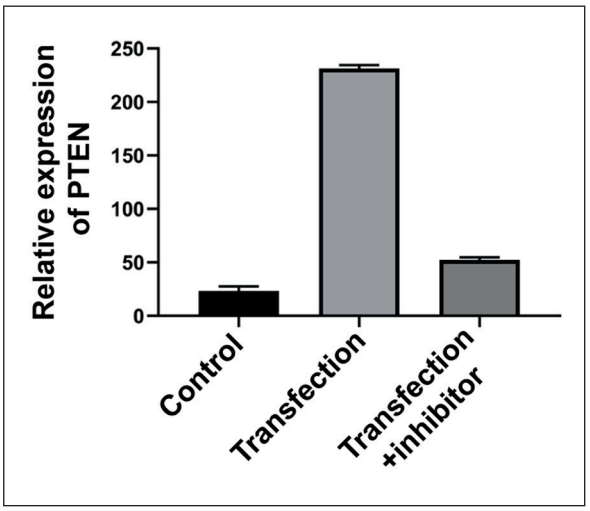

Figure 4. MRNA expression of PTEN in control group, transfection group and transfection + inhibitor group.

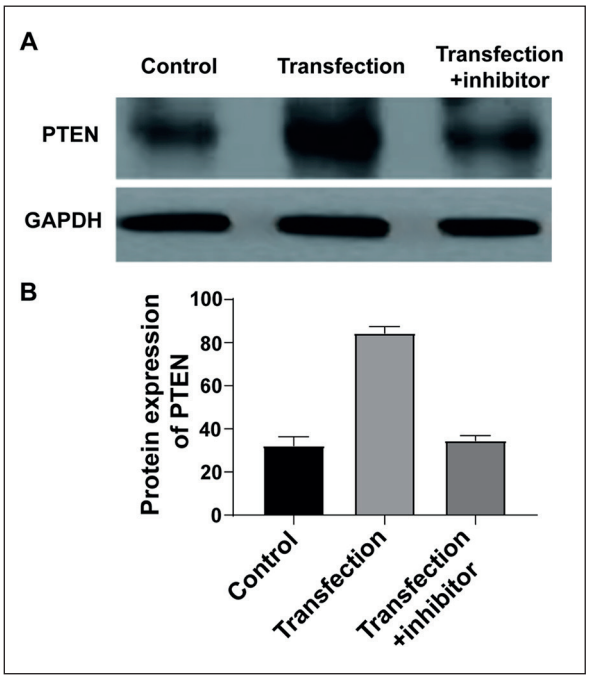

Figure 5. Protein expression of PTEN in control group, transfection group and transfection + inhibitor group.

32 inhibitor, while it was remarkably smaller after addition of the PTEN inhibitor SF1670 (Figure 7). Moreover, the expressions of pro-apoptotic proteins Bak and caspase-9 in myeloma cells were prominently increased after transfection with the miR-32 inhibitor ($p<0.05$), and notably decreased after addition of the PTEN inhibitor SF1670, while the expressions of anti-apoptotic proteins Bcl-2 and survivin were opposite to those of Bak and caspase-9 (Figure 8).

Discussion

As a common malignant tumor of the immune system, myeloma greatly damages the patient's immunity[11,12]. In addition to the uselessness of plasma cell function, myeloma affects the ability of immune cells to secrete cytokines, thereby further reducing the patient's resistance to external pathogenic microorganisms[13]. Moreover, the malignant proliferation of myeloma tissues seriously affects the growth and normal function of other cells in the hematopoietic system, and significantly reduces erythrocytes and platelets in patients, thus leading to tissue hypoxia

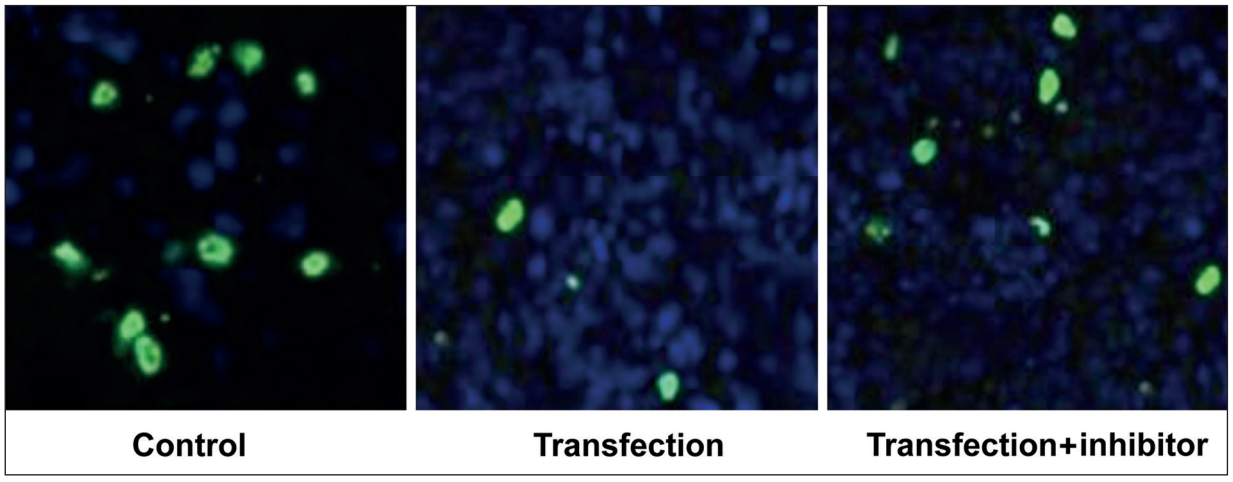

Figure 6. EdU cell proliferation assay in control group, transfection group and transfection + inhibitor group (magnification: 100×).

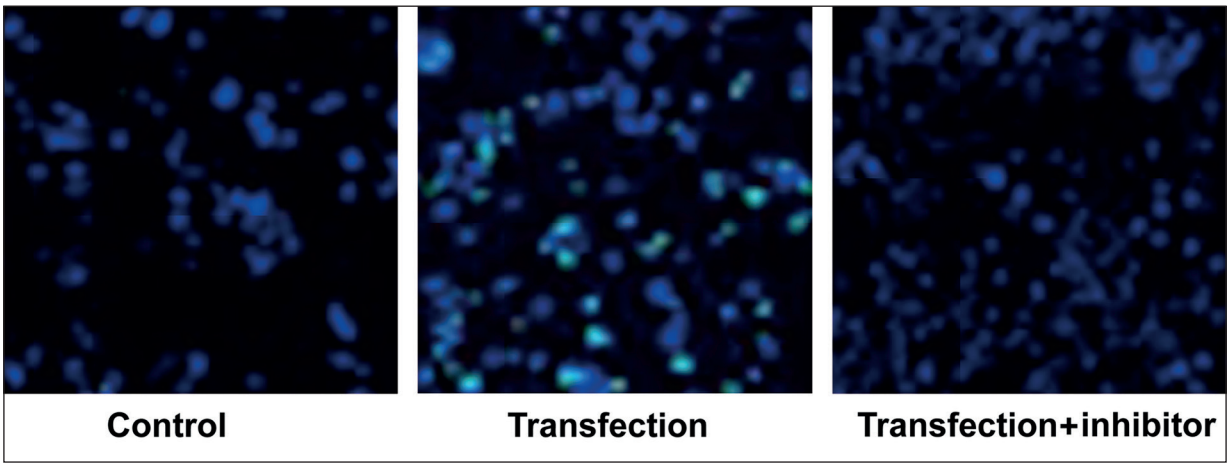

Figure 7. TUNEL apoptosis assay in control group, transfection group and transfection + inhibitor group (magnification: 100×).

and bleeding, and further aggravating the condition[14]. The mechanism of myeloma occurrence and development is not entirely clear, mainly as follows: some signaling pathways in myeloma cells are activated, so that the cell proliferation ability is substantially enhanced and becomes uncontrolled, and the number of apoptotic cells declines, thus making the growth of myeloma cells out of control[15].

In this study, according to the analysis of differentially expressed genes and miRNAs in healthy people and myeloma patients, there were a large number of highly and lowly expressed genes in myeloma patients, and these differentially expressed genes may jointly cause malignant transformation of bone marrow cells. At the same time, as key molecules regulating the post-transcriptional gene expression in the occurrence and progression of diseases by binding to the 3'-UTR of target mRNA, miRNAs are also differentially expressed in myeloma cells. In myeloma patients, miR-32, miR-126, miR-123, and miR-183 exhibit significant high expressions, while miR-5, miR-76, and miR-50 displayed evident low expressions, suggesting that miR-32, miR-126, miR-123, and miR-183 may promote tumor growth in the occurrence of myeloma, while miR-5, miR-76, and miR-50 exert an opposite effect. Therefore, designing drugs targeting these miRNAs may alter the function of myeloma cells, thus alleviating or treating the disease.

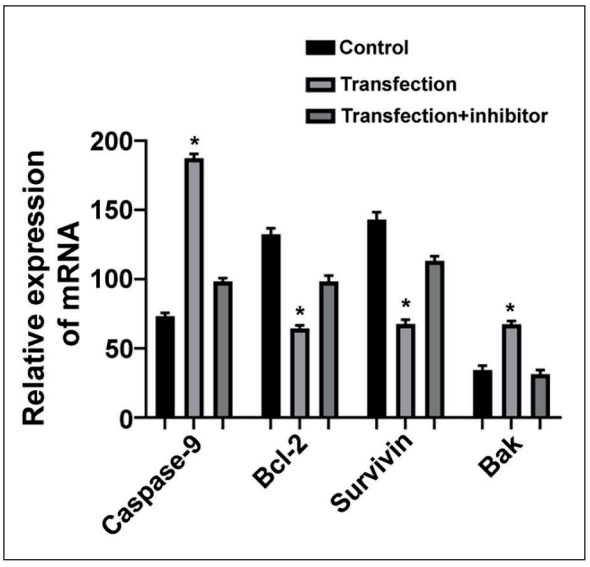

Figure 8. Expressions of apoptosis proteins in control group, transfection group and transfection + inhibitor group (*$p<0.05$ *vs.* control group and transfection + inhibitor group in *t*-test).

Among these differentially expressed miRNAs in myeloma, miR-32 had significant high expression. It has been proved that miR-32 can affect the occurrence of a variety of diseases, and its high expression can significantly worsen the survival prognosis of patients with hepatocellular carcinoma[16,17]. MiR-32 targeting PTEN can strengthen the polarization of M2 macrophages in glioma[18]. At the same time, according to the database analysis and literature reports, PTEN is the targeted regulatory gene of miR-32[18], and PTEN may have a significant effect on the tumor function. In addition, JARID2 can accelerate the progression of bladder cancer through the PTEN/Akt pathway[19], and PTEN can also affect the recurrence of prostate cancer[20]. In this study, the mRNA expression of PTEN in myeloma cells

rose after transfection with the miR-32 inhibitor, and declined after addition of the PTEN inhibitor SF1670, which were consistent with the results of Western blotting. The above results demonstrate that PTEN is the target gene of miR-32, and miR-32 functions by regulating PTEN and inhibits the expression of PTEN.

Then, the effect of miR-32 on the proliferation ability of myeloma cells was studied, and it was found that the proliferation ability of myeloma cells was evidently weakened after transfection with the miR-32 inhibitor, indicating that miR-32 can significantly enhance the proliferation ability of myeloma cells, consistent with the above conjecture that miR-32 may be a promotor of myeloma. After addition of the PTEN inhibitor SF1670, the proliferation ability was restored to a certain extent, indicating that miR-32 promotes the proliferation of myeloma cells by targeting PTEN.

The results of TUNEL assay showed that the number of apoptotic myeloma cells was remarkably larger after transfection with the miR-32 inhibitor, suggesting that miR-32 can suppress the apoptosis of myeloma cells, and once again indicating its tumor-promoting effect. The number of apoptotic myeloma cells was remarkably smaller after addition of the PTEN inhibitor SF1670, demonstrating that miR-32 affects the apoptosis of myeloma by targeting PTEN. Finally, the expressions of pro-apoptotic proteins Bak and caspase-9 in myeloma cells were evidently increased after transfection with the miR-32 inhibitor ($p<0.05$), and notably decreased after addition of the PTEN inhibitor SF1670, while the expressions of anti-apoptotic proteins Bcl-2 and survivin were opposite to those of Bak and caspase-9. The expressions of apoptosis proteins in myeloma cells were consistent with the results of TUNEL assay, also showing that the effect of miR-32 on the apoptosis of myeloma is realized by targeting PTEN.

Conclusions

In summary, miR-32 targeting PTEN will have certain effects on the proliferation and apoptosis of myeloma cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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