• Basic Research •

miRNA-145/miRNA-205 inhibits proliferation and invasion of uveal melanoma cells by targeting NPR1/CDC42

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Abstract

● **AIM:** To investigate the role of microRNA-145 (miRNA-145) and microRNA-205 (miRNA-205) in proliferation and invasion of uveal melanoma (UM) cells.

● **METHODS:** The expression level of miRNA-145 and miRNA-205 from samples of UM patients were determined by real-time polymerase chain reaction (RT-PCR). The growth and invasion inhibitory effects were observed by the transfection of UM cells with miRNA-145 and miRNA-205. Several epithelial-to-mesenchymal transition (EMT) -related proteins were screened by Western blotting. UM clinical samples from The Cancer Genome Atlas (TCGA) were applied to search for potential protein interaction. Pearson's correlation analysis was applied to estimate co-expression between genes. Dual-luciferase reporter assay was used to verify the binding sites on target protein for miRNA-145 and miRNA-205.

● **RESULTS:** The expression levels of miRNA-145 and miRNA-205 in the samples from patients with UM were significantly lower than those in the normal tissue samples. Significant growth and invasion inhibitory effects were observed in human UM cells with miRNA-145 and miRNA-205 overexpression. The miRNA-145 and miRNA-205 could decrease the expression level of cell division control protein 42 (CDC42). After database searching and sequence alignment, we identified that *Neuropilin 1* (*NRP1*) had binding sites for both miRNA-145 and miRNA-205.

● **CONCLUSION:** The miRNA-145 and miRNA-205 can reduce the proliferation, migration and invasion of UM cells by targeting the mRNA of its upstream protein NRP1 to down-regulate the expression level of CDC42.

● **KEYWORDS:** uveal melanoma; microRNA-145; microRNA-205; CDC42; NRP1

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INTRODUCTION

Uveal melanoma (UM) is a common primary intraocular tumor, which incidence increases with the age among adults[1]. Due to the lack of early symptoms, most UM patients are in advanced stage at the time of diagnosis[2]. UM is highly metastatic. According to statistics, almost 50% UM patients have metastasis within ten years of treatment, and liver is the most common distant sites for metastasis[3]. The metastasis of UM involves complex and multi-step molecular mechanisms that are still not fully understood. Therefore, it is of great clinical significance to study the metastasis mechanism of UM for effective methods of diagnosis, treatment and prognosis.

The micro-RNAs (miRNA) are small noncoding RNA molecules that function in RNA silencing and posttranscriptional regulation of gene expression. Their roles in gene expression, cell cycle, and cellular development have been evaluated, and many groups have examined miRNA dysregulation in UM[4]. In the study of UM, miRNA-9 was found to suppress the UM cell migration and invasion through the nuclear factor kappa-B1 (NF-κB1) pathway[5]. In other studies, miRNA-34a might inhibit the growth and migration of UM cells through p53 anti-cancer network and suppressed proliferation by interfering with cell cycle[6]. Ye *et al*[7] reported that overexpression of miRNA-145 could inhibit cell proliferation, migration, and invasion of breast cancer cell by altering the whole transcriptome. Duan *et al*[8] and Park *et al*[9] discovered low expression of miRNA-205 leading to poor prognosis of non-small cell lung cancer in their respective research. Both miRNA-145 and miRNA-205 were identified as anti-oncomiRNA. However, the regulation of these two miRNAs on proliferation and invasion of UM cells has not been reported and thus it is necessary to further investigate the mechanism of miRNA-145 and miRNA-205 in cancer development.

In this study, we constructed UM cells with expression of miRNA-145 and miRNA-205, respectively. We found that overexpression of these two miRNAs could significantly reduce the proliferation, migration and invasion of UM cells. Moreover, miRNA-145 and miRNA-205 might downregulate the expression level of epithelial-to-mesenchymal transition (EMT) related protein cell division control protein 42 (CDC42) by binding to target gene *Neuropilin 1* (*NRP1*). These findings provide a better understanding of development of UM and novel potential biomarkers for prognosis.

MATERIALS AND METHODS

Ethical Approval This study was approved by the Medical Ethics Committee of the Beijing Tongren Hospital.

Tissue Samples The samples of high-invasive UM (Cell type of the sample was epithelial in pathological examination), low-invasive UM (Cell type of the sample was spindle in pathological examination) and healthy controls were obtained from the Beijing Tongren Eye Bank (Beijing, China). Each group contained five samples. Total RNA containing miRNA was extracted using miRNeasy Mini Kit (Qiagen, German) according to the manufacturer's protocol. The extracted RNA was assessed by a NanoDrop spectrophotometer with measurement of 260/280 ratio.

Detection of miRNA Expression Level The All-in-One miRNA quantitative real-time polymerase chain reaction (qRT-PCR) Detection Kit (GeneCopoeia, MD, USA) was used to detected the expression level of miRNA as described by the manufacturer's recommendation. After cDNA was synthesized as template, quantitative polymerase chain reaction (qPCR) was performed on LightCycler 480II (Roche, Switzerland) for miRNA quantification.

Cell Culture and Transfection Human choroidal melanoma cell line OCM-1A and C918 were purchased from Procell (PROCELL Life Science&Technology Co., Ltd., Wuhan, China). The cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Thermo Fisher Scientifc, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio, FL, USA) and 1% penicillin-streptomycin (P/S; Gibco, Thermo Fisher Scientifc, Inc., Waltham, MA, USA) in a humidified incubator with 5% carbon dioxide at 37°C. The constructed lentiviral vectors pLenti-EF1α-EBFP-miR-145-miR-NC, pLenti-EF1α-EBFP-miR-205-miR-NC and pLenti-EF1α-mKate-miR-145-miR-205 were transfected into 293T cells. Lentivirus were collected after 48h and transfected into the polybrene (SyngenTech, Beijing, China) treated OCM-1A/C918 using Lipofectamine 3000 transfection reagent (Life Technologies, Thermo Fisher Scientifc, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The transfected cells were screened by flow cytometry.

Proliferation Assay The cells were cultured in 96-well plate with 2000 cells/100 μL for 12-24h. Cell counting kit-8 (CCK-8) reagent (ApplyGen, Beijing, China) was added with 10 μL/well 1-4h before each detection time point. Growth rate was determined by measurement of OD450 nm using spectrophotometer at each time point.

Invasion Assay Matrigel (BD Biosciences, NJ, USA) was diluted to 0.2-0.3 mg/mL and coated on the upper surface of the polycarbohydrate membrane of Transwell (Corning, NY, USA) chamber then incubated at 37°C for 1-4h. The serum-free cell suspension was added to the upper chamber with 20 000 cells/200 mL. After 24h, the membrane was fixed with polyformaldehyde and stained with giemsa (Solarbio Life Sciences, Beijing, China). The cells were observed and counted under a light microscope.

Western Blotting Proteins extracted from wild type OCM-1A cells, miRNA-145 overexpression cells, miRNA-205 overexpression cells, and miRNA-145+miRNA-205 overexpression cells were quantified with bicinchoninic acid assay and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Sigma, MA, USA). The PVDF membrane was blocked in phosphate buffered saline with Tween-20 (PBST; Solarbio Life Sciences, Beijing, China) containing 5% non-fat dry milk for one hour then incubated with rabbit anti-CDC42, rabbit anti-BMI, rabbit anti-ZEB1, rabbit anti-RhoA, rabbit anti-RAC1, rabbit anti-p-SMAD, and rabbit anti-β-actin (Abcam, Cambridge, MA, USA) at 4°C overnight. After three times washed with PBST, the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibodies (Beyotime Biotechnology, Shanghai, China) for one hour at room temperature. Target bands were visualized by enhanced chemiluminescent substrate and Image J software was used to evaluate the expression levels of target proteins.

Dual-Luciferase Reporter Assay The constructed plasmids were transfected into HEK293 cells. After 48h, cells were lysed and determined the luciferase activity using Dual-Luciferase Reporter Assay System (Beyotime Biotechnology, Shanghai, China) in Luminoskan Ascent luminometer (Thermo Fisher Scientifc, Inc., Waltham, MA, USA) according to the manufacture's recommendation.

Statistical Analysis In order to investigate CDC42 and its co-expression proteins, we used starBase[10] to search the upstream proteins. In addition, co-expression coefficient between CDC42 and its co-expression protein was estimated by using Pearson's correlation analysis based on the UM dataset from The Cancer Genome Atlas (TCGA). MiRBase[11] and Geneious software were used for miRNA-mRNA interaction sites prediction. SPSS 20.0 was used to perform statistical

analysis of the experimental data. $P<0.05$ was considered to be significant difference. Kaplan-Meier plotter (http://kmplot.com/analysis) was used to assess the overall survival of UM at high and low expression of miRNA.

RESULTS

Expression of miRNA-145 and miRNA-205 in Uveal Melanoma The expression levels of miRNA-145 in high-invasive and low-invasive UM were both significantly lower than that in healthy control (Figure 1A). The expression level of miRNA-205 in low-invasive UM was lower than that in healthy control and significantly lower in high-invasive UM (Figure 1B).

Overexpression of miRNA-145 and miRNA-205 Suppresses Proliferation of Melanoma Cells Down-regulation of miRNA-145 and miRNA-205 in UM patient samples suggested that they might play a role in tumor suppression. In order to validate the effect of miRNA-145 and miRNA-205 in UM, OCM-1A and C918 cells were used to perform proliferation assay. Cells were transfected with miRNA-145, miRNA-205, miRNA-145+miRNA-205, and negative control mimics. The cell survival curve (Figure 2) showed that overexpression of miRNA-145 could significantly reduce the proliferation of the melanoma cells, while miRNA-205 had little effect on the proliferation of cells. And the co-expression of miRNA-145 and miRNA-205 could slightly enhance the reduction of OCM-1A (Figure 2A) and C918 (Figure 2B) cell proliferation.

Overexpression of miRNA-145 and miRNA-205 Suppresses Melanoma Cell Invasiveness In order to study the role of miRNA-145 and miRNA-205 in melanoma cell, we proceeded to invasion assays using OCM-1A and C918. As shown in Figure 3, migration through Matrigel in the transwell assay showed slightly reduced with overexpression of miRNA-145 but significantly reduced with overexpression of miRNA-205. Moreover, the co-expression of miRNA-145 and miRNA-205 resulted in a much more decrease in OCM-1A (Figure 3A and 3B) and C918 (Figure 3C and 3D) invasiveness.

miRNA-145 and miRNA-205 Together Regulate CDC42 in Melanoma Cells To find the potential protein influenced by miRNA-145 and miRNA-205, we screened several proteins related to tumor invasion. Western blotting was performed to examine the candidates: CDC42, zinc finger E-box binding homeobox 1 (ZEB1), Rac family small GTPase 1 (RAC1), and phosphorylated mothers against decapentaplegic homolog 3 (p-SMAD3). As shown in Figure 4A and 4B, the co-expression of miRNA-145 and miRNA-205 could significantly reduce the expression level of CDC42 in cells. To validate this result, plasmids containing CDC42 were transfected into melanoma cells, and this add-back of CDC42 could rescue the effect of co-expression of miRNA-145 and miRNA-205 in OCM-1A (Figure 4C) and C918 (Figure 4D).

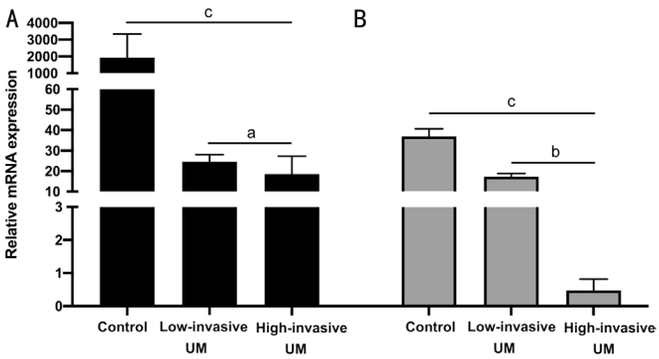

Figure 1 Expression level and survival analysis of miRNA-145 and miRNA-205 The expression levels of miRNA-145 (A) and miRNA-205 (B) were quantified by qRT-PCR. $^{a}P<0.1$; $^{b}P<0.05$; $^{c}P<0.01$.

Both miRNA-145 and miRNA-205 Suppress NRP1 To investigate the mechanism of miRNA-145 and miRNA-205 affecting CDC42 expression, we studied the potential target gene *NRP1*, which encodes protein NRP1 that is co-expression with CDC42 in the UM clinical samples from TCGA. As a result, expressions of NRP1 and CDC42 exhibit significantly positive correlation as shown in Figure 5A (pearson correlation coefficient =0.575, P=2.49e-08). The miRNA-145 and miRNA-205 binding sites with *NRP1* were predicted using miRBase and BLAST (Figure 5B). We showed significant suppression of luciferase activity of the plasmids containing *NRP1* binding sites. These results suggested that both miRNA-145 (Figure 5C) and miRNA-205 (Figure 5D) downregulated the expression of CDC42 by suppressing *NRP1*.

DISCUSSION

UM, the most common primary intraocular tumor in adults, is the second most common form of melanoma, arising from melanocytes located in the uveal tract of the eye. It is a highly aggressive disease, with a strong tendency to metastasize from the eye to other organs, most commonly to the liver. The primary tumor can be treated successfully using several options, such as enucleation, stereotactic radiotherapy, brachytherapy and proton therapy[12]. Molecular targeting therapy, which focusing to find effective therapeutic target genes or proteins, has been developed a lot in recent years. The discovery of a large number of abnormally expressed miRNA and their predicted target genes make miRNA have promising clinical application. As such, we studied the mechanism of miRNA-145 and miRNA-205 on UM cells, providing new targets for the treatment of UM.

The miRNA-145, which widely expressed in many tissues, is recently identified as tumor suppressor and a promising cancer treatment target candidate. Published data confirm that miRNA-145 expression in various tumors is significantly lower than that in normal tissues and that overexpression of miRNA-145 inhibits the growth of different tumor cells,

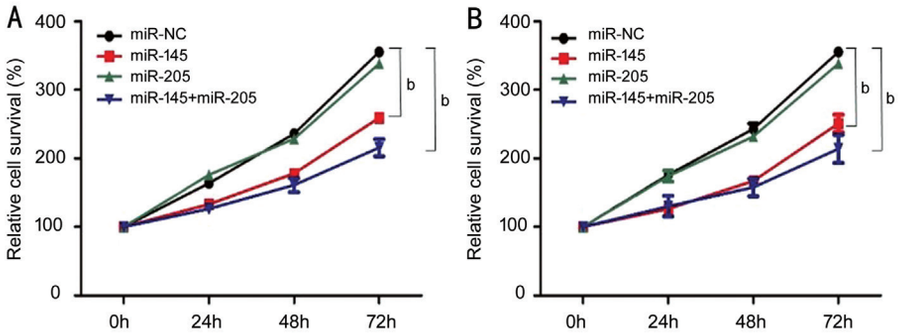

Figure 2 The effect of miRNA-145 and miRNA-205 on the proliferation of cells The survival curves showed miRNA-145 and co-expression of miRNA-145 and miRNA-205 could decrease the survival of the OCM-1A cells (A) and C918 cells (B). $^{b}P<0.05$.

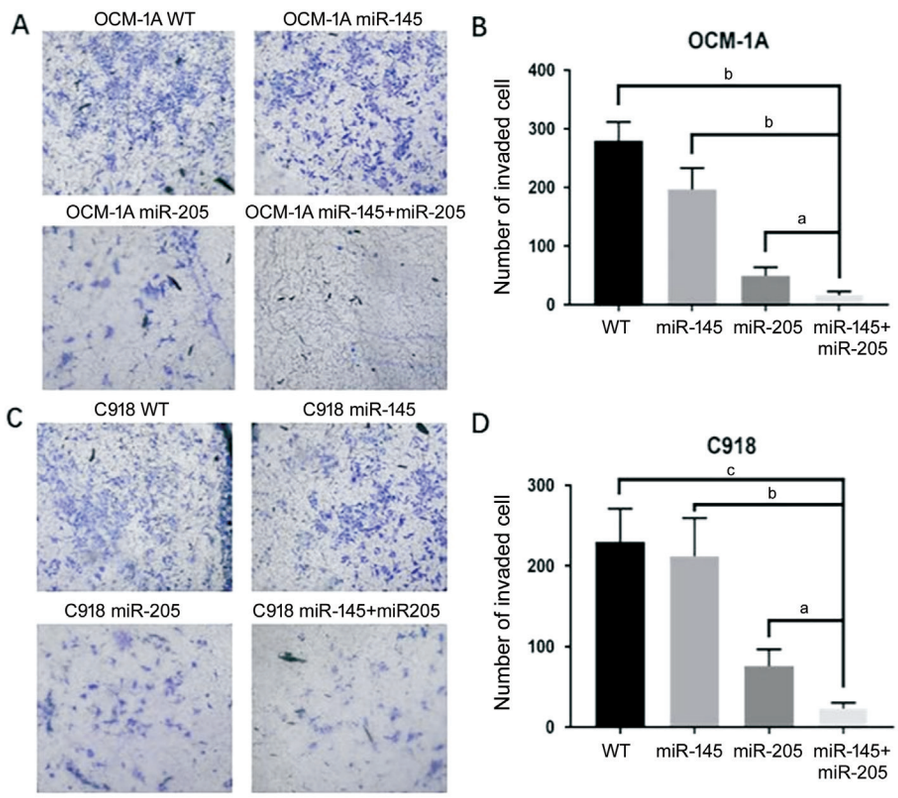

Figure 3 The effect of miRNA-145 and miRNA-205 on the invasiveness of cells Representative images of OCM-1A (A) and C918 (C) on the lower surface of the membrane in the transwell assay. OCM-1A (B) and C918 (D) migrating through the Matrigel were quantified and the results were normalized to the wildtype control. $^{a}P<0.1$; $^{b}P<0.05$; $^{c}P<0.01$.

significantly reduces the ability of tumors to spread, and improves sensitivity to chemotherapeutic drugs[13]. The miRNA-145 induces cell cycle arrest in G0-G1 phase and shorten S phase[14], which may be related to the inhibition of c-Myc expression resulting in down-regulation of Cyclin D and elE4E. However, no such arrest effects have been observed in normal tissue cells of high expression of miRNA-145. It was reported that the expression level of miRNA-145 in liver cancer was significantly lower than that in normal and adjacent tissues[15]. Sachdeva and Mo[16] showed that miRNA-145 not only inhibited the growth of tumors, but also inhibited the invasion and metastasis of tumor cells. Studies have shown that miRNA-145 can inhibit EMT of malignant tumor cells by targeting EMT-related genes, especially *N-cadherin* and *vimentin*, suppressing tumor cells invasion and metastasis in bladder cancer and rectal cancer[17-18]. The miRNA-205, however, is down-regulated in breast cancer, prostate cancer and melanoma as anti-oncogene, but also up-regulated in esophageal cancer, ovarian cancer and lung cancer as proto-oncogene[19]. The differential expression of miRNA-205 in various tumor tissues suggested that it regulates different target genes. In esophageal cancer, miRNA-205 promotes cancer metastasis by down-regulating the expression of target gene *ASPP2*; while in ovarian cancer, miRNA-205 enhances the cellular motility by targeting ZEB1 and SIPI1 thus promotes the clinical progression[20]. The role of miRNA-205

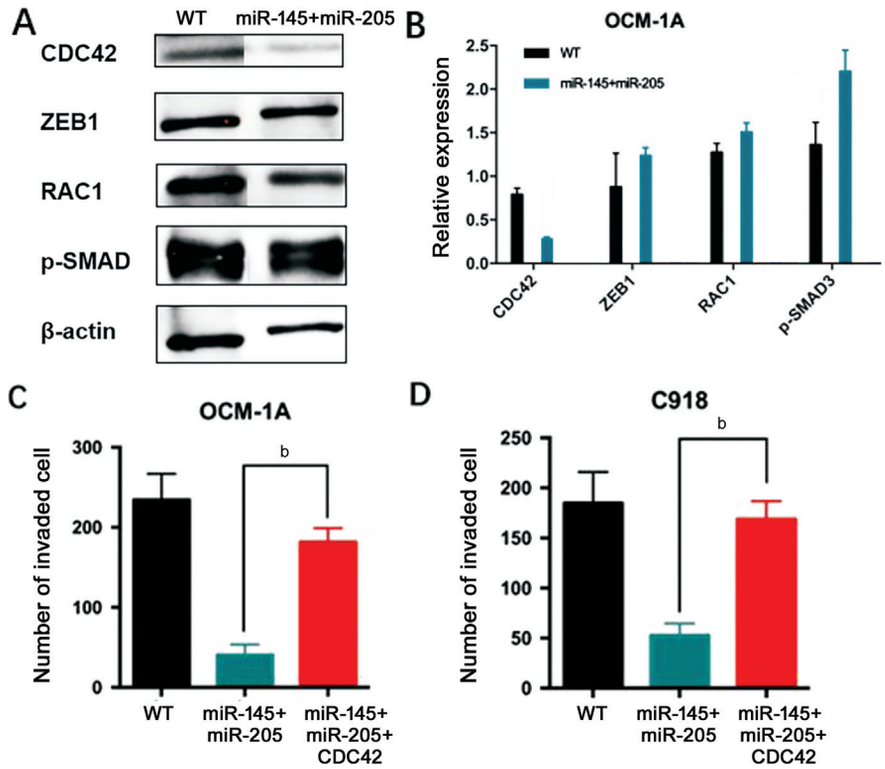

Figure 4 Proteins expression levels in melanoma cells with overexpression of miRNA-145 and miRNA-205 Western blotting (A) and quantitative assessment (B) of candidate proteins in OCM-1A cells. β-actin was used as a loading control. Add-back of CDC42 rescues the effect of co-expression of miRNA-145 and miRNA- 205 in OCM-1A (C) and C918 (D). $^{b}P<0.05$.

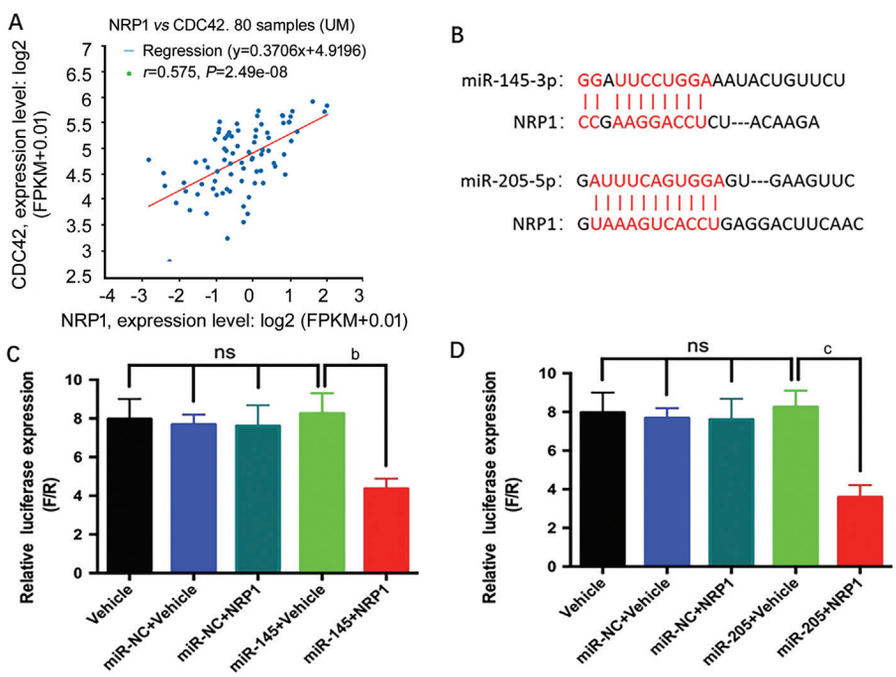

Figure 5 miRNA-145 and miRNA-205 suppress NRP1 NRP1 and CDC42 are co-expression in the UM samples (A). The predicted binding site of miRNA-145 and miRNA-205 with NRP1 (B). The miRNA-145 (C) and miRNA-205 (D) significantly suppress the luciferase activity in the dual-luciferase reporter assay. $^{b}P<0.05$; $^{c}P<0.01$.

in melanoma has been noted. Dar *et al*[21] showed that up-regulation of miRNA-205 could decrease E2F1 and E2F5 protein levels and inhibit the proliferation of melanoma cells through E2F-regulated AKT phosphorylation. In a study of using quantitative *in situ* hybridization to detect the expression of miRNA-205 in 105 cases of primary melanoma, Hanna *et al*[22] showed that decreased level of miRNA-205 was associated with reduced specific survival of melanoma. In

our study of miRNAs in UM, we found that miRNA-145 could significantly reduce the proliferation of UM cells, while miRNA-205 had little effect on cell proliferation, and there was slightly additive effect when both two miRNAs expression were elevated together. We also found in the clinical samples, the expression of miRNA-205 in high-invasive UM was much lower than that in low-invasive UM, while the expression levels of miRNA-145 in both high-invasive and low-invasive UM were equally lower than that in normal samples. This may be due to the inhibition of cell proliferation by miRNA-145 leading to suppress the process of metastasis. In addition, we showed that miRNA-205 could significantly reduce the invasion of UM cells in transwell migration assay, while miRNA-145 was overexpressed alone slightly affected the invasiveness of UM cells. However, stronger inhibitory effects on invasiveness were observed in both miRNAs expression elevated in UM cells. Thus, we suspect that miRNA-145 prefers to inhibit cell proliferation, while miRNA-205 is more likely to inhibit cell invasion. Our findings are consistent with previous reports that miRNA-145 and miRNA-205 play a role as tumor suppressors in UM.

In order to study the mechanism of miRNA-145 and miRNA-205 affecting UM cell proliferation and invasion, we screened several EMT-related proteins and found the co-expression of miRNA-145 and miRNA-205 could downregulate the expression level of CDC42, which helps cancer cells adhere to the blood vessels. But no binding site of *CDC42* gene sequence to miRNA-145 or miRNA-205 was found. However, we discovered that NRP1 had binding sites both to miRNA-145 and miRNA-205 and thus concluded that miRNA-145 and miRNA-205 may regulate CDC42 by targeting its upstream protein. NRP1 is a transmembrane receptor of the vascular endothelial growth factor (VEGF) family members and is involved in angiogenesis, tumor proliferation and migration. Numerous studies have shown that NRP1 is widely expressed in tumor cells and associated with poor prognosis of cancers[23]. NRP1 also interacts with various membrane receptors such as c-met, integrin and transforming growth factor receptor (TGFR) to regulate the occurrence and development of tumors. It has been reported that NRP1 is required for extracellular matrix-induced CDC42 activation in human endothelial cells, and loss of NRP1 and inhibition of CDC42 similarly impair angiogenesis[24]. Kiso *et al*[25] showed the long isoform of VEGF produced NRP1 signal resulting in activation of CDC42-mediated filopodia formation thus increased cell migration. Our finding confirmed the suppression of NRP1 leading to down-regulation of CDC42. Our study had limitations. Although we proved miRNA-145 and miRNA-205 could downregulate the expression of EMT-related protein CDC42 by suppressing its upstream gene *NRP1*, the mechanisms of EMT in UM have not yet been elucidated in this study. In the future, EMT in UM would be taken more research.

The abnormally expressed miRNA regulated by epigenetics participate in tumorigenesis, proliferation, invasion and metastasis by inhibiting or promoting the expression of downstream targeted genes in UM. In summary, our study successfully proved that the expression levels of miRNA-145 and miRNA-205 in UM cells were significantly lower than those in normal cells. Increased miRNA-145 expression could reduce the UM cell proliferation and the combined transfection with miRNA-145 and miRNA-205 could significantly suppress cell invasion. Moreover, we explored the mechanism underlying the miRNAs and showed miRNA-145 and miRNA-205 could down-regulate the expression of EMT-related protein CDC42 by suppressing its upstream gene *NRP1*, hampering migration of UM cells. Further study will be needed to develop drugs targeting miRNAs for new anticancer strategies of UM.

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