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miR-26 inhibits proliferation and promotes apoptosis of multiple myeloma cells by targeting BNIP3

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ABSTRACT

This study was carried out to investigate the molecular mechanism of microRNA-26 (miR-26) targeting BNIP3 to mediate proliferation and apoptosis of multiple myeloma (MM) cells. The expression of miR-26 and BNIP3 in MM and normal tissues was detected by qRT-PCR and Western blot. According to the average expression of miR-26 and BNIP3, the patients were divided into 12 cases with high miR-26 expression group, 18 cases with low miR-26 expression group, 20 cases with BNIP3 high expression group, and 10 cases with BNIP3 low expression group. The correlation between the expression of miR-26 and BNIP3 and the clinicopathological characteristics of MM patients was compared and analyzed. The effect of up-regulation of miR-26 expression and BNIP3 overexpression on the proliferation of multiple myeloma cells RPMI8226 was examined by MTT assay. Flow cytometry was used to detect the effect of miR-26 expression and BNIP3 overexpression on the apoptosis of RPMI8226 cells. The dual luciferase reporter assay validated the targeted regulation of miR-26 on BNIP3. The expression level of miR-26 in MM tissues was lower than that in normal tissues (P<0.05), and the expression level of BNIP3 in MM tissues was higher than that in normal tissues (P<0.05). miR-26 was closely related to clinical stage, M protein type and light chain type (P<0.05), while BNIP3 was closely related to M protein type and light chain type (P<0.05). After up-regulating miR-26 expression, cell viability was significantly decreased (P<0.05), apoptosis rate was significantly increased (P<0.05) Dual luciferase reporter experiments confirmed that miR-26 could target BNIP3 and negatively regulate the expression of BNIP3 (P<0.05). Overexpression of BNIP3 reversed the effect of up-regulation of miR-26 expression on proliferation and apoptosis of RPMI8226 cells. Up-regulation of miR-26 expression inhibits MM cell proliferation and promotes apoptosis by targeting BNIP3.

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Introduction

Multiple myeloma (MM) is a common hematological malignancy in clinic. The incidence rate of MM patients in China is increasing year by year (1). MicroRNAs (miRNAs) can participate in the occurrence and development of MM (2,3). miR-26 is low expressed in other malignant tumors such as acute myeloid leukemia and can participate in tumor occurrence and development (4,5). TargetScan target Gene prediction shows that BCL2/adenovirus E1B 19 kDa protein interacting protein 3 (BNIP3) may be the target gene of miR-26. Research shows that BNIP3 is highly expressed in Glioma, salivary adenoid cystic carcinoma and other tumors, and silencing BNIP3 expression can promote tumor cell apoptosis (6,7). This study mainly explores the expression status of miR-26 and BNIP3 in MM cells, analyzes their effects on MM cell proliferation and apoptosis, and aims to lay a theoretical foundation for revealing the pathogenesis of MM.

Materials and Methods

Materials and reagents

Human multiple myeloma cell RPMI8226 (Shanghai Kelei biological, Shanghai, China); RPMI 1640 medium (Hyclone, South Logan, UT, USA); Fetal bovine serum (Nanjing senbega biological, Nanjing, China); Trypsin (Shanghai Yubo biological, Shanghai, China); Lipofectamine2000 (Shanghai Hengfei biological, Shanghai, China); miR-26 mimics, miR NC, anti-miR-26, anti-miR NC (Shanghai Jima, Shanghai, China); PcDNA3.1 (Beijing synbiotic gene, Beijing, China); MTT (Shanghai Zeye Biology, Shanghai, China); Apoptosis Kit (Shanghai Yisheng biological, Shanghai, China); TRIzol reagent (Beijing all gold bio, Beijing, China); Both the reverse transcription kit and the fluorescent quantitative PCR kit (Beijing Tiangen, Beijing, China); Rabbit anti human BNIP3 antibody (Beijing Huaxia Ocean Technology Co., Ltd., Beijing, China); Rabbit anti human CyclinD1, Bcl-2, p21, Bax antibodies (CST, Danvers, MA, USA); Secondary antibody (Beijing Zhongshan Jinqiao biological, Beijing, China).

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Sources of MM tissues and normal tissues: 30 mm patients admitted to our hospital from December 2016 to June 2018 were selected as the research objects, including 16 males and 14 females, aged 40-70 years, with an average age of (58.64 ± 8.52) years. At the same time, 30 healthy volunteers who underwent physical examination in our hospital during the same period were selected as the control group, including 18 males and 12 females, aged 38-71 years, with an average age of (60.12 ± 8.54) years. Bone marrow biopsy samples of the two groups were taken during the operation and stored in -80°C ultra-low temperature refrigerator. MM patients were pathologically confirmed as multiple myeloma, and all patients did not receive other treatment before surgery; the clinicopathological data were complete.

Methods

Cell transfection and experimental grouping

RPMI8226 cells were inoculated in 6-well plates and divided into the following groups according to different transfectants. miR-26 mimics (miR-26 group), miR-NC (miR-NC group), miR-26 mimics with pcDNA-BNIP3 (miR-26+pcDNA-BNIP3 group), miR-26 mimics with pcDNA (miR-26+pcDNA group) were transfected into RPMI8226 cells, and untransfected RPMI8226 cells were used as NC group. miR-NC (miR-NC group), miR-26 mimics (miR-26 group), anti-miR-NC (anti-miR-NC group), and anti-miR-26 (anti-miR-26 group) were transfected into RPMI8226 cells, respectively.

qRT-PCR detection of miR-26, BNIP3 mRNA expression level

Total RNA was extracted from bone marrow tissues by the TRIzol method, and total RNA was extracted from RPMI8226 cells of NC group, miR-NC group and miR-26 group by TRIzol method, according to the instructions of the kit. The RNA was used as a template for reverse transcription to synthesise cDNA, which was carried out in strict accordance with the reverse transcription kit instructions. miR-26 and BNIP3 mRNA relative expression was detected in accordance with the fluorescence quantitative PCR reagent instructions.

MTT assay for cell proliferation

Collect each group of transfected RPMI8226 cells (5×10^4 cells/mL) and inoculate them into 96-well plates (100 μL/well), follow the instructions of the kit and detect the absorbance (OD 490 nm) value of each well by using an enzyme labeller.

Detection of apoptosis by flow cytometry

Collect RPMI8226 cells from each group and add 500 μL binding buffer, and detect the apoptosis rate according to the apoptosis detection kit instructions.

Dual luciferase reporter gene detection of miR-26 target gene

The wild-type reporter gene vector WT-BNIP3 and mutant reporter gene vector MUT-BNIP3 were constructed, and log-phase RPMI8226 cells were taken, and WT-BNIP3 and miR-NC, WT-BNIP3 and miR-26 mimics, MUT-BNIP3 and miR-NC, and MUT-BNIP3 and miR-26 mimics were co-transfected into RPMI8226 cells and cultured for 48 h to detect relative luciferase activity.

Western blot detection of BNIP3, CyclinD1, Bcl-2, P21, Bax protein expression

Total protein was extracted, protein concentration was detected, protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoresis products were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), closed for 1 h, placed in primary antibody dilution for overnight incubation (4°C), secondary antibody dilution was added, incubated for 1 h at room temperature, electrochemiluminescence (ECL) was added dropwise to develop the colour, and grey value of each protein band was analysed by using gel analysis system.

Statistical analysis

Statistic Package for Social Science (SPSS) 21.0 statistical software (IBM, Armonk, NY, USA) was used to analyse the data, and the measurement information was expressed as ($\bar{x}\pm s$), the independent sample *t*-test was used for comparison between two groups, one-way ANOVA was used for comparison between multiple groups, and the LSD-*t* test was used for two-by-two comparisons between groups, and the difference was considered to be statistically significant at $P < 0.05$.

Results

Expression of miR-26 and BNIP3 in MM patients

The experimental results showed that the expression level of miR-26 was significantly lower ($P < 0.05$) and the expression level of BNIP3 mRNA and protein was significantly higher ($P < 0.05$) in MM tissues compared with normal tissues, as shown in Figure 1 and Table 1.

Correlation analysis between miR-26, BNIP3 expression and clinicopathological characteristics of MM patients

According to the average expression of miR-26 and BNIP3, the patients were divided into miR-26 high expression group (12 cases) and miR-26 low expression group (18 cases); there were 20 cases in the BNIP3 high expression group and 10 cases in the BNIP3 low expression group. The correlation between the expression of miR-26 and BNIP3 and the clinicopathological characteristics of MM patients was compared and analyzed. The results showed that miR-26 was closely related to clinical stage, M protein type and light chain type ($P < 0.05$), while BNIP3 was closely related to M protein type and light chain type ($P < 0.05$), see Table 2.

Effect of up-regulating miR-26 expression on RPMI8226 cell proliferation

Compared with the miR-NC group, the viability of RPMI8226 cells in the miR-26 group was reduced ($P < 0.05$), and it could promote P21 expression and inhibit

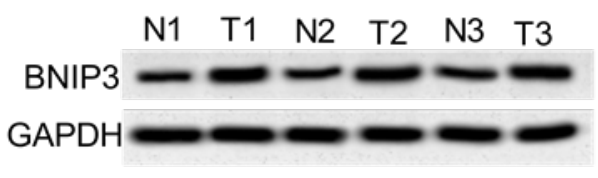

Figure 1. Immunoblot of BNIP3 protein.

Table 1. miR-26, BNIP3 expression in MM patients ($\bar{x}$±s, n=30).

Group	miR-26	BNIP3 mRNA	BNIP3protein
Normal Tissue	1.01±0.10	1.02±0.13	0.56±0.03
MM tissue	0.52±0.05*	2.36±0.21*	1.03±0.11*
t	24.005	29.717	22.578
P	<0.001	<0.001	<0.001

Note: *$P < 0.05$ compared to normal tissue.

Table 2. Correlation Analysis between miR-26, BNIP3 expression and clinicopathological characteristics of MM patients.

Clinicopathological parameters	n	miR-26		x^2, *P*	BNIP3		x^2, *P*
		High expression group (n=12)	Low expression group (n=18)		High expression group (n=20)	Low expression group (n=10)	
Age				0.201,0.654			0.268,0.605
<55 years	16	7	9		10	6	
≥55 years	14	5	9		10	4	
Clinical Stage				7.751,0.005			0.287,0.592
I~II stage	11	8	3		8	3	
Stage III-IV	19	4	15		12	7	
M protein type				7.824,0.020			18.050,0.000
IgG type	9	7	2		1	8	
IgA type	15	4	11		14	1	
IgD type	6	1	5		5	1	
Light chain type				13.032,0.000			8.213,0.004
K Chain	13	10	3		5	8	
λ Chain	17	2	15		15	2	

Table 3. Effect of up-regulation of miR-26 expression on the proliferation of RPMI8226 cells ($\bar{x}$±s, n=9).

Group	OD (490 nm)			CyclinD1 protein	P21 protein	miR-26
	24 h	48 h	72 h			
NC group	0.42±0.03	0.68±0.06	1.22±0.11	0.75±0.08	0.52±0.03	0.55±0.06
miR-NC group	0.45±0.05	0.70±0.07	1.25±0.13	0.71±0.10	0.55±0.06	0.58±0.09
miR-26 group	0.22±0.04*	0.35±0.04*	0.55±0.07*	0.46±0.05*	1.02±0.11*	2.13±0.27
F	84.420	103.277	124.752	35.286	127.898	260.628
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Note: *$P < 0.05$ compared to miR-NC group.

CyclinD1 expression ($P < 0.05$), as shown in Figure 2 and Table 3.

Effect of up-regulation of miR-26 expression on apoptosis of RPMI8226 cells

Compared with the miR-NC group, the apoptosis rate of cells in the miR-26 group was elevated ($P < 0.05$) and could promote Bax expression and inhibit Bcl-2 expression ($P < 0.05$), as shown in Figure 3 and Table 4.

miR-26 targets and regulates BNIP3 expression

The 3'UTR of BNIP3 contains nucleotide sequences complementary to miR-26, see Figure 4A. Transfection of miR-26 mimicxs reduced the luciferase activity of WT-BNIP3 ($P < 0.05$), see Table 5. miR-26 was able to negatively regulate the expression of BNIP3 ($P < 0.05$), see Figure 4B, Table 6.

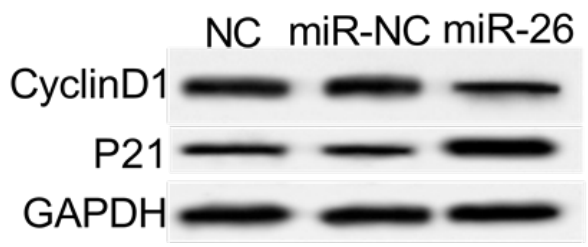

Figure 2. Immunoblot of cell proliferation-associated proteins.

BNIP3 overexpression reverses upregulation of miR-26 expression on RPMI8226 cell proliferation and apoptosis

Compared with the miR-26+pcDNA group, the relative expression of BNIP3 protein in RPMI8226 cells in the miR-26+pcDNA-BNIP3 group was elevated (P < 0.05)

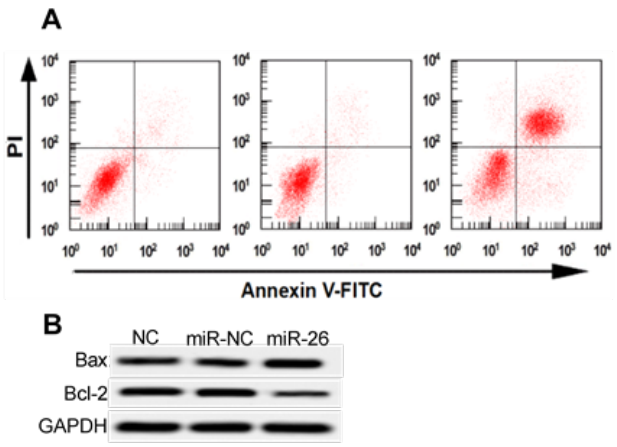

Figure 3. Effect of up-regulation of miR-26 expression on apoptosis in RPMI8226 cells; (A) apoptosis detection by flow cytometry; (B) immunoblotting of apoptosis-related proteins.

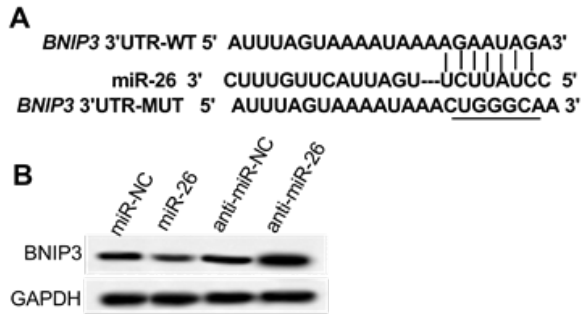

Figure 4. miR-26 targets and regulates BNIP3 expression; (A) TargetScan prediction shows that the 3'UTR of BNIP3 contains a nucleotide sequence complementary to miR-26; (B) Immunoblotting of BNIP3 protein.

and could promote cell proliferation and inhibit apoptosis, and also promoted CyclinD1 and Bcl-2 expression while inhibiting P21 and Bax expression ($P < 0.05$), as shown in Figure 5, Tables 7 and 8.

Discussion

Mir-26 is lowly expressed in liver cancer cells, and upregulation of its expression can promote liver cancer cell apoptosis (8). Inhibiting mir-26 expression can promote gastric cancer cell invasion (9). Relevant reports pointed out that mir-26 inhibited renal cell carcinoma progression through targeted downregulation of coronin-3 expression (10). The results of this study showed that the expression level of mir-26 in mm tissues was significantly reduced, and mir-26 was closely related to clinical stage, M protein type, and light chain type, suggesting that the reduced expression of mir-26 may promote the occurrence of mm. Studies have shown that abnormal expression of CyclinD1 and p21 is closely related to cell proliferation (11,12). The results of this study showed that upregulation of mir-26 expression could inhibit cell proliferation and CyclinD1 expression, while promoting the expression of p21, suggesting that upregulation of mir-26 expression can inhibit MM cell proliferation. The pro-apoptotic protein Bax is low expressed in tumor cells, and the anti-apoptotic protein Bcl-2 is highly expressed in tumor cells and can promote apoptosis (13,14). The results of this study showed that up-regulating mir-26 expression could promote apoptosis and Bax expression, while inhibiting bcl-2 expression, suggesting that up-regulating mir-26 could induce apoptosis in MM cells.

BNIP3 is highly expressed in ovarian cancer cells, and mir-182-5p inhibits the proliferation and migration of ovarian cancer cells by targeting BNIP3 (15,16). Studies

Table 4. Effect of up-regulation of miR-26 expression on apoptosis in RPMI8226 cells ($\bar{x}$±s, n=9).

Group	Apoptosis rate (%)	BCL2-Associated X	B-cell lymphoma-2
NC group	8.57±1.13	0.56±0.08	0.96±0.13
miR-NC group	8.55±1.15	0.55±0.10	0.92±0.17
miR-26 group	28.57±2.36*	0.93±0.15*	0.43±0.06*
F	441.132	32.553	47.605
P	<0.001	<0.001	<0.001

Note: *$P < 0.05$ compared to the miR-NC group.

Table 5. Dual luciferase reporter assay ($\bar{x}$±s, n=9).

Group	WT-BNIP3	MUT-BNIP3
miR-NC group	1.03±0.15	1.02±0.17
miR-26 group	0.59±0.08*	1.00±0.15
t	7.765	0.265
P	<0.001	0.795

Table 6. miR-26 negatively regulates BNIP3 expression ($\bar{x}$±s, n=9).

Group	BNIP3 protein
miR-NC group	0.88±0.16
miR-26 group	0.41±0.05*
anti-miR-NC group	0.86±0.18
anti-miR-26 group	1.10±0.20#
F	30.027
P	<0.001

Note: *$P < 0.05$ compared with miR-NC group; #$P < 0.05$ compared with anti-miR-NC group.

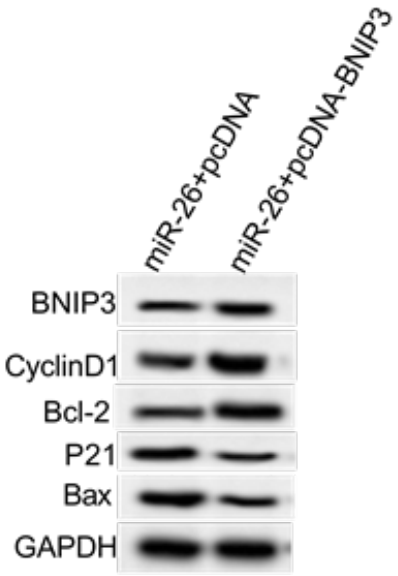

Figure 5. Immunoblotting of BNIP3 protein with cell proliferation and apoptosis-related proteins.

have shown that elevated BNIP3 expression predicts poor prognosis in patients with uveal melanoma (17). Relevant reports have pointed out that BNIP3 is highly expressed in cholangiocarcinoma and can promote tumorigenesis and development (18). The results of this study showed that the expression level of BNIP3 in mm tissues was significantly increased, and BNIP3 was closely related to M protein type and light chain type. Further studies showed that mir-26 could target BNIP3 and negatively regulate the expression of BNIP3. Up-regulation of mir-26 expression could inhibit the proliferation of MM cells and promote apoptosis by targeting the expression of BNIP3.

In conclusion, mir-26 expression is downregulated in mm and BNIP3 expression is upregulated. Upregulation of mir-26 expression in MM cells can target to inhibit BNIP3 expression, thereby weakening the proliferation ability of MM cells and inducing apoptosis, which can provide a potential target for MM-targeted therapy. However, clinical studies and in vivo experiments are still needed to verify the relevant mechanism of mir-26 and BNIP3 in the occurrence and development of mm.

Conflict of Interests

The authors declared no conflict of interest.

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Table 7. BNIP3 overexpression reversibly upregulates miR-26 expression on proliferation and apoptosis of RPMI8226 cells ($\bar{x}$±s, n=9).

Group	OD(490 nm)			Apoptosis rate (%)
	24 h	48 h	72 h	
miR-26+pcDNA group	0.25±0.02	0.37±0.04	0.56±0.05	27.58±1.52
miR-26+pcDNA-BNIP3 group	0.46±0.07*	0.63±0.06*	1.03±0.11*	13.25±2.3*
t	8.654	10.817	11.669	15.547
P	<0.001	<0.001	<0.001	<0.001

Note: *$P < 0.05$ compared to miR-26+pcDNA group.

Table 8. BNIP3 protein expression associated with cell proliferation and apoptosis ($\bar{x}$±s, n=9).

Group	BNIP3 protein	CyclinD1 protein	BCL2-Associated X	P21 protein	B-cell lymphoma-2
miR-26+pcDNA group	0.45±0.08	0.43±0.06	0.45±0.07	0.86±0.15	0.84±0.17
miR-26+pcDNA-BNIP3 group	0.87±0.13*	1.01±0.05*	0.88±0.09*	0.45±0.03*	0.42±0.05*
t	8.255	22.278	11.314	8.041	7.111
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: *$P < 0.05$ compared to miR-26+pcDNA group.

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