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ORIGINAL ARTICLE

MiR-26a inhibits proliferation and apoptosis of uveal melanoma cells *via* regulating p53/MDM2 pathway

Yang Guo, Ye Tian

Department of Dermatology, Dongzhimen Hospital Beijing University of Chinese Medicine, Beijing, China.

Summary

Purpose: *To study the effect of micro ribonucleic acid (miR)-26a on the proliferation and apoptosis of uveal melanoma (UM) cell lines, and to explore the potential signaling pathway.*

Methods: *UM SP6.5 cells were used in this study, and were transfected with miR-26a mimic (miR-26a mimic group) and miR-26a small-interfering RNA (siRNA) (miR-26a siRNA group) using Lipofectamine 2000 transfection reagent, with miR-26a negative control (NC) as the blank controls (miR-26a NC group). The level of miR-26a in SP6.5 cells was detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and the effects of miR-26a on the viability, proliferation and apoptosis of SP6.5 cells were detected via cell counting kit-8 (CCK-8) assay and colony formation assay.*

Results: *Compared with those in the miR-26a NC group, SP6.5 cells in the miR-26a siRNA group had significantly enhanced viability and proliferation, a significantly decreased apoptosis rate, reduced mRNA and protein levels of p53, and obviously increased mRNA and protein levels of MDM2. Moreover, in comparison with those in the miR-26a NC group, SP6.5 cells in.the miR-26a mimic group had evidently weakened viability and proliferation, an evidently higher apoptosis rate, increased mRNA and protein levels of p53, and markedly lower mRNA and protein levels of MDM2.*

Conclusions: *Highly expressed miR-26a can inhibit the proliferation and promote apoptosis of SP6.5 cells, whose potential mechanism may be related to the regulation on the p53/MDM2 pathway.*

Key words: *miR-26a, uveal melanoma, cell proliferation, apoptosis, p53/MDM2 pathway*

Introduction

Uveal melanoma (UM) is the most common intraocular malignant tumor in adults, and its morbidity rate ranks first among intraocular tumors in foreign countries and second only to retinoblastoma in China, also with a high mortality rate [1.2]. UM mainly originates from UM cells characterized by strong proliferative activity and proneness to metastasis. UM cells easily metastasize through blood flow, and 85% of them metastasize to the liver. However, tumor metastasis during treatment indicates the poor prognosis of patients. Despite some therapeutic drugs in the clinic, the survival rate of patients fails to be significantly improved [3.4]. Therefore, considering its high grade of malignancy, it is of great significance to find biomarkers for early prevention and detection of UM.

Micro ribonucleic acids (miRs) are a class of non-coding small RNA molecules with about 21-25 nucleotides in length. They can cause degradation or inhibit translation of target genes through targeting mRNA 3'-untranslated region (3'UTR), thereby regulating cell proliferation, differentiation and apoptosis [5,6]. According to a recent study, the abnormal expression of miRs is closely related to tumors, and they play a similar role to tumor suppressor genes or proto-oncogenes *in vivo*, thus regulating the occurrence, development or outcome of tumors [7]. MiR-26a is a widely-studied

Corresponding author: Ye Tian, BM. Department of Dermatology, Dongzhimen Hospital, Beijing University of Chinese Medicine, No.5 Haiyuncang Rd, Dongcheng District, 100700 Beijing, China.
Tel: +86 013552689991, Email: tianyepk822@126.com
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miR. In the literature it is reported that miR-26a is lowly expressed in liver cancer, lung cancer and prostate cancer, which is closely related to tumor recurrence, metastasis and poor prognosis [8.9]. However, the regulatory effect of miR-26a on UM is rarely reported.

There is a large number of studies showing that the tumor suppressor gene p53 plays an important role in physiological and pathological processes, such as senescence and tumor, but inactivation of p53 plays a key role in tumor formation [10]. It has been confirmed that p53 mutation occurs in about 50% of malignant tumors. The protein encoded by p53 serves as a transcription factor regulating proliferation, survival, metastasis and apoptosis of tumor cells [11]. However, p53 mutation does not coexist with murine double minute 2 (MDM2) mutation, and the amplification of MDM2 is closely associated with tumor metastasis [12]. The above findings also suggest that modulating the p53/MDM2 signaling pathway will be a new treatment approach for UM.

In this paper, therefore, SP6.5 cells were used as the experimental objects, the regulatory effects of miR-26a on proliferation and apoptosis of SP6.5 cells were explored, and its potential regulatory mechanism was further detected.

Methods

Reagents

MiR-26a negative control (NC), mimic and small-interfering RNA (siRNA) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), penicillin/streptomycin (P/S), fetal bovine serum (FBS) and 1640 medium from Hyclone (South Logan, UT, USA), 4% paraformaldehyde, methyl thiazolyl tetrazolium (MTT) kit and radioimmunoprecipitation (RIPA) lysis buffer from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China), 96-well plates and culture dishes from Corning (Corning, NY, USA), miR-26a and β-actin primers from Thermo (Waltham, MA, USA), 5-Ethynyl-2'- deoxyuridine (EdU) staining kit from Nanjing KeyGEN Biotech Co., Ltd. (Nanjing, China), p53, MDM2 and β-actin primary antibodies from CST (Danvers, MA, USA), horseradish peroxidase (HRP)-labeled secondary antibodies from Abcam (Cambridge, MA, USA), high-fidelity polymerase and SYBR Green PCR Master Mixture from TaKaRa (Tokyo, Japan), and Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA).

Instruments

Pure water system was purchased from Millipore (Billerica, MA, USA), inverted fluorescence microscope from Olympus (Tokyo, Japan), high-speed centrifuge from Eppendorf (Hamburg, Germany), CO_2 incubator from SANYO (Osaka, Japan), qPCR instrument from Agilent (Santa Clara, CA, USA), super clean bench from Beijing First Semiconductor Factory (Beijing, China), gel imager and Western blotting membrane transfer instrument from BD (Franklin Lakes, NJ, USA), and NanoDrop instrument from Thermo (Waltham, MA, USA)

Cells

SP6.5 cells purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in the 1640 medium containing 1% P/S and 10% FBS. After resuscitation for 24 h, the medium was replaced. Upon reaching 90% confluence, the cells were subcultured. In the experiment, SP6.5 cells were randomly divided into miR-26a NC group, miR-26a mimic group and miR-26a siRNA group.

Culture and transfection of SP6.5 cells

SP6.5 cells were inoculated into 6-well plates (3×10^5 cells/well). Upon reaching 80% confluence, the cells were directly transfected with miR-26a NC, miR-26a mimic and miR-26a siRNA (20 μM) using Lipofectamine 2000 transfection reagent, and cultured in an incubator at 37°C for 24 h. Then, the level of miR-26a in SP6.5 cells was detected *via* qRT-PCR, with β-actin as an internal reference. The total RNA was extracted using TRIzol from cells in each group (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions, followed by PCR amplification. The total reaction system was 25 μL for a total of 35 cycles, with three replicates. The expression of miR-26a was detected using $2^{-\Delta\Delta Ct}$.

Detection of the effect of miR-26a on viability of SP6.5 cells via CCK-8 assay and colony formation assay

SP6.5 cells were inoculated into a 96-well plates (5×10^3 cells/well) for 3 times. After culture for 24 h, 10 μL of MTT reagent was added into the medium for incubation for another 4 h. After the medium was discarded, the crystal violet dissolved in dimethyl sulfoxide (DMSO) was added and the mixture was shaken on a shaker for 15 min. Then the absorbance was measured at 450 nm using a microplate reader. SP6.5 cells in the

Table 1. Primers used in the study

List	*Primer*
p53	F: CTTTGAGGTGCGTGTTT
	R: CAGTGCTCGCTTAGTGC
miR-26a	F: GACTGTTCAAGTAATCCAGGATA
	R: GTGCAGGGTCCGAGGTATTC
MDM2	F: GTGAATGATTCCAGAGAGT
	R: TACTGAGAGTTGATGGC
U6	F: GCGCGTCGTGAAGCGTTC
	R: GTGCAGGGTCCGAGGT
β-actin	F: GGCTGTATTCCCCTCCATCG
	R: CCAGTTGGTAACAATGCCATGT

logarithmic growth phase were inoculated into 6-well plates (1×10^5 cells/well), cultured in the incubator, fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Finally, the cells were observed and photographed.

Detection of the effect of miR-26a on the proliferation of SP6.5 cells via EdU staining

SP6.5 cells were inoculated into 6-well plates and transfected with miR-26a NC, siRNA and mimic. After 24 h, they were fixed with 4% paraformaldehyde, and incubated with 1 mL of EdU staining solution in each well for 30 min in the dark. Finally, the staining was examined under an inverted fluorescence microscope.

Detection of the effect of miR-26a on apoptosis of SP6.5 cells using TUNEL assay

After the medium in the plate was discarded and the plate was washed with phosphate buffered saline (PBS), the cells were added with 4% paraformaldehyde and TUNEL staining solution for incubation for 20 min in the dark. After the plates were washed with PBS, the staining was observed under fluorescence microscope, and the apoptosis rate was determined.

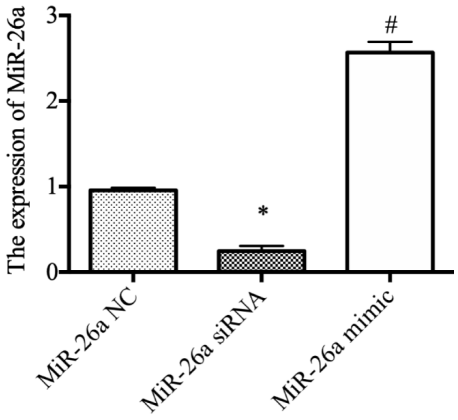

Figure 1. MiR-26a level in SP6.5 cells in each group (*p<0.05: miR-26a siRNA group *vs.* miR-26a NC group) (#p<0.05: miR-26a mimic group *vs.* miR-26a NC group).

Determination of the effects of miR-26a on mRNA levels of p53 and MDM2 in SP6.5 cells using qRT-PCR

The total RNA was extracted from cells in each group, and reversely transcribed into cDNA according to the instructions, followed by PCR amplification. The reaction system was the same as that in "culture and transfection of SP6.5 cells", for a total of 30 cycles. The primers are shown in Table 1. The mRNA levels of p53 and MDM2 in cells were determined.

Determination of the effects of miR-26a on protein levels of p53 and MDM2 in SP6.5 cells using Western blotting

After the medium was discarded, the plates were washed with PBS and added dropwise with RIPA lysis buffer. The lysate was collected and loaded for 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the samples were transferred onto a membrane, sealed with 5% skim milk powder for 1 h, and incubated with p53, MDM2 and β-actin primary antibodies (1:1000) overnight. In the next morning, the samples were incubated again with secondary antibodies (1:3000), followed by color development *via* electrochemiluminescence (ECL). Finally, the optical density of bands was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Statistics

GraphPad 6.0 software (La Jolla, CA, USA) was used for the systematic analysis of the experimental results in this study. One-way analysis of variance (ANOVA) was used for the comparison of means among groups. P<0.05 suggested that the difference was statistically significant.

Results

MiR-26a level in SP6.5 cells detected using qRT-PCR

At 24 h after transfection, the total RNA was extracted in each group, and the level of miR-26a in SP6.5 cells was detected *via* qRT-PCR. As shown in Figure 1, compared with that in miR-26a NC group, the level of miR-26a in SP6.5 cells significantly declined in miR-26a siRNA group (p<0.05), but it significantly rose in miR-26a mimic group (p<0.05).

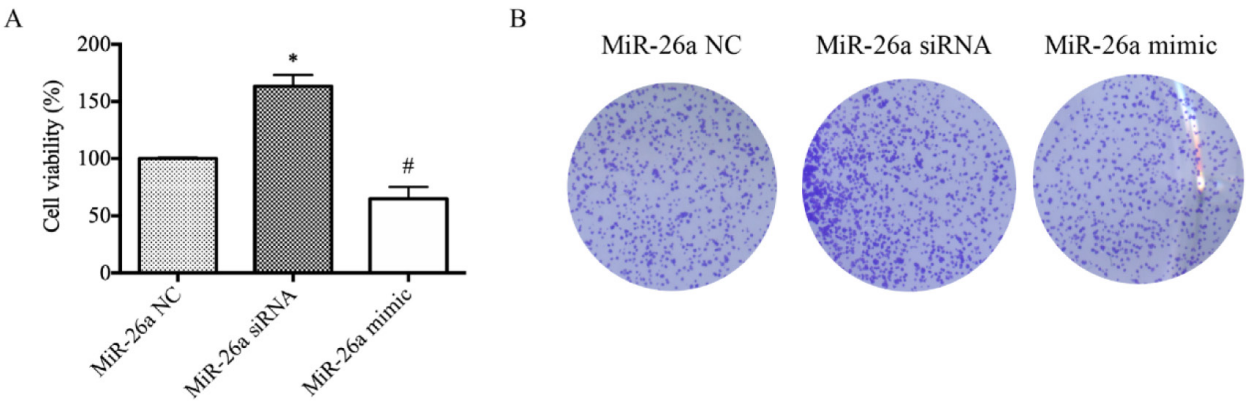

Figure 2. SP6.5 cell viability in each group. **A:** MTT assay results. **B:** Crystal violet staining results (magnification: 10×) (*p<0.05: miR-26a siRNA group *vs.* miR-26a NC group, #p<0.05; miR-26a mimic group *vs.* miR-26a NC group).

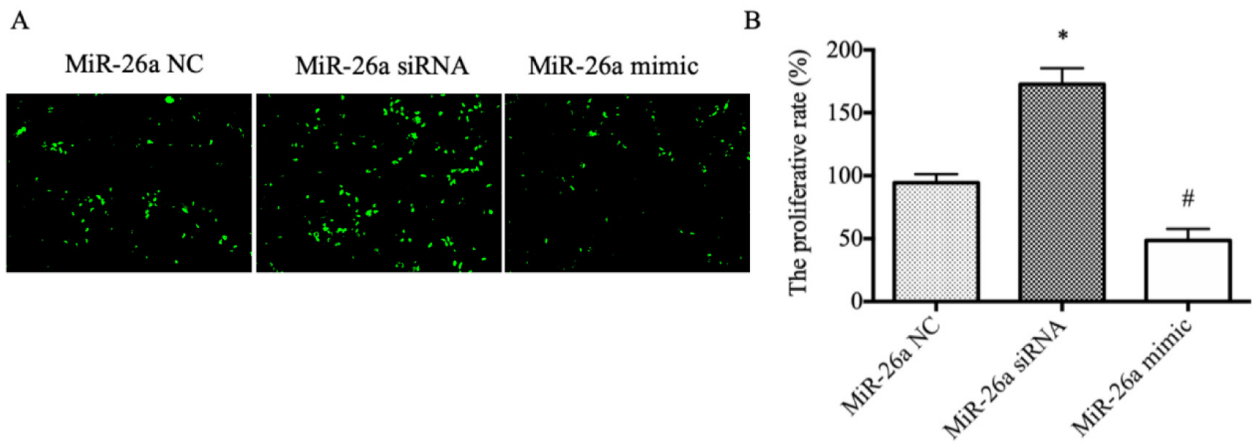

Figure 3. Proliferation of SP6.5 cells in each group. **A:** EdU staining (magnification: 200×). **B:** Cell proliferation rate (*p<0.05: miR-26a siRNA group *vs.* miR-26a NC group; #p<0.05: miR-26a mimic group *vs.* miR-26a NC group).

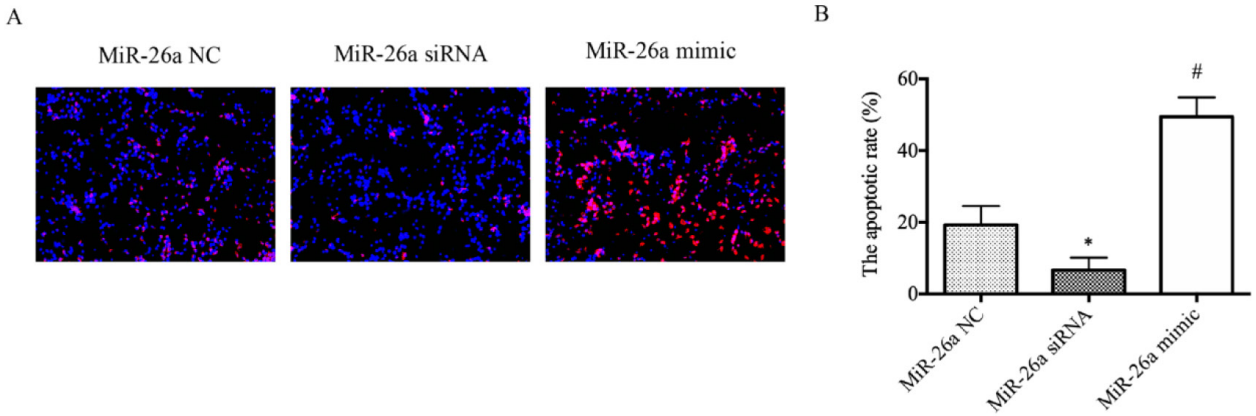

Figure 4. Apoptosis of SP6.5 cells in each group. **A:** TUNEL staining. (magnification: 200×). **B:** Apoptosis rate (*p<0.05: miR-26a siRNA group *vs.* miR-26a NC group, #p<0.05: miR-26a mimic group *vs.* miR-26a NC group).

MiR-26a mimic could inhibit the viability of SP6.5 cells

The effect of miR-26a mimic on the viability of SP6.5 cells was determined through MTT assay and colony formation assay. The results of MTT assay revealed that compared with that in miR-26a NC group, the viability of SP6.5 cells was obviously enhanced in miR-26a siRNA group (p<0.05), but it was obviously weakened in miR-26a mimic group (p<0.05) (Figure 2A). The results of colony formation assay revealed that miR-26a mimic group had an obviously smaller number of colonies than miR-26a NC group (Figure 2B), indicating that miR-26a mimic can markedly inhibit the viability of SP6.5 cells.

MiR-26a mimic could suppress the proliferation of SP6.5 cells

It was found *via* EdU staining that the proliferation rate of SP6.5 cells was markedly higher in the miR-26a siRNA group (p<0.05), while it was markedly lower in the miR-26a mimic group than in the miR-26a NC group (p<0.05) (Figure 3). Therefore, miR-26a mimic can markedly suppress the proliferation of SP6.5 cells.

MiR-26a mimic could promote apoptosis of SP6.5 cells

The effect of miR-26a on the apoptosis of SP6.5 cells was determined using TUNEL staining. As shown in Figure 4, the apoptosis rate of SP6.5 cell declined in miR-26a siRNA group (p<0.05), while it evidently rose in miR-26a mimic group compared with that in miR-26a NC group (p<0.05). The above results demonstrate that miR-26a mimic can evidently promote the apoptosis of SP6.5 cells.

MiR-26a mimic could promote p53 mRNA expression but inhibit MDM2 mRNA expression in SP6.5 cells

According to the results of qRT-PCR, compared with miR-26a NC group, miR-26a siRNA group had decreased p53 mRNA expression (p<0.05) but increased MDM2 mRNA expression (p<0.05), while miR-26a mimic group had the opposite expressions (p<0.05) (Figure 5).

MiR-26a mimic could promote p53 protein expression but inhibit MDM2 protein expression in SP6.5 cells

According to the results of Western blotting, miR-26a siRNA group had lower p53 protein expression ($p<0.05$) but higher MDM2 protein expression than miR-26a NC group ($p<0.05$), while miR-26a mimic group had the opposite expressions ($p<0.05$) (Figure 6).

Discussion

As a rare intraocular malignant tumor in adults, UM occurs most frequently in the choroid, followed by the ciliary body and iris. Although the effective rate of local control of UM is over 90% after local radiotherapy, distant metastasis, mainly hepatic metastasis, still occurs in half of the patients [13, 14]. According to the epidemiological research, the occurrence of UM is related to gender, age, geography and genetics. Most patients will not seek treatment until blurred vision, metamorphopsia and visual hallucination occur so that they have missed the best opportunity for treatment [15]. Therefore, how to raise the survival rate and improve the quality of life of UM patients is a challenging focus for medical researchers.

With the constant advance in biological technology, it has been found that non-coding RNAs can regulate more than 90% of genes, and people have gradually had a better understanding of miRs [16]. Studies have shown that the expression of miRs varies in different stages of UM, suggesting that miRs are involved in regulating the occurrence, development and invasion of UM [17,18]. Moreover, it is well documented in the literature that miR-26a is involved in the regulation of proliferation, migration and apoptosis of tumor cells [19]. However, there are few reports about miR-26a in UM. Therefore, this paper aimed to explore the regulatory effect of miR-26a in UM and its mechanism.

To explore the regulatory effect of miR-26a in UM, SP6.5 cells were transfected with miR-26a mimic and inhibitor using Lipofectamine 2000 transfection reagent to reduce and raise the miR-26a expression, with miR-26a NC as the blank control. Then the miR-26a level was detected *via* qRT-PCR, and the effect of miR-26a on the viability of SP6.5 cells was detected using MTT assay and crystal violet staining. It was found that miR-26a mimic could significantly suppress the viability of SP6.5 cells. The effect of miR-26a on the proliferation of SP6.5 cells was determined using EdU staining. The results manifested that after treatment with miR-26a mimic, the number of proliferating cells (green fluorescence) was obviously smaller in miR-26a mimic group than that in miR-26a NC group. It can be seen that miR-26a mimic can

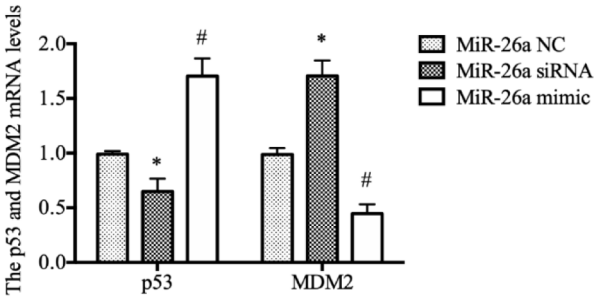

Figure 5. P53 and MDM2 mRNA levels in SP6.5 cells. (*p<0.05: miR-26a siRNA group *vs.* miR-26a NC group; #p<0.05: miR-26a mimic group *vs.* miR-26a NC group).

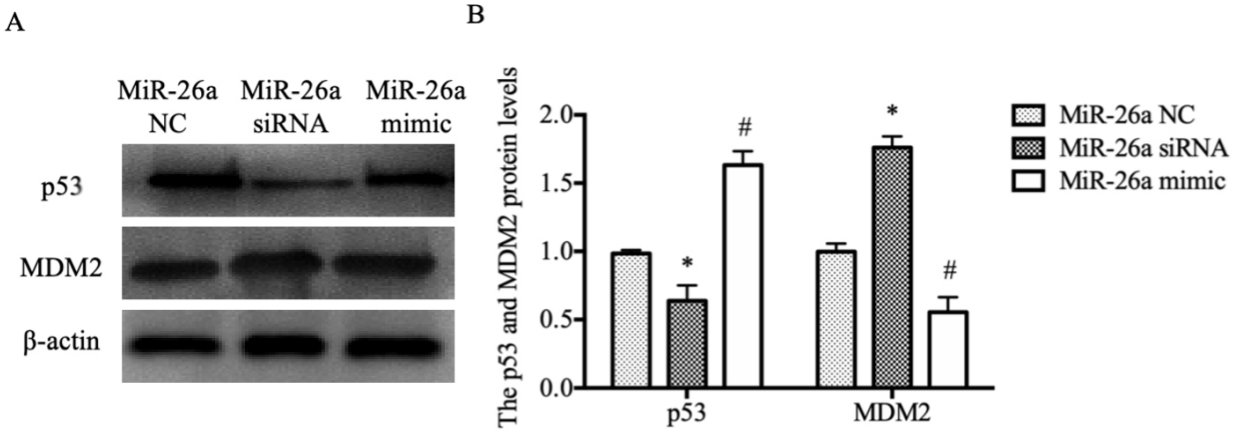

Figure 6. P53 and MDM2 protein levels in SP6.5 cells. **A:** The results of Western blotting showed that miR-26a siRNA group had lower p53 protein expression than miR-26a NC group ($p<0.05$), while miR-26a mimic group had opposite expression ($p<0.05$). **B:** The results of Western blotting showed that miR-26a siRNA group had lower MDM2 protein expression than miR-26a NC group ($p<0.05$), while miR-26a mimic group had opposite expression ($p<0.05$) (*p<0.05: miR-26a siRNA group *vs.* miR-26a NC group; #p<0.05: miR-26a mimic group *vs.* miR-26a NC group).

obviously inhibit the proliferation of SP6.5 cells. Moreover, the effect of miR-26a on the apoptosis of SP6.5 cells was detected *via* TUNEL staining, and it was confirmed that miR-26a mimic could evidently promote apoptosis.

To explore the regulatory mechanism of miR-26a, emphasis was put on the most widely studied tumor suppressor gene p53. Previous studies have found that the expression product of p53, p53 protein, can promote cancer cell apoptosis, thereby preventing cancerization and tumor growth. In addition, p53 protein is able to help gene defect repair, thus protecting the human body. P53 is mainly regulated by the E3 ubiquitin ligase MDM2 *in vivo*, and ubiquitylated p53 is then degraded by the proteasome, inhibiting the transcriptional activity of p53. The level of p53 protein remains stable in cells under normal conditions, but its activity is inhibited when tumors occur. Liapis et al [20] found that in the case of neurofibroma in children, there is increasing p53 mutation, indicating that abnormal expression of p53 is an important biological factor for poor prognosis of tumor. Therefore, the levels of p53 and MDM2 were determined at the gene and protein levels, and the results showed that miR-26a mimic could greatly raise the p53 level and lower the MDM2 level. To sum up, miR-26a mimic can weaken the proliferation and enhance the apoptosis of UM cells, and its mechanism may be related to the regulation on the p53/MDM2 signaling pathway.

Conclusions

In conclusion, as a tumor suppressor gene, miR-26a is closely involved in regulating the proliferation and apoptosis of UM cells, which provides a theoretical basis for miR-26a as a biomarker for UM. Analyzing in depth the molecular mechanism of miR-26a will contribute to the treatment of UM with miR-26a.

Conflict of interests

The authors declare no conflict of interests.

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