

ORIGINAL ARTICLE

MiR-320a inhibits malignant phenotype of melanoma cells via targeting PBX3

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Summary

Purpose: To study the expression of micro ribonucleic acid-320a (miR-320a) in melanoma cells and its influence on the biological functions of these cells.

Methods: MiR-320a expression data and clinical data in melanoma tissues were downloaded from The Cancer Genome Atlas (TCGA) database. Real time-quantitative polymerase chain reaction (RT-qPCR) was used to detect miR-320a expression in melanoma tissues, malignant melanoma cell lines (A375, SKMEL-28 and A2058) and human epidermal melanoma (HEM) cells. The miR-320a mimic was transfected into A375 cells, and the functions of cells were detected. The luciferase reporter gene assay was employed to verify the miR-320a downstream target protein predicted by the biological information prediction software.

Results: The differential analysis of miRNAs in melanoma tissues from TCGA database showed that miR-320a expression in melanoma tissues was significantly lower than that in adjacent tissues, and low expression of miR-320a ex-

hibited a severe poor prognosis ($p < 0.01$). MiR-320a mimic could significantly enhance the expression level of miR-320a ($p < 0.01$). The absorbance at 490 nm of A375 cells overexpressing miR-320a decreased remarkably and their proliferation ability was weakened ($p < 0.01$). Overexpression of miR-320a in A375 cells inhibited cell migration to wound parts and epithelial-mesenchymal transition (EMT), invading malignant phenotype ($p < 0.05$). Flow cytometry was employed and it was denoted that after transfecting with miR-320a, the apoptosis rate of A375 cells was elevated overtly ($p < 0.01$). The dual luciferase report test indicated that the luciferase activity in wild type pre-B-cell leukemia transcription factor 3 (PBX3) was markedly lower than that of mutant type PBX 3 ($p < 0.05$).

Conclusions: Targeted binding of miR-320a to PBX3 protein can inhibit the malignant phenotype of cells and affects the occurrence and development of melanoma.

Key words: melanoma, miR-320a, TCGA database

Introduction

Malignant melanoma (MM) is the most lethal skin malignant tumor. According to the latest statistics, MM ranks seventh in the incidence rate of female malignant tumors. Moreover, MM is commonly seen in males [1]. MM developed by malignant transformation of melanocytes has shown obvious clinicopathological features, such as invasiveness, poor prognosis and resistance to chemo-

therapy or radiotherapy [2,3]. Although progress has been made in chemotherapy and immunotherapy to treat tumors, the available treatment options for MM are limited and the prognosis of patients with metastatic melanoma is still unfavorable [4,5]. The root cause of unsatisfactory results is the lack of basic knowledge about the biological mechanism of carcinogenesis and progression of

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MM. In order to provide effective treatment strategies for MM, it is necessary to clearly understand and explain the important molecular mechanisms involved in the pathogenesis of MM. Micro ribonucleic acids (miRs) are highly conserved, with no more than 25 bases, and are mainly combined with 3'-untranslated region (3'-UTR) of the target gene, thus inhibiting translation of the target gene and eventually leading to expression silencing or reduction [6]. MiRs play a key role in almost all regulatory pathways of cells (such as proliferation and differentiation, apoptosis and fat storage), in which these biological processes are changed in tumors [7]. Some studies have also revealed that RNAs related to malignant tumors play an important role in tumor metastasis [8,9].

The function of miR-320a on chromosome 8p21.3 has been studied previously. MiR-320a plays an anticancer role in many cancers including hematological malignancies and solid tumors. Xishan et al. [10] pointed out that miR-320a maintains a low level in the peripheral blood of chronic myeloid leukemia (CML), and its expression level is reduced. MiR-320a combined with oncogenes [breakpoint cluster region protein (BCR)/abelson murine leukemia viral oncogene homolog (ABL)] of target protein, inhibiting the expression of oncoproteins and ultimately impeding the malignant phenotype of CML cells. In diseases, pre-B-cell leukemia transcription factor 3 (PBX3) is also a target protein of miR-320a, which significantly inhibits the proliferation and induces the apoptosis of cells after combined with miR-320a [11]. MiR-320a is down-regulated and has important functions in many solid tumors, like anti-cancer effects in colorectal cancer, nasopharyngeal carcinoma, breast cancer and bladder cancer. Additionally, overexpression of miR-320a can partially inhibit the malignant behavior of tumors [12-15]. However, the potential mechanism of downregulation of these miRs is still unclear. Previous studies have indicated that miR-320a expression is down-regulated in MM, but its function on melanocytes has not been explored yet.

Methods

Experimental materials

MM cell lines (A375, SKMEL-28 and A2058) and human epidermal melanocytes (HEM) purchased from the American Type Culture Collection (ATCC) cell bank (Manassas, VA, USA), TRIzol reagent (Invitrogen, Carlsbad, CA, USA), polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), MTS reagent (Santa Cruz, CA, USA), horseradish peroxidase (HRP) coupled secondary antibody (Beyotime Co., Ltd., Beijing, China), Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and primary antibody (Abcam, Cambridge, MA, USA).

Research subjects

In the experiment, MM tissues and paracancer tissues in The Cancer Genome Atlas (TCGA) database were selected as study objects and divided into MM group and Paracancer group. The A375 cells transfected with miR-320a mimic were enrolled into miR-320a mimic group and the A375 cells transfected with miR-320a control were enrolled into miR-320a control group.

Real-time fluorescence quantitative polymerase chain reaction (PCR)

Immediately after the collected cells were precipitated, RNAs were extracted and purified. A proper volume of TRIzol reagent was added according to the number of cells, the A_{260}/A_{280} ratio was detected to evaluate the purity of the extracted RNAs, and the ratio was ensured to be between 1.8 and 2.0. Qualified samples were used for subsequent complementary deoxyribonucleic acid (cDNA) synthesis and their concentrations were recorded with a detection box. With a 20 μ L system containing 1 μ L of upstream primer, 1 μ L of downstream primer, 1 μ L of cDNA with the same concentration, 0.5 μ L of dNTP, 10 μ L of SYBR Green and 6.5 μ L of double-distilled water (ddH_2O), quantitative amplification was carried out based on the normal procedure, and 35 cycles were set. Cycle threshold (CT) values in each well were recorded after the experiment. The quantitative data of all messenger RNAs (mRNAs) were normalized to U6. The primer sequences were as follows: MiR-320a: F: ACTATGGAAAAGCTGGCTTGAGAG; R: ATTCGTTGAGAGATCACAAGCGT. U6: F: ATTGGAACGATACAGAGAAGAT; R: GGAACGCTTCACGAATTT

The expression changes of miR-320a were calculated by $2^{-\Delta\Delta CT}$ method.

Cell transfection

Cells were inoculated into 6-well plates in advance, and the cell density of each well was ensured to be consistent, which was about 3×10^5 . Transfection was carried out after incubation for 24 h. MiR-320a mimic and miR-320a control were prepared according to instructions and transfected with Lipofectamine 2000. The transfection efficiency was observed under the fluorescence microscope 48 h after transfection, and the cells were collected for subsequent experimental procedures.

Measurement of the proliferation and apoptosis of cells in vitro

After preparing the medium containing 10% cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan), the transfected cells were inoculated into 96-well plastic petri dish containing 10% CCK-8. The optical density (OD) value at 490 nm was measured with enzyme assay in 6 consecutive days after transfection. All the experiments were carried out three times. The steps of detecting apoptosis were as follows: the precipitation of cells digested by trypsin was collected, resuspended and washed with phosphate buffered saline (PBS), and transferred to the flow tube, Annexin V-FITC and PI (propidium iodide) were added respectively, and the interval of each staining solution was 10 min. Finally, the proportion of apoptotic cells was detected and analyzed by a flow cytometer.

Protein expression changes detected by Western blotting analysis

After RIPA cell lysis buffer was added, the total protein in the cells was extracted. The extraction process was ensured to be operated on ice. Bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA) was used to detect the protein concentration. After protein samples

were separated, they were subjected to wet membrane transfer for 1 h and added with blocking reagent to block non-specific sites for 5-10 min. Next, the membrane was washed tris buffered saline tween (TBST) three times, and incubated with specific primary antibodies for 11-16 h and then secondary antibodies. The strip analysis results were gained via membrane scanning.

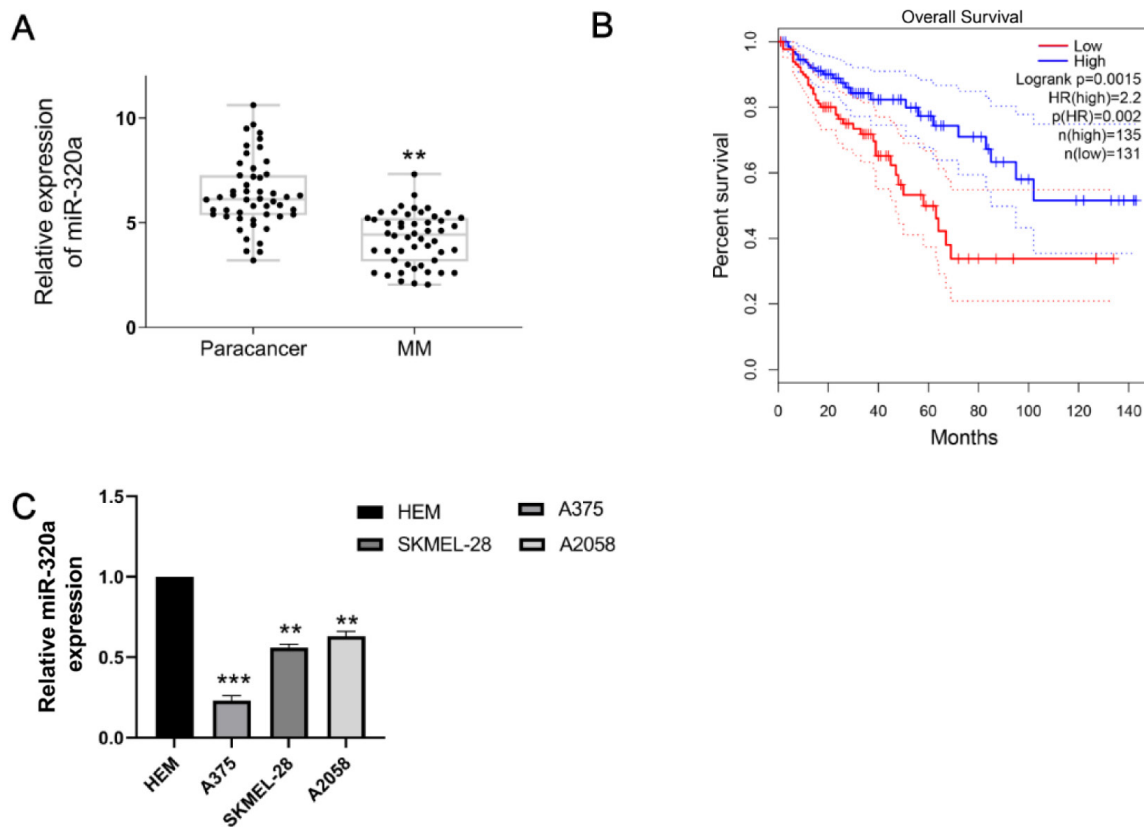


Figure 1. Expression of miR-320a in MM tissues and cells. **A:** Expression of miR-320a in MM tissues from TCGA database. The expression of miR-320a is overtly lower in MM group than that of Paracancer group. **B:** Influence of miR-320a expression on the prognosis of patients. The overall survival rate of MM patients in low miR-320a expression group is significantly lower than that in high expression group ($p < 0.01$). **C:** Expression of miR-320a in melanoma cells [the expression of miR-320a is markedly lower in A375, SKMEL-28 and A2058 cells than that in HEM ($p < 0.05$) (** $p < 0.01$, *** $p < 0.001$).

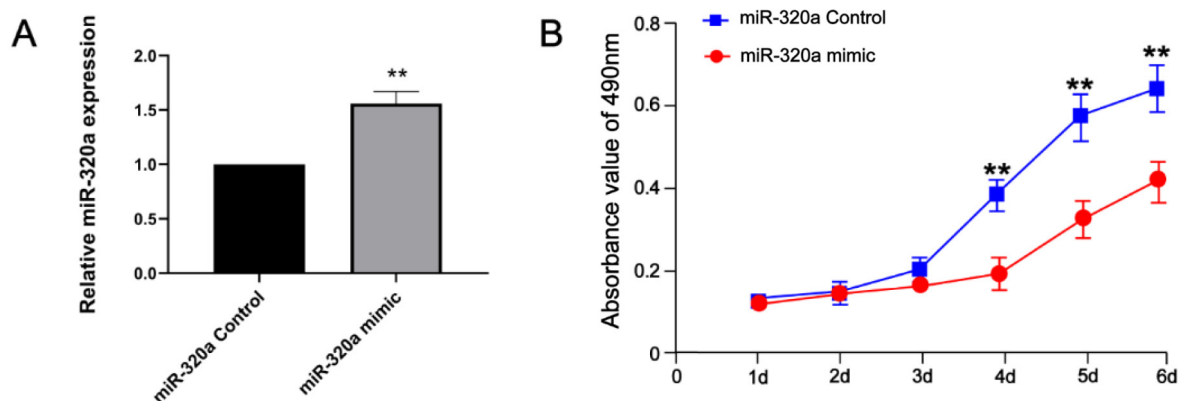


Figure 2. Effect of miR-320a expression on proliferation of melanoma cells. **A:** Expression of miR-320a in A375 cells transfected with miR-320a mimic. Compared with that in miR-320a control group, the expression of miR-320a in miR-320a mimic is distinctly higher ($p < 0.01$). **B:** Effect of miR-320a on the proliferation of melanoma cells. The absorbance at 490 nm of miR-320a mimic group is obviously reduced on the fourth day ($p < 0.01$) (** $p < 0.01$).

Wound healing assay

Six-well plates were inoculated with 4×10^5 cells. 24 h later, after the cells covered the bottom of the well, the Dulbecco's Modified Eagle's medium (DMEM) was replaced with the serum-free one and a 20 μ L tip was adopted to draw several straight lines at the bottom of the well to mimic the wound. DMEM was replaced with modified Eagle's medium (MEM) or 1640 (HyClone, South Logan, UT, USA) containing 2% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). After incubation for 24 h, the area occupied by migrating cells in scratches was evaluated.

Dual luciferase assay

Lipofectamine 3000 was selected to transfect miR-320a mimic, miR-320a control, wild type PBX3 (ACAGCUUUA) or mutant type PBX3 (ACACCCUUA) into A375 cells and the changes of enzyme activity were explored after 48 h.

Statistics

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was chosen to process the experimental data. Chi-square test and t-test were adopted as statistical methods. $P < 0.05$ indicated that the difference was significant.

Results

MiR-320a expression in MM tissues and cells

Differential analysis of miR expression in MM tissues and corresponding adjacent tissues in TCGA database showed that miR-320a expression was downregulated in MM group ($p < 0.01$), and survival analysis of MM group revealed that patients with high expression of miR-320a had a high survival rate and good surgical prognosis ($p < 0.05$). In addition, the expression of miR-320a in A375, SKMEL-28 and A2058 cells was remarkably lower than that in HEM cells ($p < 0.01$), and the expression level in A375 cells was the lowest ($p < 0.001$). Therefore, A375 cells were adopted for upregulation of miR-320a expression (Figure 1).

High expression of miR-320a overtly inhibited the proliferation of melanoma cells

Compared with miR-320a control group, MiR-320a mimic distinctly increased the expression of A375 cells ($p < 0.01$). CCK-8 experimental data revealed that the absorbance of cells at 490 nm in

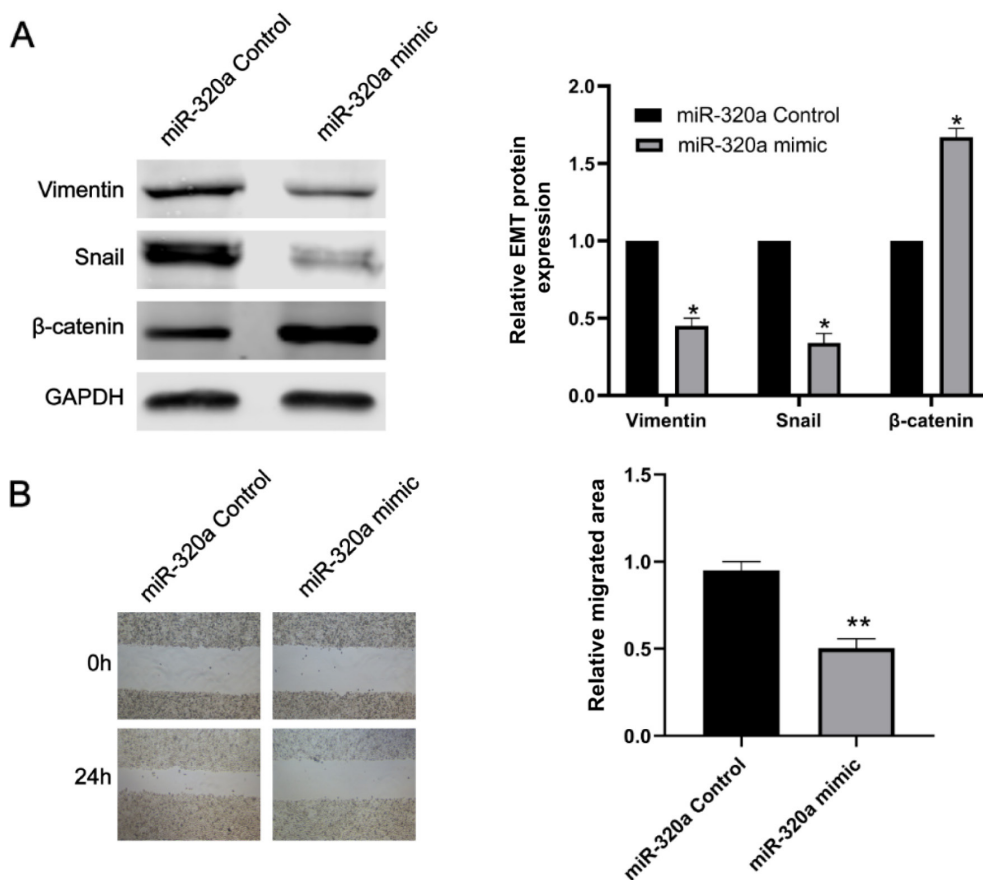


Figure 3. Influence of miR-320a on invasion and metastasis of melanoma cells. **A:** Influence of miR-320a on expression of EMT protein. Compared with those in miR-320a control group, the expressions of Vimentin and Snail proteins are reduced and the expression of β -catenin protein is markedly increased in miR-320 mimic group ($p < 0.05$). **B:** Influence of miR-320a on melanoma cell metastasis. The migration area in miR-320a mimic group is significantly smaller than that of miR-320a control group after 24 h ($p < 0.01$) (* $p < 0.05$ ** $p < 0.01$).

the mimic group was remarkably lower than that in the control group ($p < 0.01$). The results suggested that upregulation of miR-320a can impede the proliferation changes in melanoma cells (Figure 2).

High expression of miR-320a inhibited the invasion and metastasis of melanoma cells

The results of Western blotting analysis presented that the expressions of epithelial proteins

Vimentin and Snail in miR-320a mimic group were lower than those in miR-320a control group, while the expression of periplasmic protein β -catenin was markedly enhanced ($p < 0.05$), which indicated that the invasion ability of melanoma cells is weakened after high expression of miR-320a. Wound healing assay suggested that the migration distance of A375 cells with upregulation of miR-320a was overtly lower than that of miR-320a control group

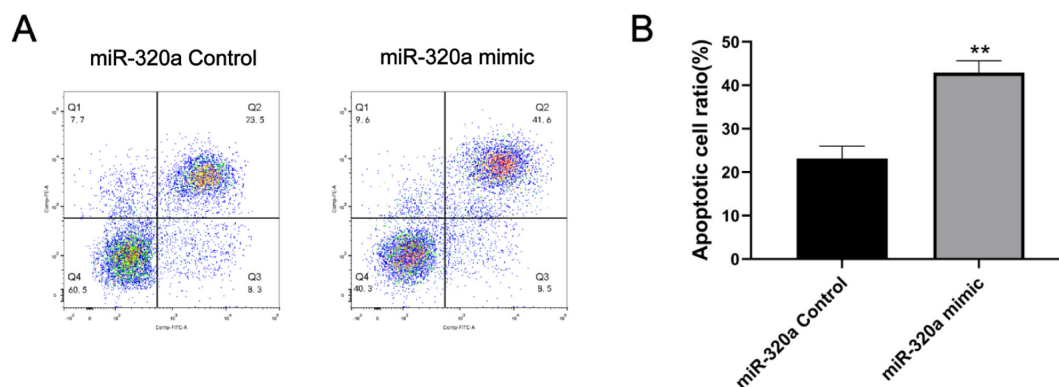


Figure 4. Influence of miR-320a on the apoptosis of melanoma cells. **A:** Apoptosis of melanoma cells detected via flow cytometry. **B:** Proportion of apoptotic melanoma cells. Compared with that in miR-320a control group, the apoptosis rate of melanoma cells in miR-320a mimic group is overtly elevated (** $p < 0.01$).

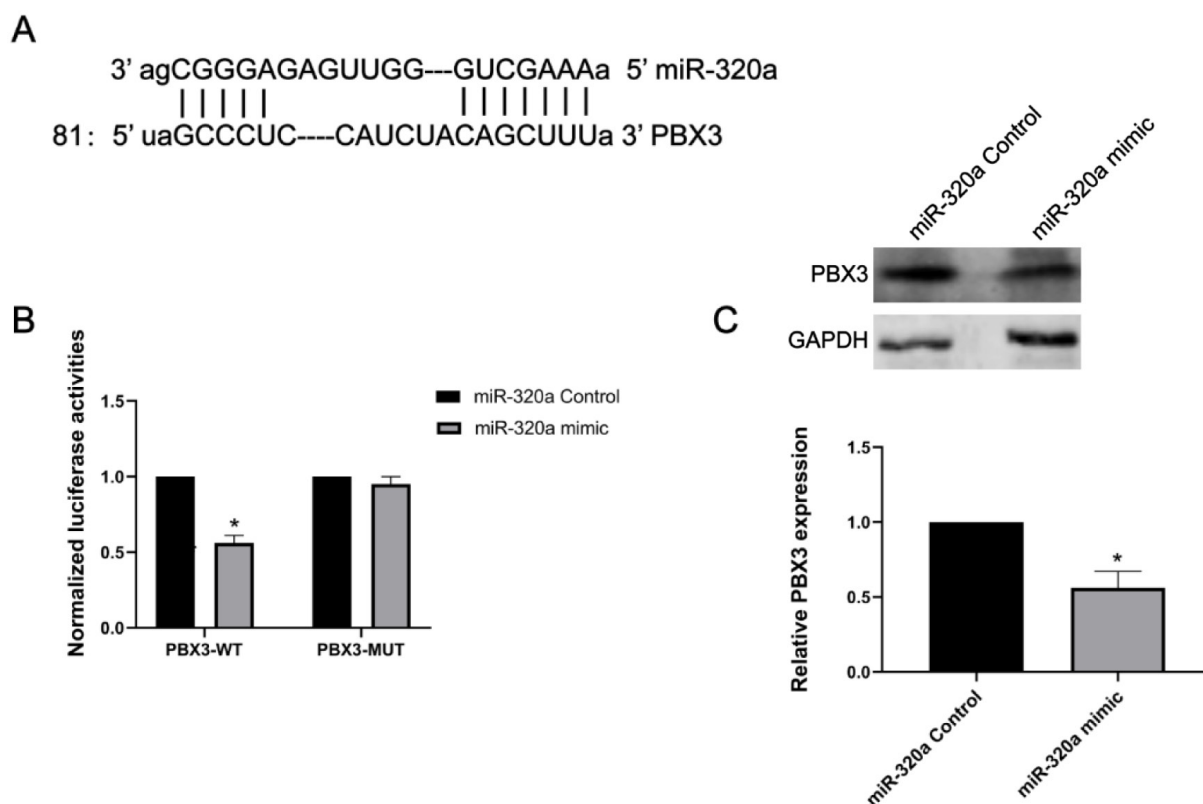


Figure 5. PBX3 is the direct target of miR-320a. **A:** Binding site of miR-320a and PBX3. **B:** Relative luciferase activity in A375 cells transfected with wild type and mutant type PBX3 genes. Compared with that in mutant type PBX3, the relative luciferase activity in wild type PBX3 is significantly decreased ($p < 0.05$). **C:** Expression changes of PBX3 protein in transfected cells. Expression of PBX3 protein in miR-320a mimic group is significantly lower than that in miR-320a control group ($p < 0.05$) (** $p < 0.05$).

after 24 h ($p < 0.01$). It was noticed by these data that upregulation of miR-320a can impede the invasion and metastasis of melanoma cells (Figure 3).

MiR-320a induced the apoptosis of melanocytes

Flow cytometry was chosen to detect the proportion of apoptotic cells 48 h after transfection. The experimental results demonstrated that the proportion of apoptotic cells in the miR-320a mimic group raised remarkably, and the expression level of miR-320a in cells promoted apoptosis (Figure 4).

MiR-320a directly targeted oncogene PBX3 in melanoma

In order to examine the potential mechanism of miR-320a in MM, online software programs (TargetScanHuman7.1, miRDB and microRNA.org) were employed to predict the target gene of miR-320a. The interaction between miR-320a and 3'-UTR of PBX3 was predicted using these three databases. Dual luciferase assay confirmed that PBX3 was the direct target gene of miR-320a. Besides, the expression of PBX3 protein in miR-320a mimic group was distinctly lower than that in miR-320a control group ($p < 0.05$) (Figure 5).

Discussion

Patients with MM that is a highly invasive malignancy have a low cure rate, and their 5-year survival rate has gradually decreased in recent years [16]. It has been proved that miRs play a leading role in RNA silencing [17]. MiRs have dual functions i.e. inhibiting or promoting cancer and play a role in many malignant tumors [18]. Changes in miRs expression often occur in cancer cells and are usually related to tumorigenesis and cancer development [19]. As miRs play an important role in tumor progression and development, more and more researchers begin to explore miRs [6]. In this study, the expression of miR-320a in MM cell lines and tissues was overtly reduced, and the malignant phenotype of MM cells was alleviated after over-expression of miR-320a.

Recent studies have pointed out that miR-320a expression is reduced in breast cancer and ovarian cancer, and miR-320a participates in a variety of biological functions by regulating specific target genes [10-12,14,15]. A research by Xie et al [20] suggested that miR-320a markedly declines in hepatocellular carcinoma cells, while the lack of miR-320a can stimulate the metastasis and invasion of these cells. Decreased expression of miR-320a promotes the proliferation and invasion of

non-small cell lung cancer by targeting voltage-dependent anion channel 1 [21]. These data have proved that miR-320a is related to cancer. In this study, the expression changes of miR-320a in 180 cases of MM tissues from TCGA public data and the guiding significance for prognosis were analyzed. The data results revealed that miR-320a expression in MM tissues was overtly higher than that in adjacent tissues. The survival results suggested that the survival rate of MM patients with high expression of miR-320a was improved and the prognosis was poor, which indicated that miR-320a has guiding significance for MM prognosis. Compared with that in human epidermal melanocytes, miR-320a in MM cells A375, SKMEL-28 and A2058 had remarkably lower expression, and the lowest in A375 cells, which was detected by quantitative polymerase chain reaction (qPCR). The evaluation of the effect of miR-320a on the malignant biological behavior of MM cells revealed that, compared with that of A375 control cells, the proliferation and invasion ability of A375 cells with overexpressed miR-320a were obviously weakened, and the proportion of apoptotic cells was elevated, which is the purpose of treating MM. These data showed that miR-320a plays an anticancer role in MM. However, a larger number of samples is needed to confirm these findings.

PBX3 located on chromosome 9q33.3 belongs to a highly conserved PBX protein family and PBX is a member of the triple amino acid loop extension superclass of Homeodomain protein [22]. PBX3 protein is often markedly overexpressed during tumor occurrence, which can activate important signaling pathways to participate in the regulation of tumor processes [23-25]. These findings display the powerful functions of PBX3. PBX3 promotes tumor growth and malignant phenotype formation of cells. Its expression is related to the clinicopathological characteristics of MM patients [11]. In this study, it was predicted with bioinformatics prediction software that one of the target proteins of miR-320a is PBX3, and the 3'-UTR of PBX3 can bind to miR-320a. It was detected by dual luciferase reporter gene that the luciferase activity in wild type PBX3 was lower than that of mutant type PBX3, and the expression of PBX3 protein in A375 cells transfected with miR-320a mimic was obviously lower than that of A375 cells. The results implied that miR-320a directly interacts with 3'-UTR of PBX3, and downregulates the expression of PBX3 protein in MM, suggesting the potential mechanism of miR-320a controlling PBX3 after transcription. However, whether PBX3 is regulated by miR-320a in GC is still unknown.

Conclusions

In summary, the above research data show that miR-320a is downregulated by regulating PBX3 oncogene in MM and inhibits the proliferation, invasion and metastasis of MM cells inducing their

apoptosis. As a result, miR-320a may be a potential therapeutic target in MM.

Conflict of interests

The authors declare no conflict of interests.

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