

## ORIGINAL ARTICLE

# MiR-125b acts as a tumor suppressor of melanoma by targeting NCAM

Tao Wang, Wei Li, Shuang Yin, Weihua Chen

Department of Plastic and Aesthetic Surgery, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, China.

### Summary

**Purpose:** To investigate the effects of micro ribonucleic acid (miR)-125b on the growth and apoptosis of malignant melanoma (MM) cells and related mechanisms.

**Methods:** The differences in the expressions of miR-125b and neural cell adhesion molecule-120 (NCAM-120), NCAM-140 and NCAM-180 in 5 cases of MM tissues (MM group) and corresponding adjacent tissues (Paracancer group) as well as MM cells were detected via quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry, respectively. Next, malignant melanoma A375 cells were selected to exogenously overexpress miR-125 (miR-125 mimic group). Then, in vitro functional assays were conducted to determine the influences of miR-125b on the proliferation, migration and apoptosis of MM cells, and the changes in the binding of miR-125b to the 3'-untranslated region (3'-UTR) of wild-type and mutant NCAM messenger RNAs (mRNAs) were verified using dual-luciferase assay.

**Results:** MiR-125b was significantly lowly expressed in MM tissues and cells ( $p < 0.01$ ), while NCAM-120, NCAM-140 and NCAM-180 were clearly highly expressed in MM tissues and cells ( $p < 0.05$ ). After miR-125b was exogenously overexpressed in A375 cells, the proliferation and migration ability of A375 cells was decreased, whereas the proportion of apoptotic cells rose ( $p < 0.05$ ). Besides, the results of dual-luciferase reporter assay revealed that the luciferase activity of A375 cells in the 3'-UTR of wild-type NCAM mRNA was overtly lowered compared with that of mutant NCAM mRNA ( $p < 0.05$ ).

**Conclusions:** MiR-125b acts as a tumor suppressor in MM cells by targeting and binding to NCAM.

**Key words:** malignant melanoma, mechanism, apoptosis, miR-125b, NCAM

### Introduction

Micro ribonucleic acid (miR)-125b is a highly conserved small RNA consisting of 21-25 nucleotides, which cannot encode proteins but can bind to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs) to regulate their expressions at the transcription level [1,2]. MiRs are particularly crucial in restricting carcinogenicity by suppressing the translation of target mRNAs. In fact, according to the genetic maps known of miRs, miRs are mainly located in chromosomal loci that are prone to deletion or amplification [3]. These research findings suggest that miRs are

located in the loci that are frequently deleted and mutated in oncogenes, and their chromosomal regions can be negatively regulated to weaken the ability of miRs to repress cancers. A study manifested that miR expression dysregulation has an association with the development, progression and metastasis of human malignancies [4]. In contrast, abnormal expression or mutation of miRs increases the possibility of metastasis [5]. Actually, research revealed that targeted changes in miR expressions are able to inhibit carcinogenicity of tissues [6].

Corresponding author: Weihua Chen, MD. Department of Plastic and Aesthetic Surgery, the Fourth Affiliated Hospital of Harbin Medical University, 37 Yiyuan Street, Nangang District, Harbin, Heilongjiang, 15001, China.  
Tel: +86 013703689959, Email: 001787@hrbmu.edu.cn  
Received: 10/10/2020; Accepted: 19/11/2020

Cutaneous malignant melanoma (MM) is highly metastatic and thus becomes the most aggressive cutaneous malignancy. MM is mainly detected in fair-skinned Caucasians, characterized by canceration of pigment-producing melanocytes [7]. Traditionally, cutaneous MM has been proven to be resistant to chemotherapy and radiation therapy [8]. Since the induction of cell senescence has a great potential in treating malignant tumors, understanding the mechanism by which miRs affect the proliferation of cells will be conducive to the development of new therapies for cutaneous MM. The role of miR-125b in malignancies remains controversial. In other words, some studies have verified that miR-125b serves as an oncogene participating in malignant tumors like megakaryoblastic leukemia and prostate cancer [9,10]. Another study denoted that miR-125b facilitates the sensitivity of breast cancer cells to chemotherapeutic drugs and impedes the formation of drug-resistant phenotypes [11]. However, it is found in a study that miR-125b acts as a tumor suppressor in bladder cancer and MM, repressing the proliferation and invasion of cancer cells [12]. The exact role of miR-125b as an inhibitor in malignant tumors, especially in MM, and invasive diseases needs to be described. Therefore, it is necessary to discover the exact functioning mechanism of miR-125b in metastatic melanoma to target and regulate miRs, thereby treating melanomas. Neural cell adhesion molecule (NCAM) belongs to the immunoglobulin-like CAM family and has three major subtypes, namely, NCAM120, NCAM140 and NCAM180 [13], originally discovered to mediate cell adhesion in nerve cells [14]. However, it is reported that NCAM is expressed in various cancer cells, mainly plays the role of signal transmission, and participates in the proliferation,

apoptosis and differentiation in various signaling pathways [15,16]. A study conducted by Zhang et al [17] showed that miR-125b targets NCAM to promote tau phosphorylation, thus participating in the progression of Alzheimer's disease. In this study, therefore, it was speculated that miR-125b is involved in the progression of MM *via* targeted regulation on NCAM.

## Methods

### Experimental materials used

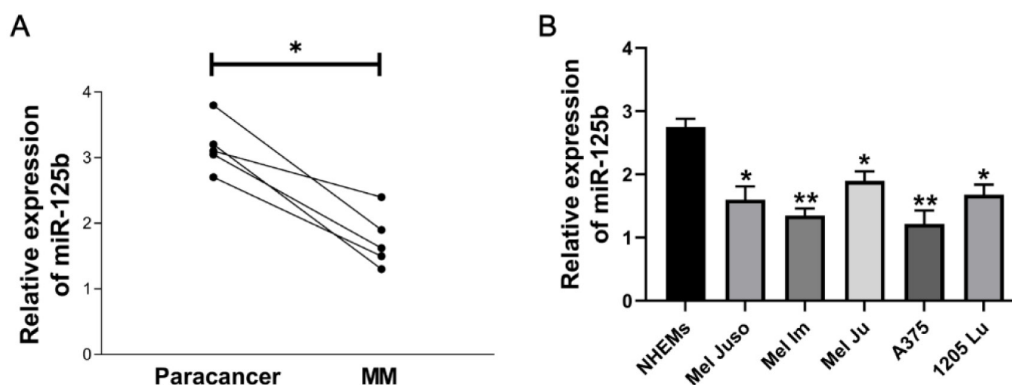
MM tissues (collected in our hospital), A375 cell lines, miR isolation kits (Ambion, Austin, TX, USA), Lightcycler (Roche, Basel, Switzerland), HiPerFect transfection reagent (Qiagen, Hilden, Germany), NCAM-140, NCAM-120 and NCAM-180 antibodies (Abcam, Cambridge, MA, USA), and TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA).

### Objects of study

MM tissues were selected as study subjects, with cancer tissues as MM group, and adjacent tissues as Paracancer group. Besides, A375 cells were chosen and transfected with miR-125b mimic (miR-125b mimic group) or mimic control (mimic control group). This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University. Signed informed consents were obtained from all participants before the study entry.

### RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA samples were extracted using TRIzol reagent according to manufacturer's instructions. Next, qRT-PCR was performed on Lightcycler using a 20 µL reaction system containing 500 ng of complementary deoxyribose nucleic acid (cDNA) template, 0.5 µL of forward and reverse primers (20 µM) and 10 µL of Sybr Premix Ex Taq under the following PCR conditions: 95°C for 30



**Figure 1.** MiR-125b expression changes in MM tissues and cells. **A:** Compared with that in Paracancer group, the expression of miR-125b is remarkably lowered in MM group ( $p < 0.05$ ). **B:** The expression of miR-125b is significantly lower in Mel Juso, Mel Im, Mel Ju, A375 and 1205 Lu cells than that in neonatal human epidermal melanocytes (NHEMs) (\* $p < 0.05$  vs. Paracancer group/NHEMs, \*\* $p < 0.01$ ).

s (initial denaturation) and 40 cycles of 95°C for 3 min, 60°C for 5 s, and 72°C for 16 s at a temperature transition rate of 20°C/s.  $\beta$ -actin was used as an endogenous reference of target genes. The primer sequences used in this study are as follows: MiR-125b-F: 5'-AAAAA-GCTTGCTGCCACACAGTCACATG-3'; MiR-125b-R: 5'-AAAACGCGTCCATCATCTGTGTAGAAGCAGG-3'.  $\beta$ -actin-F: 5'-CTACGTCGCCCTGGACTTCGAGC-3';  $\beta$ -actin-R: 5'-GATGGAGCCGCCGATCCACACGG-3'.

#### Western blotting

Total proteins were extracted from cells in miR-125b mimic group and mimic control group using RIPA lysis cell buffer *via* ultrasonication, of which the concentration was then detected with bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA). Thereafter, the target proteins were separated through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) by wet transfer method, blocked on a shaker at room temperature for 1 h, and incubated with NCAM-120, NCAM-140 and NCAM-180 primary antibodies in a refrigerator for 12-16 h. Afterwards, the gel was

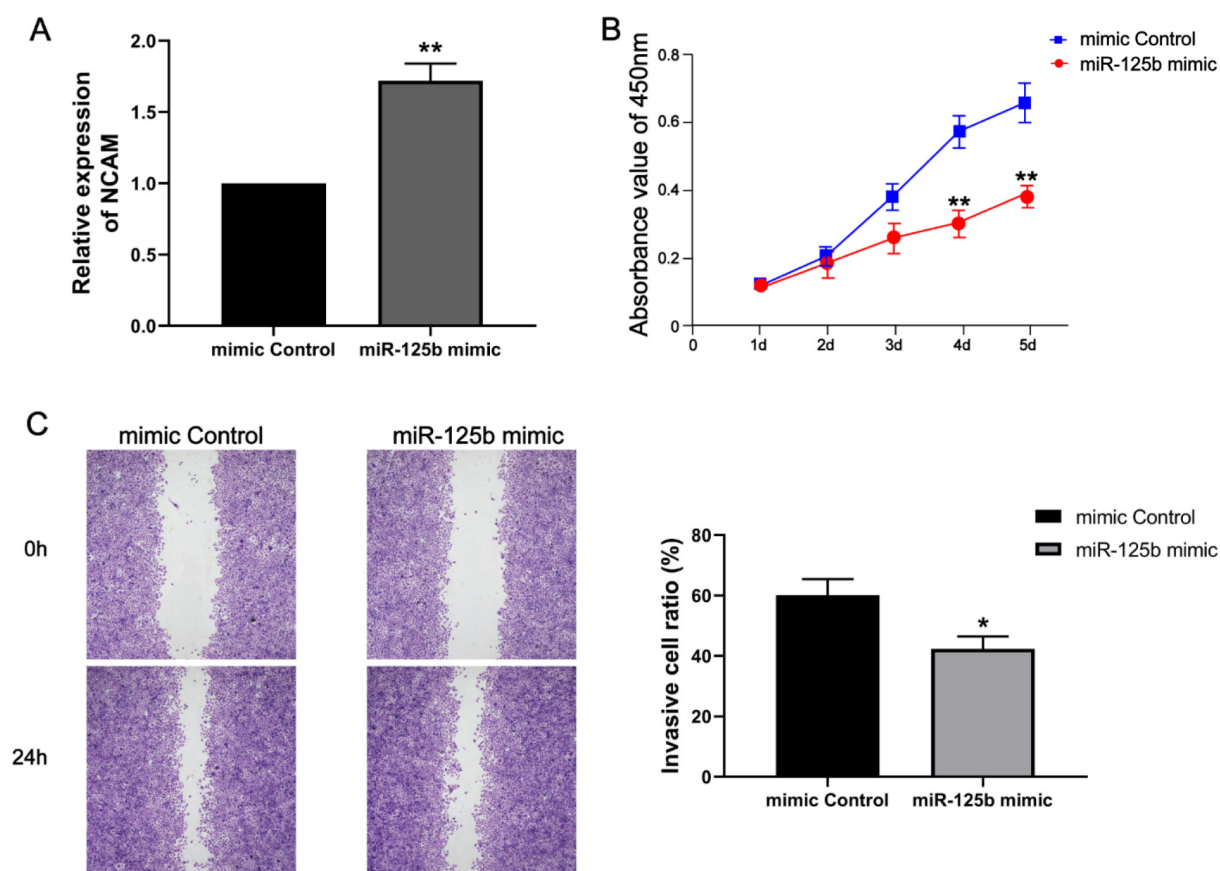
added with horse radish peroxidase (HRP)-conjugated secondary antibody, and washed with phosphate buffered saline-tween (PBST) for three times, followed by observation of the brightness of bands with enhanced chemiluminescence solution. Lastly, ImageJ software was employed to determine the protein expression level *via* densitometry

#### Detection of apoptosis through flow cytometry

According to the instructions of apoptosis detection reagent, the cell pellet in miR-125b mimic group and mimic control group was collected and added with Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) staining solution (2: 1) for incubation in the dark for 30 min. Finally, cell apoptosis was analyzed by flow cytometry (Portec AG, Arlesheim, Switzerland).

#### Cell counting kit-8 (CCK-8) proliferation assay

The proliferation of different cell lines was determined *via* CCK-8 assay (Dojindo, Kumamoto, Japan) as per the manufacturer instructions. Briefly, 3000 MM cells were seeded in 100  $\mu$ L of Dulbecco's Modified Eagle's Medium (DMDM) in 96-well plates for triplicate



**Figure 2.** Effects of miR-125b on the proliferation and migration of MM cells. **A:** Changes in miR-125b expression in A375 cells transfected with miR-125b mimic/mimic control. Compared with mimic control group, miR-125b mimic group displays a clearly raised miR-125b expression ( $p < 0.05$ ). **B:** Effect of miR-125b on the proliferation of A375 cells. The absorbance is significantly lower in miR-125b mimic group than that in mimic control group. **C:** Effect of miR-125b on the migration of A375 cells (magnification: 400 $\times$ ). The proportion of migrated cells in miR-125b mimic group is overtly lower than that in mimic control group ( $p < 0.05$ ) (\* $p < 0.05$  vs \*\* $p < 0.01$  mimic control group).



experiments, followed by incubation with DMEM containing 10% CCK-8 in a 5% CO<sub>2</sub> incubator at 37°C for 2 h. Afterwards, the absorbance at 450 nm was read by a microplate reader (Bio-Pad, Hercules, CA, USA) to determine the changes in cell optical density (OD).

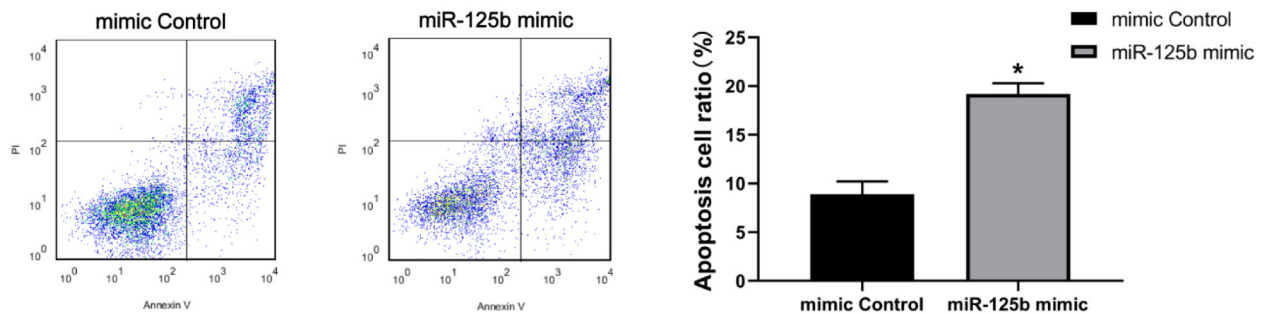
#### Wound healing assay

Cells were evenly seeded in 6-well plates and cultured in an incubator. When cells were confluent, they were scratched with a pipette tip to create wounds.

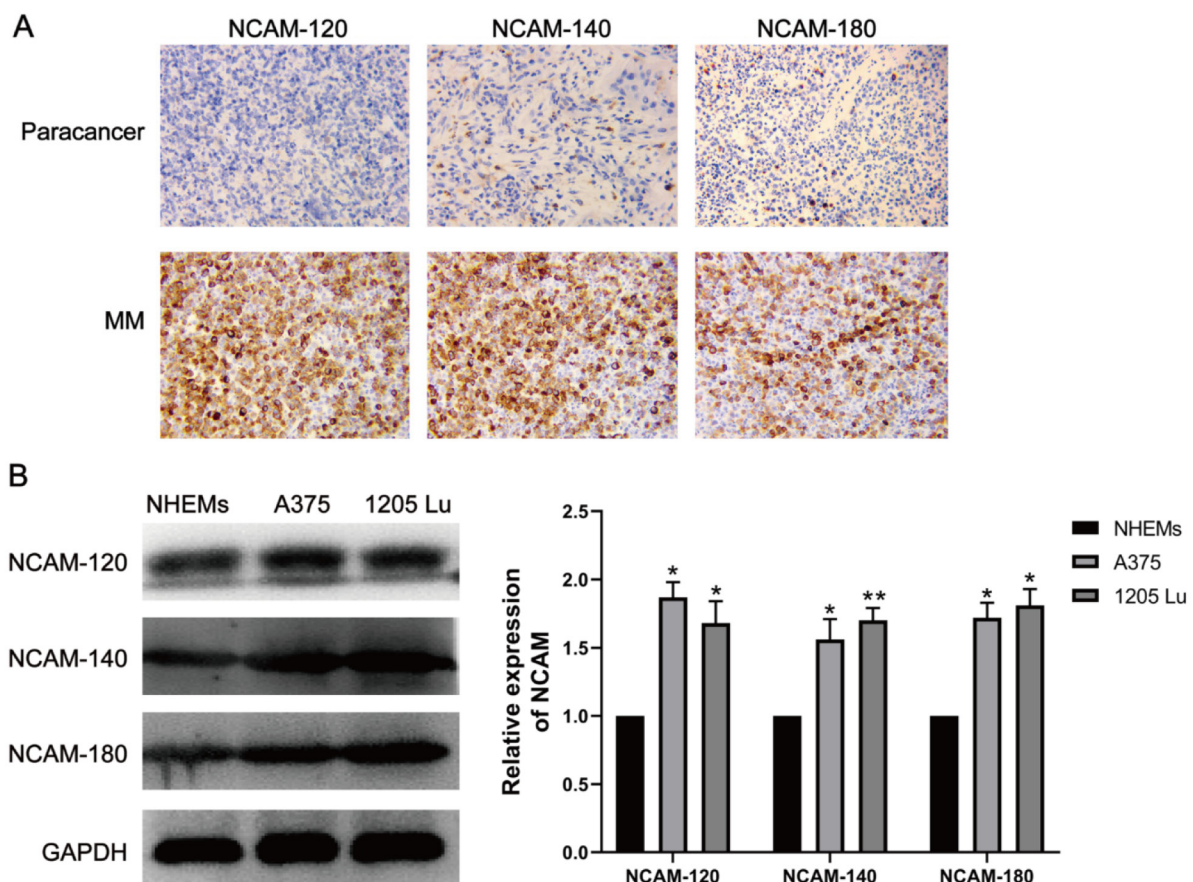
Next, the cells were rinsed with phosphate buffered saline (PBS) to remove cell debris. The cell-free area was photographed at 0 and 24 h after scratching. T-Scratch software was used to calculate the area of wound closure.

#### Dual-luciferase reporter assay

A375 cells were co-transfected with 50 nM miR-125b mimic or mimic control and 500 nM psiCHECK-2-NCAM-3'-UTR-WT/NCAM-3'-UTR-MUT. Next, the



**Figure 3.** Influence of miR-125b on the apoptosis of MM cells. Compared with that in mimic control group, the proportion of apoptotic cells was significantly raised in miR-125b mimic group ( $p < 0.05$ ) (\* $p < 0.05$  vs. mimic control group).



**Figure 4.** NCAM expression changes in MM tissues and cells. **A:** NCAM expression in MM tissues detected via immunohistochemistry (magnification: 400×). Compared with Paracancer group, MM group exhibits enhanced staining intensity of NCAM protein ( $p < 0.05$ ). **B:** NCAM protein expression in cells detected through Western blotting. The protein expression levels of NCAM-120, NCAM-140 and NCAM-180 are notably higher in MM cells than those in NHEMs cells ( $p < 0.05$ ) (\* $p < 0.05$  vs. \*\* $p < 0.01$  vs. Paracancer group/NHEMs).

changes in luciferase activity in both groups of cells were detected using a detection system.

### Statistics

SPSS Statistics v17.0 (IBM, Armonk, NY, USA) was adopted to process and analyze data and graphs. T-test was utilized for analysis of significant differences, and  $p < 0.05$  indicated a statistical difference.

## Results

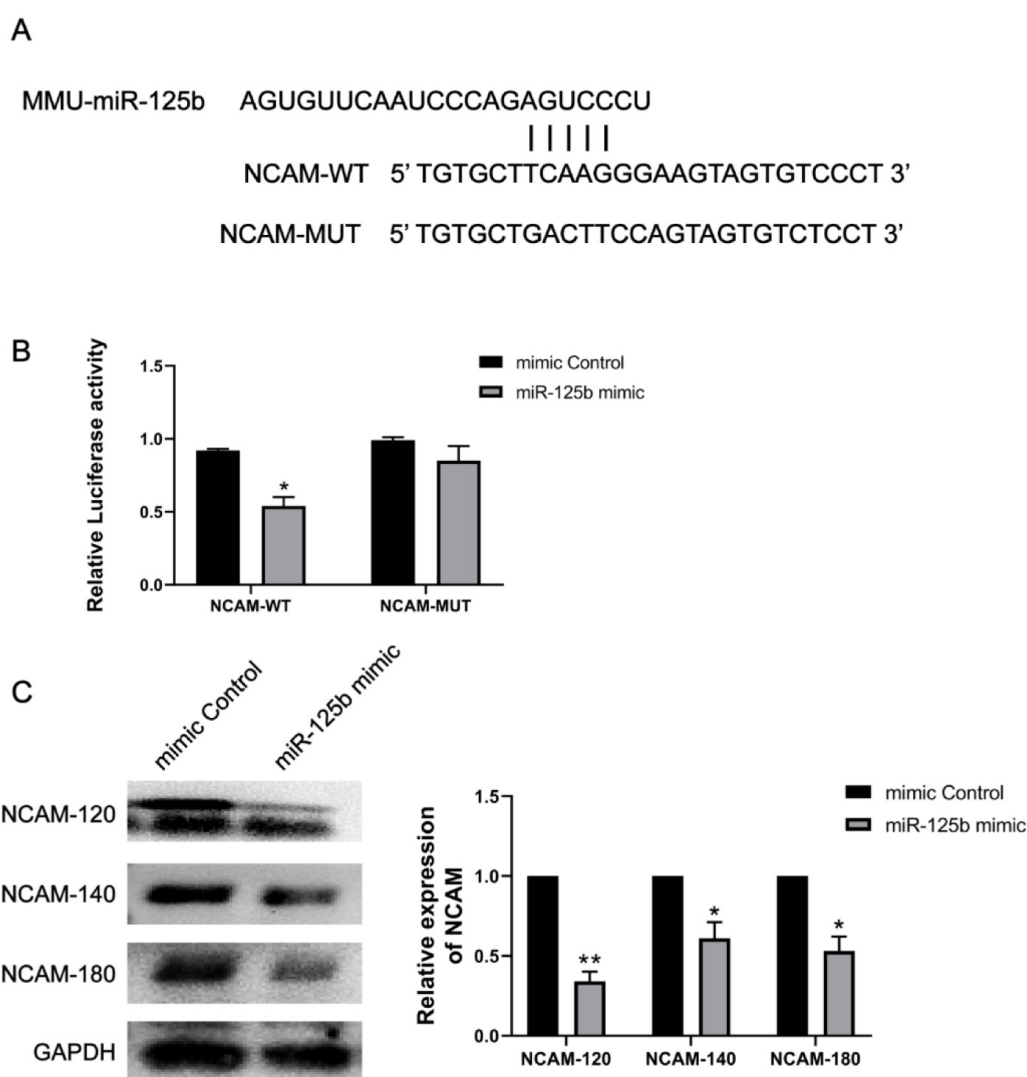
*MiR-125b had a notably decreased expression in MM tissues and cells*

Based on the detection of the changes in miR-125b expression in 5 cases of MM tissues and malignant melanocytes by qRT-PCR, miR-125b ex-

pression was evidently decreased in MM tissues ( $p < 0.01$ ) and cells ( $p < 0.05$ ) (Figure 1).

*MiR-125b repressed the proliferation and migration of MM cells*

To explore the role of miR-125b in MM, A375 cells were selected to exogenously overexpress miR-125b, and CCK-8 proliferation assay and wound healing assay were performed to detect the changes in the proliferation and migration of cells. It was discovered that compared with mimic control group, exogenous overexpression of miR-125b distinctly decreased the absorbance of cells at 450 nm and the healing area ( $p < 0.05$ ) (Figure 2), suggesting that cell proliferation and migration are inhibited after up-regulation of miR-125.



**Figure 5.** Interaction between miR-125b and NCAM determined *via* dual luciferase assay. **A:** SEED sequence cloned into the psiCHECK-2 vector. **B:** Changes in luciferase activity after co-transfection. In comparison with the cells transfected with NCAM-MUT, luciferase activity was significantly reduced in the cells transfected with NCAM-WT ( $p < 0.05$ ). **C:** Changes in NCAM expression in A375 cells exogenously expressing miR-125b. The expressions of NCAM-120, NCAM-140 and NCAM-180 were remarkably lower in miR-125b mimic group than those in mimic control group ( $p < 0.05$ ) (\* $p < 0.05$  vs. mimic control group, \*\* $p < 0.01$  vs. mimic control group).

### *MiR-125b impeded the apoptosis of MM cells*

In accordance with the results of flow cytometry used to detect the influence of miR-125b on the apoptosis of MM cells, the proportion of apoptotic cells was evidently reduced after exogenous overexpression of miR-125b ( $p < 0.05$ ) (Figure 3), implying that miR-125b serves as a tumor suppressor in MM.

### *NCAM expression was significantly high in MM tissues and cells*

Immunohistochemical staining assay was conducted to measure the expression level of NCAM proteins in MM tissues. The results revealed that NCAM-120, NCAM-140 and NCAM-180 were mainly located in the cytoplasm of MM tissues, and the staining intensity was stronger in MM group than that in Paracancer group. Besides, statistics of gene expression in the two groups demonstrated that NCAM was significantly overexpressed in MM tissues ( $p < 0.05$ ). In addition, Western blotting results uncovered that the expressions of NCAM-120, NCAM-140 and NCAM-180 were also distinctly higher in MM (A375 and 1205 Lu) cells than those in NHEMs ( $p < 0.05$ ) (Figure 4), indicating that NCAM is significantly overexpressed in MM tissues and cells.

### *MiR-125b participated in the progression of MM by regulating NCAM protein expression*

To further investigate the relationship between miR-125b and NCAM, the dual luciferase reporter detection system was adopted to detect the inhibitory effect of miR-125b on NCAM 3'-UTR "SEED sequence". The SEED sequence was amplified into the psiCHECK-2 vector, and then A375 cells were co-transfected with miR-125b mimic/mimic control and psiCHECK-NCAM-MUT/NCAM-WT. It was discovered that 48 h after co-transfection, the Renilla luciferase activity was significantly lower in cells transfected with NCAM-WT than in those transfected with NCAM-MUT ( $p < 0.05$ ), and NCAM expression was overtly lowered in MM cells with exogenously overexpressed miR-125b ( $p < 0.05$ ) (Figure 5). These results suggest that miR-125b represses NCAM expression by targeting the NCAM 3'-UTR sequence.

## **Discussion**

As an aggressive tumor, MM tends to metastasize in the early stage and is resistant to the majority of current treatments [18]. The abnormal expression of some miRs may contribute to the development and progression of melanoma [19].

Several miRs are known as "carcinomas" (miRs with carcinogenic or suppressive effects). It is demonstrated that miRs are involved in cell proliferation and differentiation through specific gene regulatory networks. It is believed that hundreds of genes are regulated by a single miR, and one-third of protein-coding genes are targets of miRs [20]. Calin et al [21] speculated that miR-125b is a major regulator of breast cancer, lung cancer, ovarian cancer, and cervical cancer. However, the regulatory process of miRs, as well as the possible regulatory potential of miR-125b in MM, is poorly understood at present.

In this study, the experimental data indicated that the proliferation and migration potential of melanoma cells was affected by miR-125b. Based on *in vitro* functional assays, the proliferation and migration capability of A375 cells with over-expressed miR-125b was markedly inhibited, while their apoptosis capacity was enhanced. These findings suggest that the miRs play a vital role in MM. In addition, it was determined in this study that miR-125b expression was downregulated in melanoma cells and tissues (primary melanoma and metastases) compared with that in NHEMs, implying that miR-125b may be a potential target for the treatment of MM. Further research confirmed that miR-125b is downregulated in cutaneous MM cells and has an impact on cell survival and senescence [22]. The experimental data in this study revealed that miR-125b inhibited the growth in MM and acted as a potential tumor suppressor.

Previous reports have denoted that the over-expression of NCAM is correlated with inadequate response of many cancers to the treatment, which reduces the survival of cancer patients [23,24]. Moreover, a phase I study evaluating the efficacy of NCAM-targeted antibody-drug conjugates in treating patients with NCAM-positive solid tumors uncovered that there are clinical benefits [25]. This study findings suggest that NCAM can be used as a potential marker for the diagnosis and treatment of various malignancies. However, the role of NCAM in human melanoma and its underlying mechanisms remain unclear despite these recent advances. A study conducted by Zhang et al [17] showed that miR-125b targets NCAM to promote tau phosphorylation, thus participating in the progression of Alzheimer's disease. In this study, therefore, it was speculated that miR-125b can also target NCAM to participate in the progression of MM. It was detected that the expression of NCAM was notably high in the 5 cases of MM tissues collected and MM cells. For this reason, it was speculated that NCAM may be one of the targets of miR-125b. To verify such a speculation, double



luciferase reporter assay was performed, and the results showed that the Renilla luciferase activity was significantly lower in the cells transfected with NCAM-WT than that in the cells transfected with NCAM-MUT, indicating that miR-125b inhibits Renilla luciferase activity by targeting NCAM 3'-UTR sequence. NCAM expression was overtly decreased in A375 cells exogenously transfected with miR-125b, suggesting that miR-125b negatively regulates NCAM expression. However, the effects of NCAM on the proliferation and migration of MM cells still need to be verified.

## Conclusions

In conclusion, miR-125b is under-expressed in MM and negatively modulates NCAM expression, resulting in abnormal expression of NCAM and affecting the proliferation and migration of cells. This study provides a basis for miR-125b and NCAM as potential therapeutic targets for MM.

## Conflict of interests

The authors declare no conflict of interests.

## References

- Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer* 2003;3:859-68.
- Zhan C, Wang T, You H, Si C. Different expressions of miR-125b and SOX30 in malignant lymphomas and their significance. *JBUON* 2018;23:1179-84.
- Sur D, Cainap C, Burz C et al. The role of miRNA -31-3p and miR-31-5p in the anti-EGFR treatment efficacy of wild-type K-RAS metastatic colorectal cancer. Is it really the next best thing in miRNAs? *JBUON* 2019;24:1739-46.
- Ding D, Qi Z. Clinical significance of miRNA-195 expression in patients with laryngeal carcinoma. *JBUON* 2019;24:315-22.
- Shaulian E, Karin M. AP-1 in cell proliferation and survival. *Oncogene* 2001;20:2390-2400.
- Yang H, Shen C. MicroRNA-29c induces G1 arrest of melanoma by targeting CDK6. *JBUON* 2019;24:819-25.
- Papaconstantinou I, Karakatsanis A, Gazouli M, Polymeneas G, Voros D. The role of microRNAs in liver cancer. *Eur J Gastroenterol Hepatol* 2012;24:223-8.
- Le XF, Merchant O, Bast RC, Calin GA. The Roles of MicroRNAs in the Cancer Invasion-Metastasis Cascade. *Cancer Microenviron* 2010;3:137-47.
- Ender C, Meister G. Argonaute proteins at a glance. *J Cell Sci* 2010;123:1819-23.
- Deng Y, Deng H, Bi F et al. MicroRNA-137 targets carboxyl-terminal binding protein 1 in melanoma cell lines. *Int J Biol Sci* 2011;7:133-7.
- Zeng Y, Yi R, Cullen BR. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci U S A* 2003;100:9779-84.
- Janga SC, Vallabhaneni S. MicroRNAs as post-transcriptional machines and their interplay with cellular networks. *Adv Exp Med Biol* 2011;722:59-74.
- Hansen SM, Berezin V, Bock E. Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-cadherin. *Cell Mol Life Sci* 2008;65:3809-21.
- Ronn LC, Hartz BP, Bock E. The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system. *Exp Gerontol* 1998;33:853-64.
- Fukuda M, Kusama K, Sakashita H. Cimetidine inhibits salivary gland tumor cell adhesion to neural cells and induces apoptosis by blocking NCAM expression. *BMC Cancer* 2008;8:376.
- Francavilla C, Cattaneo P, Berezin V et al. The binding of NCAM to FGFR1 induces a specific cellular response mediated by receptor trafficking. *J Cell Biol* 2009;187:1101-16.
- Zhang L, Dong H, Si Y et al. miR-125b promotes tau phosphorylation by targeting the neural cell adhesion molecule in neuropathological progression. *Neurobiol Aging* 2019;73:41-9.
- Russo AE, Torrisi E, Bevelacqua Y et al. Melanoma: molecular pathogenesis and emerging target therapies (Review). *Int J Oncol* 2009;34:1481-9.
- Lichthardt S, Kersch A, Dietz UA et al. Original article: role of adjuvant chemotherapy in a perioperative chemotherapy regimen for gastric cancer. *BMC Cancer* 2016;16:650.
- Chen J, Feilotter HE, Pare GC et al. MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. *Am J Pathol* 2010;176:2520-9.
- Calin GA, Sevignani C, Dumitru CD et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A* 2004;101:2999-3004.
- Glud M, Rossing M, Hother C et al. Downregulation of miR-125b in metastatic cutaneous malignant melanoma. *Melanoma Res* 2010;20:479-84.
- Cho EY, Choi Y, Chae SW, Sohn JH, Ahn GH. Immunohistochemical study of the expression of adhesion molecules in ovarian serous neoplasms. *Pathol Int* 2006;56:62-70.
- Raspadori D, Damiani D, Michieli M et al. CD56 and PGP expression in acute myeloid leukemia: impact on clinical outcome. *Haematologica* 2002;87:1135-40.
- Shah MH, Lorigan P, O'Brien ME et al. Phase I study of IMGN901, a CD56-targeting antibody-drug conjugate, in patients with CD56-positive solid tumors. *Invest New Drugs* 2016;34:290-9.