Melanoma is the most aggressive skin cancer, and accounts for the major part of skin cancer-related deaths in the world. In addition, the underlying mechanism of tumor progression in melanoma remains far from being elucidated. In this study, we have evaluated the function of miR-25 in melanoma. First, we examined the expression of miR-25 in four melanoma cell lines (A875, MV3, M14 and uacc-257) and in a normal melanocyte cell line (HEM-a). Then, we overexpressed miR-25 in M14 cells. Our results show that miR-25 promotes M14 cell proliferation and migration. We found that miR-25 up-regulates the PI3K/Akt/mTOR signaling pathway in these tumor cells. Furthermore, a luciferase-based reporter gene assay showed that miR-25 could directly target the RNA-binding motif protein 47 (RBM47). Taken together, our findings suggest that RBM47 is a promising target for the treatment of melanoma.

Key words: melanoma cell, migration, miR-25; RBM47; proliferation.

Introduction

Skin cancer is one of the most common cancer in the world. Basal cell carcinoma, squamous cell carcinoma and melanoma are all skin cancers [1-3]. Melanoma rarely occurs and only accounts for 2.3% of all skin cancers, but it is the most dangerous. 75% of skin cancer-related deaths are caused by melanoma [4]. Melanomas are usually related to ultraviolet radiation and arise in skin. Superficial spreading, acral lentigious, nodular and lentigomaligna melanomas are four clinical categories [5]. Despite major surgery can remove melanoma successfully, the identity of primary melanoma cells invading surrounding tissues and the treatment efficacy for metastatic melanoma are poor [6, 7]. In addition, cancer cells always develop drug resistance to clinical treatments. Hence, there is an urgent need to find novel strategies to ameliorate melanoma treatment.

Many studies have shown that miRNAs regulate various processes in tumorigenesis, including cell proliferation, migration and invasion. miR-25 has been found significantly overexpressed in various tumors [8, 9]. Previous studies have demonstrated that miR-25 promotes cancer cell proliferation, migration and invasion by regulating the expression of various protein in tumor-associated signaling pathways [10-13]. However, another study demonstrated that miR-25 suppresses cell growth and mobility of osteosarcoma via targeting SOX4 [14]. In addition, other studies have shown that miR-25 inhibits cell apoptosis in various cancers, including gastric adenocarcinoma, lung cancer and pancreatic cancer [15-18]. Thus, despite many reports have demonstrated the effects of miR-25 in tumorigenesis, the involvement and impact of miR-25 in the progression of melanoma remain unexplored in details.

RNA binding motif protein 47 (RBM47) is critical for posttranscriptional regulation of RNA during embryonic development and tumor progression. Recent studies have shown that RBM47 exerts diverse effects in embryonic development. It has been reported that knockdown of RBM47 gene causes headless phenotype during zebrafish head development [19]. In addition, RBM47 contributes to somatic cell reprogramming through the control of alternative splicing (AS) or binding to transcription factors [20, 21]. RBM47 is considered to be an epithelial cell state-associated gene and its down-regulation increases the metastatic traits of colorectal, breast and lung cancer [22-24]. RBM47 also inhibits cancer cell growth through the targeting of Nrf2 in lung adenocarcinoma [25]. So far, the relationship between RBM47 gene and melanoma development is unclear.
In the present study, we have investigated the function of miR-25 in melanoma by targeting RBM47. First, we evaluated the expression of miR-25 in melanoma cell lines (A875, MV3, M14 and UACC-257). Then we overexpressed miR-25 in M14 cells. Our results show that miR-25 promotes M14 cell proliferation and migration. It also up-regulates the PI3K/AKT/mTOR signaling pathway in melanoma. We also demonstrate herein that RBM47 is a direct target of miR-25. All of these findings demonstrated that RBM47 might be a new drug target for the treatment of melanoma.

Materials and Methods

Cell culture
Human melanoma cell lines, M14, A875, MV3, uacc257 and the normal melanocyte HEM-a were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All cells were cultured in RPMI 1640 medium (Hyclone) containing 10% fetal bovine serum (Gibco), 0.1 mg/mL streptomycin and 100 U/mL penicillin (Sigma). Cells were cultured in a 5% CO2 incubator at 37 °C. Cells were used for further experiment while in their exponential growth phase. Cells were washed with PBS for 3 times and then treated with trypsin (Solarbio), after that resuspended cells were seeded into 6-well plates. The transfection was performed when cell density reached 80%.

Transfection
DNA transfection was performed by using Lipofectamine 2000 Transfection Reagent (Invitrogen) following the manufacturer’s instructions. Cells on 6-well plate were changed with fresh completed medium 2 hours before transfection. 10 µL Lipofectamine 2000 reagents were diluted in 250 µL serum-free medium, mixed well gently and incubated for 5 min at room temperature. 2.5 µg pCMV-MIR-miR25 plasmid and control vector pCMV-MIR plasmid (Negative control, NC) were diluted in 250 µL serum-free medium. Then diluted DNA was added into Lipofectamine 2000 reagent, mixed well and incubated for 30 minutes at room temperature. The medium of 6-well plates were discarded, and the cells were washed with PBS for 3 times. Then about 500 µL mixture solutions were added into 6-well plates. After 6 hours culture, the cells were changed to the complete medium. The following experiments were performed after 24 hours culture.

RNA extraction and quantitative real-time PCR assay
Total RNA was extracted by using Trizol reagent (CWBio) following the manufacturer’s instructions. 1 microgram of total RNA was used for cDNA synthesis. miR-25 expression was assessed by quantitative real-time PCR with the SYBR Green PCR Master Mix (CWBio) according to the manufacturer’s protocol. The qRT-PCR reactions were performed at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 sec and 60 °C for 45 sec, and, finally, maintained at 72 °C for 30 min. The U6 was used to normalize expression: U6 forward, 5’-CTCGCTTCG GCAGCACA-3’ and U6 reverse, 5’-AACGCTTCACGAATTTGCGT-3’. miR-25 primers were purchased from Guangzhou RiboBio Co. All reactions were performed in triplicate. Data analysis was performed according to the 2−ΔΔCt method.

Western blot
M14 melanoma cells were harvested 48 hours after transfection. Cells were washed with cold PBS and lysed in RIPA buffer (CWBio) with protease inhibitors.
was added into each well of the plate every 2–3 days. Cells were then fixed with 4% paraformaldehyde for 30 minutes and stained with 0.1% crystal violet for another 30 minutes. Cell clones were counted and analyzed after washing.

Transwell migration assay
M14 cells were resuspended in serum-free medium 24 hours after transfection. For the migration assay, 105 cells in 100 μL serum-free medium were seeded into the upper chamber. 500 μL complete medium was added to the bottom wells to stimulate migration or invasion. After incubation, the non-invasive cells in the upper chamber were removed with cotton swabs. The invading cells were fixed with 4% paraformaldehyde for 30 minutes and stained with 0.1% crystal violet for another 30 minutes. Cell clones were counted and analyzed after washing.

Dual-luciferase reporter assay
The wild-type (wt) or mutated RBM47 3’UTR were inserted into pmirGLO vector. The pmirGLO-RBM47 3’UTR (wt) or pmirGLO-RBM47 3’UTR (mut) vectors were co-transfected with pCMV-MIR-miR25 or with a negative control (pCMV-MIR) into M14 cells by lipofectamine 2000. Forty-eight hours after transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Cell Counting Kit-8 assay
M14 cells were cultured for 24 hours after transfection. The cells were then treated with trypsin, resuspended and counted. 1,000 cells were seeded into 96-well plates in 100 μL medium and cultured in a 5% CO2 incubator at 37 °C. Cell viability was tested every 24 hours. For each test, 10 μL CCK-8 (Solarbio) reagent was added into the wells and incubated for 1.5 hours at 37 °C. Finally, the absorbance (optical density, O.D.) was measured with a microplate reader at 450 nm.

Plate clone formation assay
M14 cells were resuspended and counted 24 hours after transfection. They were then seeded into 60 mm plates (500 cells/plate) in 5 mL pre-warmed medium. The plates were incubated for 2 weeks in a 5% CO2 incubator at 37 °C. During this period, complete medium (1 mL)
after miR-25 or NC plasmid transfection. The CCK8 assay showed that the proliferation of M14 cells transfected with miR-25 increased after 72 hours transfection compared to cells transfected with the control vector (P<0.05, Figure 2A). Similarly, the plate clone formation assay showed that the M14 cell clone number is significantly increased when miR-25 is overexpressed as compared with cells transfected with the control vector (P<0.05, Figure 2B). Thus, the overexpression of miR-25 promotes M14 cell proliferation.

A strong expression of miR-25 is associated with an increase in melanoma M14 cell migration

A common feature of cancer cells is their aberrant motility, leading to cell migration. To further investigate the effect of miR-25 in regulating cell motility, we performed Transwell assays to evaluate the role of miR-25 on M14 cell migration. As shown in Figure 3A and B, the migration ability of miR-25 transfected M14 cells was significantly increased as compared to the negative control group (P<0.05). These results suggest that miR-25 is able to increase the ability of melanoma cell migration.

miR-25 up-regulates the PI3K/Akt/mTOR signaling pathway in melanoma M14 cells

We then investigated which signaling pathway could be involved in the effects on proliferation and migration observed when miR-25 expression is increased in M14 melanoma cells. The PI3K/Akt/mTOR signaling pathway is an important regulator of physiological cell processes which include proliferation, motility, differentiation, metabolisms and cell death. Figure 4 shows that the activation of PI3K/Akt/
Discussion

Melanoma is the most aggressive skin cancers and is the first leading cause of skin cancer-related death in the world [4]. Advances in diagnosis and clinical therapy have enable to reach a 5-year survival of melanoma patients, but the therapy of metastatic melanoma is extremely limited [5]. In addition, melanoma progression still remains poorly understood. In order to find more effective therapeutic strategy for treating melanoma cancer, it is important to identify novel melanoma-related factors which are responsible for cancer incidence and progression and to unravel the underlying mechanisms. Hence, there is an urgent need to find novel factors associated with melanoma and strategies to improve melanoma treatment. Previous studies have shown that miR-25 plays a critical role in tumorigenesis and in gastric carcinogenesis. The expression of miR-25 in many gastric cancer patient tissues and plasma is significantly increased [29, 30]. Interestingly, cyclin-dependent kinase 6 (CDK6) plays a key role in mammalian cell proliferation and previous studies indicate that miR-25 regulates CDK6 directly in vascular smooth muscle cell, hence, inhibiting cell proliferation and ki-67 expression [31]. In addition, another cell cycle-related protein p27 is regulated by miR-25 negatively, and was shown to promote proliferation of osteosarcoma cells [32]. Abnormal expression of miR-25 in several types of cancer suggests its potential association with tumorigenesis. To date, the association between miR-25 and melanoma remains unclear. RNA binding motif protein 47 (RBM47) is critical for epithelial mesenchymal transition during embryonic development and tumor progression. RBM47 together with other factors such as PI3K/AKT/mTOR signaling pathway plays a critical role in melanoma cell proliferation and motility.
with its cofactor catalytic polypeptide-1 (APOBEC1) are required for cellular processes such as the transendothelial migration and the C to U RNA editing [33-36]. Our study indicates for the first time that miR-25 directly targeted to RBM47.

The A875, MV3, M14 and uacc257 melanoma cancer cells and the normal melanocytes HEM-a were selected to perform the present study. We first evaluated the expression level of miR-25 in the cells from the five cell lines (Figure 1). Our results showed that miR-25 expression is up-regulated in melanoma cancer cells compared with normal melanocytes. Since the expression level of miR-25 in M14 cells was the lowest among melanoma cell lines, we overexpressed miR-25 in these cells to further investigate miR-25 functions (Figure 1). We found that M14 cell proliferation is increased in cells transfected with a miR-25-encoding expression vector (Figure 2). Similarly, results of plate clone formation assay showed that the cell clone number was significantly increased with miR-25 overexpression (Figure 2). Western blots showed that a high amount of miR-25 is associated with the activation of the PI3K/Akt signaling pathway marked by an increase of the expression of phosphorylated Akt (p-Akt), phosphorylated mTOR (p-mTOR), Cyclin D1 and P70 (Figure 4). Finally, we showed that a high level of miR-25 is associated with an increase in melanoma cell M14 proliferation and motility by directly targeting and inhibiting RBM47 (Figure 5).
In conclusion, our study demonstrates that a strong expression of miR-25 in M14 cells is associated with an increased proliferation and migration by directly targeting and inhibiting RBM47. Inhibition of miR-25 may be therefore a candidate approach for treatment of melanoma and new drug targeting RBM47 is worthy of further study and exploration.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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