Original Research

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miR-5100 mediates migration and invasion of melanomatous cells in vitro via targeting SPINK5

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Abstract

The mechanism of miR-5100 and his target gene spink5 in the development of melanoma are rarely reported. Many data from targetscan and oncomine data base suggest that miR-5100 may regulate spink5 and mediate the biological behavior of melanoma. In this study, we used miR-5100's agomir and antagomir to interfere with the A375 cell line. Experimental data indicate that miR-5100 Agomir significantly reduces the expression of SPINK5, and miR-5100 Antagomir substantially increases the expression of SPINK5. Wound Healing assay showed that Compared with the control group, the healing distance of the SPINK5 inhibition group was shortened considerably, and the healing range of the SPINK5 overexpression group was substantially longer. Wound Healing assay showed that compared to the control group, migration of A375 cell line decreased during wound healing on high mRNA expression levels of SPINK5 and vice versa. Transwell assays further demonstrated that SPINK5 knockdown by Agomir significantly enhanced while SPINK5 overexpression markedly suppressed the invasion of A375 cells. Transwell assays also demonstrated that SPINK5 knockdown by Agomir significantly increased. In contrast, SPINK5 overexpression markedly suppressed the invasion of A375 cells. Western blot analysis showed that after overexpression of miR-5100, the expression of spink5 decreased, and the decrease of spink5 significantly promoted the expression of ERK1/2, p-ERK1/2, and p-AKT; the expression of spink5 increased after inhibiting the expression of miR-5100, and the expression of spink5 significantly inhibited ERK1/2, p-ERK1/2, p-AKT expression. Wound healing assay showed that compared to the control group, migration of A375 cell line decreased during wound healing on the MK-2206 group and SCH772984 group after using MK-2206, a p-Akt inhibitor, SCH772984, an ERK1/2 and p-ERK1/2 Inhibitor. Transwell assays suggest that compared with the control group, the invasive ability of groups MK-2206 and SCH772984 was significantly reduced. In conclusion, miR-5100 mediates migration and invasion of melanomatous cells in vitro via targeting SPINK5.

Keywords: miR-5100; SPINK5; melanomatous; migration; invasion

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Introduction

Malignant melanoma (MM) is a highly malignant tumor that originates from neurosaki melanocytes[1,2]. It has the characteristics of radon invasion, metastasis, and high lethality. In recent years, the incidence of MM has been increasing, and its annual growth rate is about 3% -5%[3]. Therefore, the regulatory mechanism of melanoma cell migration and invasion was explored, and the target of the treatment of migration and invasion was found to prolong the survival of melanoma patients, Improving the therapeutic effect of melanoma is of great significance[4].

microRNA is a class of small non-coding RNAs (17–25 nucleotides), which regulates the expression of mRNA in cells to play a biological role. Many recent studies have shown that microRNA plays an essential role in the occurrence and development of malignant tumors[5]. MicroRNAs have been used as biological markers and Potential for therapeutic targets. Many research reports indicate that miR-5100 is overexpressed in different nausea tumor tissues[6,7], which suggests that miR-5100 can be used as a biomarker to measure the prognosis of patients with malignant tumors[8], but it is unclear how it regulates target genes play a role in the migration and invasion of malignant tumors.

Serine Protease Inhibitor Kazal-type 5 (SPINK5) is closely related to the growth and development of epidermal cells, keratinogenesis, and proteolysis during terminal cell differentiation[9,10]. Deletion and mutation of the SPINK5 gene can cause skin barrier functional factors. In previous studies of head and neck squamous cell carcinoma, it was found that the expression of SPINK5 in cancer tissues is lower than that in adjacent tissues[11]. The lower the expression of SPINK5 in patients with head and neck squamous cell carcinoma, the more prone to regional lymph node metastasis[12]. The overall survival of lung cancer and breast cancer patients with high expression of SPINK5 is significantly higher than those with low expression of SPINK5[13,14]. The above studies suggest that SPINK5 may have the effect of inhibiting tumor migration and invasion. Oncomine analysis of SPINK5 gene expression in melanoma patients suggests the inhibitory effect of spink5 on melanoma (Figs.1A-C). The targetscan database predicts that spink5 may be one of the target genes of miR-5100, so their regulatory relationship in malignant melanoma cells has attracted our attention.

The purpose of our research is to verify the regulatory relationship between miR-5100 and SPINK5 and to study how the miR-5100/spink5 axis plays a role in the migration and invasion of melanoma cells, which will provide a therapeutic strategy for malignant melanoma.

Material and Methods

Cells

Human melanoma cell line A375, purchased from Kunming Cell Bank, Chinese Academy of Sciences.A375 cell line was cultured in RPMI 1640 medium containing 10% (volume fraction) calf serum under 5% CO_2 and 37℃.

Cells transfection

A375 cell line was cultured in RPMI1640 containing 10% fetal bovine serum. After passaging, log-phase cells were taken for transfection, 24h before transfection, adjust the cell density to 4×10^5/mL with DMEM (containing 10% FBS, 100 U/ mL penicillin, and 100μg/mL streptomycin) and inoculate it on 24-well plates. Prepare A and B solutions in two sterile EP tubes. Includes 1 μL Lipofectamine 2000, 50 μL serum-free culture medium, and place at room temperature 5 min; add 20 pmol/L miR-5100 agomir/antagomir to 50 μL serum-free medium

and mix well; Add 100 μL miR-5100 agomir or antagomir/lipofectamine complex to a 24-well plate containing A375 cells and shake gently. Cells were then cultured in a 37℃ and 5% CO_2 incubator for 48 h before subsequent experiments.

Migration and Transwell assays

A375 cells were seeded in a six-well plate. Approximately 48 h later, when cells were 95~100% confluent, cells were incubated overnight in RPMI 1640 without FBS. Wounding was performed by scraping through the cell monolayer with a 10-μL pipette tip. Medium and non-adherent cells were re-moved, and cells were washed twice with PBS, and fresh serum-free medium was added. Cells were permitted to migrate into the area of clearing for 24 h. Wound healing was photographed microscopically.

For Transwell migration assay, A375 cells in exponential growth were harvested, washed, and suspended in DMEM containing 20% FBS and 10 ng/mL EGF. Cells (5×10^5/200 μL) were seeded into polycarbonate membrane inserts (8 μm pore size) in 24-Transwell cell culture dishes. Cells were allowed to attach to the membrane for 30 min. The lower chamber was filled with 600 μL DMEM with 10 ng/mL EGF (R&D Systems, Minneapolis, MN, USA). Cells were permitted to migrate for 6 h. After the incubation, stationary cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed in 4% formal formalin and stained with Giemsa. The number of stained cells in three random fields was counted under an inverted microscope (×100) and photographed with Nikon TS100 (Tokyo, Japan).

RT-PCR assays

Total RNAs were isolated with TRIzol reagent (Invitrogen). Equal amounts of RNA (1 μg) from each sample were used for complementary DNA (cDNA) synthesis and quantitative PCR was performed on the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using RevertAid™ M-MuLV ReverseTransciptase (Promega) and 2× SYBR Green Master Mix and analyzed using StepOne Software v2.1 (Applied Biosystems). $2^{-\Delta\Delta CT}$ method was used to calculate gene expression levels. The following primers were used in the PCR: F：TCCAAGCAGAAGAAGAAGA; R：GCGGTATTCACTACACAAC.

Luciferase Assay

Remove the growth medium from the cells to be assayed. Rinse the cells twice in PBS buffer, being careful not to dislodge any of the cells. Add a minimal volume to cover the cells. (250 μL for a 60 mm dish) and incubate for a short period at room temperature. Scrape the attached cells free from the culture dish and transfer the cells and solution to a microcentrifuge tube. Centrifuge for 5 secs at 12,000 rpm to pellet the cell debris. Transfer the supernatant (cell extract) to a new tube and discard the pelleted cell debris. Mix 20 μL of room temperature cell extract with 100 μL room temperature Luciferase Assay Reagent. Place the reaction in a luminometer. Measure the light produced for 10 seconds. Record data results transfected cells. Record the activity of your groups transfection in light units (LU). The results of all the transfections will be discussed when all the data has been compiled.

Western blot analysis

Cells were lysed using RIPA and cellular lysates were centrifuged, and after removal of the pellet, the protein content of the lysate was determined using the BCA method. Western blotting assays were run as previously described. Antibodies against the following proteins were used: ERK1/2 (1:1000), p-ERK1/2 (1:1000), AKT (1:1000), and p-AKT (1:1000) (all from Sigma, St. Louis, MO, USA). After incubation with anti-HRP secondary antibody, the protein bands were visualized by ECL. Densitometry was performed using the ImageJ software.

Statistical analysis

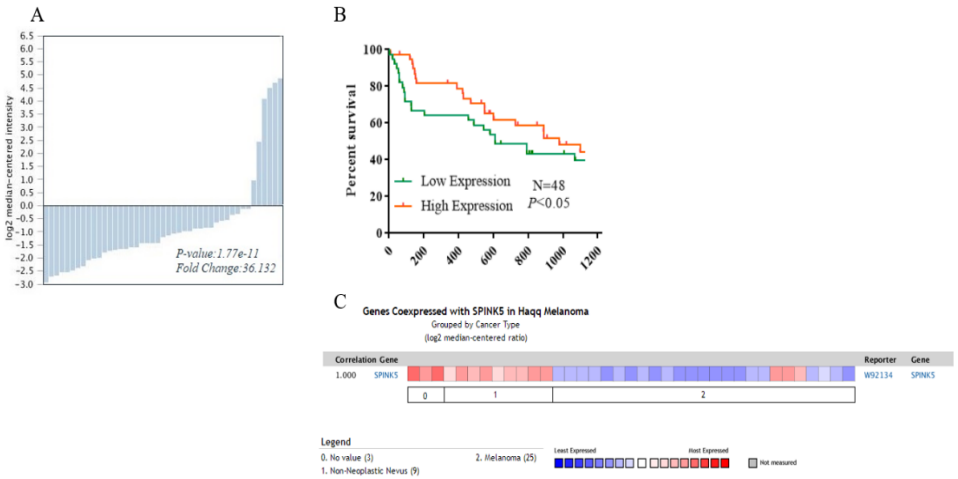

Figure 1 Analysis of SPINK5 gene expression in Melanoma patients with Oncomine, an online survival analysis platform.

All data were presented as mean±SD and analyzed by SPSS 22.0 software. The comparison was analyzed by ANOVA and post hoc paired t test if necessary. $P<0.05$ indicated significant difference.

Results

miR-5100 Targets Spink5 Expression

The targetscan database indicates that the 3′UTR region of SPINK5 is complementary to the sequence of miR-5100 (Figs.2A). qPCR experiments suggest that in the miR-5100 Agomir group, the expression of SPINK5 mRNA is lower than that in the control group. In the miR-5100 Antagomir group, the expression of SPINK5 mRNA is higher than that in the control group.

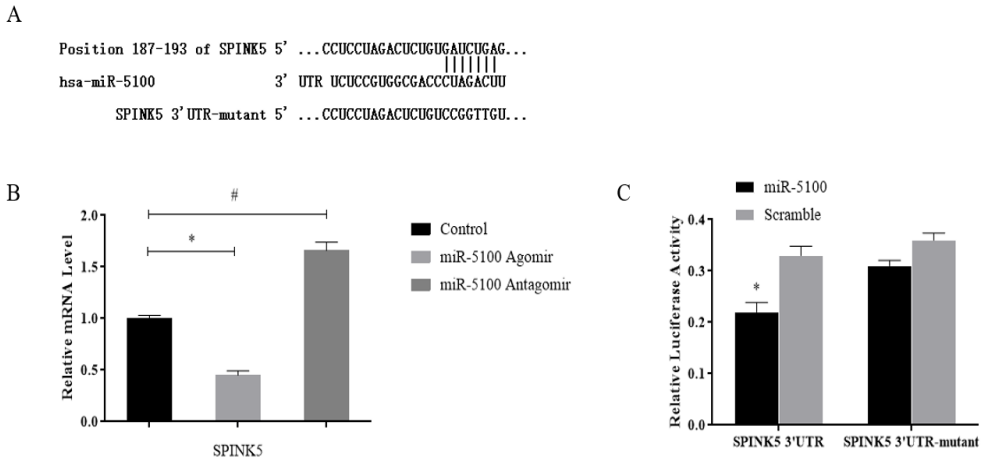

Figure 2 A) The targetscan database indicates that the 3′UTR region of SPINK5 is complementary to the sequence of miR-5100; B) qPCR experiments suggest that in miR-5100 Agomir group, the expression of SPINK5 mRNA is lower than that in the control group, and in miR-5100 Antagomir group, the expression of SPINK5 mRNA is higher than that in the control group. C) Luciferase experiments confirmed that miR-5100 could bind to the 3'UTR region of SPINK5 and inhibit protein expression. Mutant refers to a vector obtained by mutating the complementary binding sequence of miR-5100 in the 3'UTR area of SPINK5.

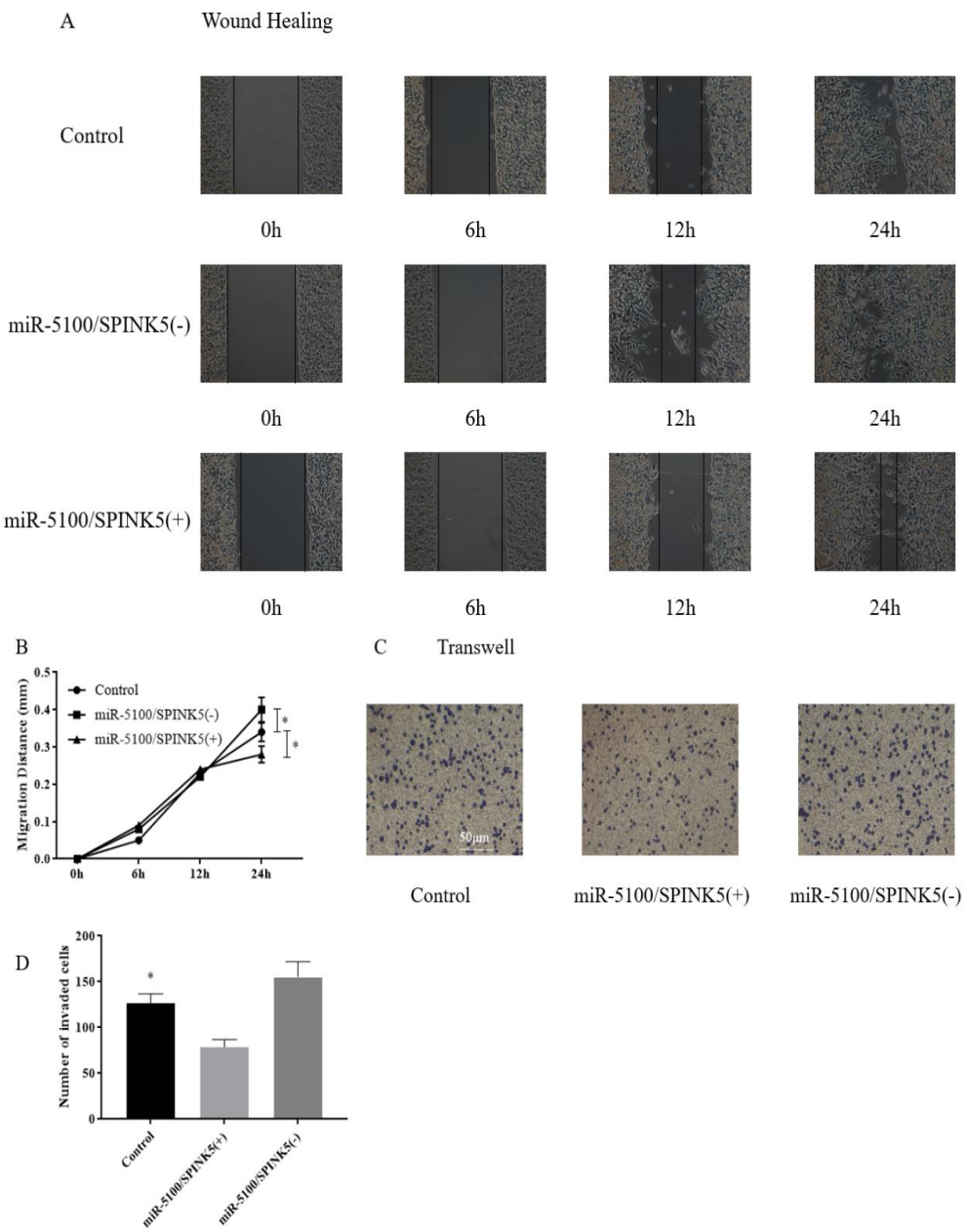

Figure 3 A), B) Wound assays in groups of Control, miR-5100/SPINK5(+), and miR-5100/SPINK5(-) at 0, 6, 12, and 24 h. (#P < 0.01 vs. Control, *P < 0.05 vs. Control) Data represent means ± SD (n=10). C), D) Transwell assays in groups of Control, miR-5100/SPINK5(+), and miR-5100/SPINK5(-) ($^{\#}P < 0.05$ vs. Control, $^{*}P < 0.01$ vs. Control) Data represent means ± SD (n=10).

Figs.2B. These results demonstrate that miR-5100 Agomir significantly reduces the expression of SPINK5, and miR-5100 Antagomir significantly increased the expression of SPINK5(Figs.2B). Luciferase experiment confirmed that miR-5100 could bind to the 3'UTR region of SPINK5 and inhibit protein expression. Mutant refers to a vector obtained by mutating the complementary binding sequence of miR-5100 in the 3'UTR area of SPINK5(Figs.2C). These data suggest that SPINK5 is a direct target gene of miR-5100.

SPINK5 mediates migration and invasion of melanoma tumor cells

Wound Healing assay showed that Compared with the control group, the healing distance of the SPINK5 inhibition group was significantly

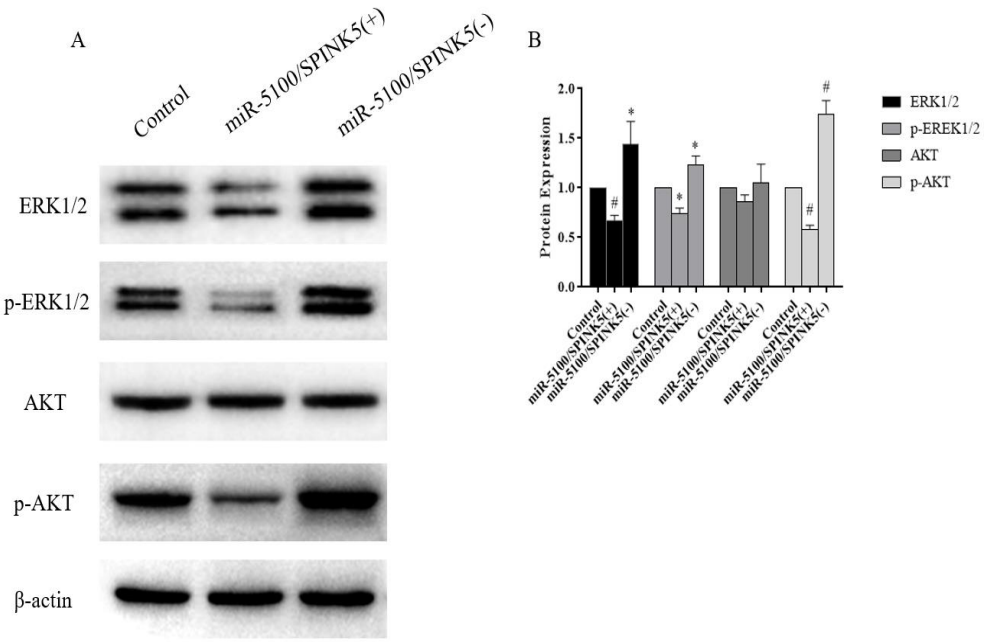

Figure 4 A), B) ERK1/2, p-ERK1/2, AKT, and p-AKT expression in groups of Control, miR-5100/SPINK5(+), and miR-5100/SPINK5(-) detected by Western blot. ($^{\#}P < 0.01$ vs. Control, $^{*}P < 0.05$ vs. Control) Data represent means ± SD.

shortened, and the healing range of the SPINK5 overexpression group was substantially longer. Wound Healing assay showed that compared to the control group, migration of A375 cell line decreased during wound healing on high mRNA expression levels of SPINK5 and vice versa (Figs.3A-B). Transwell assays further demonstrated that SPINK5 knockdown by Agomir significantly enhanced while SPINK5 overexpression markedly suppressed the invasion of A375 cells (Figs.3C-D).

miR-5100/spink5 axis works by promoting phosphorylation of ERK1/2 and AKT

Western blot analysis showed that after overexpression of miR-5100, the expression of spink5 decreased, and the decrease of spink5 significantly promoted the expression of ERK1/2, p-ERK1/2, and p-AKT; the expression of spink5 increased after inhibiting the expression of miR-5100, and the expression of spink5 significantly inhibited ERK1/2, p-ERK1/2, p-AKT expression (Figs.4A-B). The decrease of spink5 significantly promoted the expression of ERK1/2, p-ERK1/2, and p-AKT. Wound healing assay showed that compared to the control group, migration of A375 cell line decreased during wound healing on the MK-2206 group and SCH772984 group after using MK-2206, a p-Akt inhibitor, SCH772984, an ERK1/2 and p-ERK1/2 Inhibitor. Transwell assays suggest that compared with the control group, the invasive ability of groups MK-2206 and SCH772984 was significantly reduced (Figs.5C-F).

Discussion

Melanoma is an aggressive skin malignant tumor that originates from dysentery pigment cells. The 5-year survival rate of patients with malignant melanoma is only 16%[15]. In recent years, the incidence of melanoma has been increasing, and in-depth research on the molecular mechanism of melanoma occurrence and development will help melanoma diagnosis and treatment drug development[16,17].

microRNAs, as non-protein-encoded small RNAs, can bind to RNA-mediated silencing complexes,

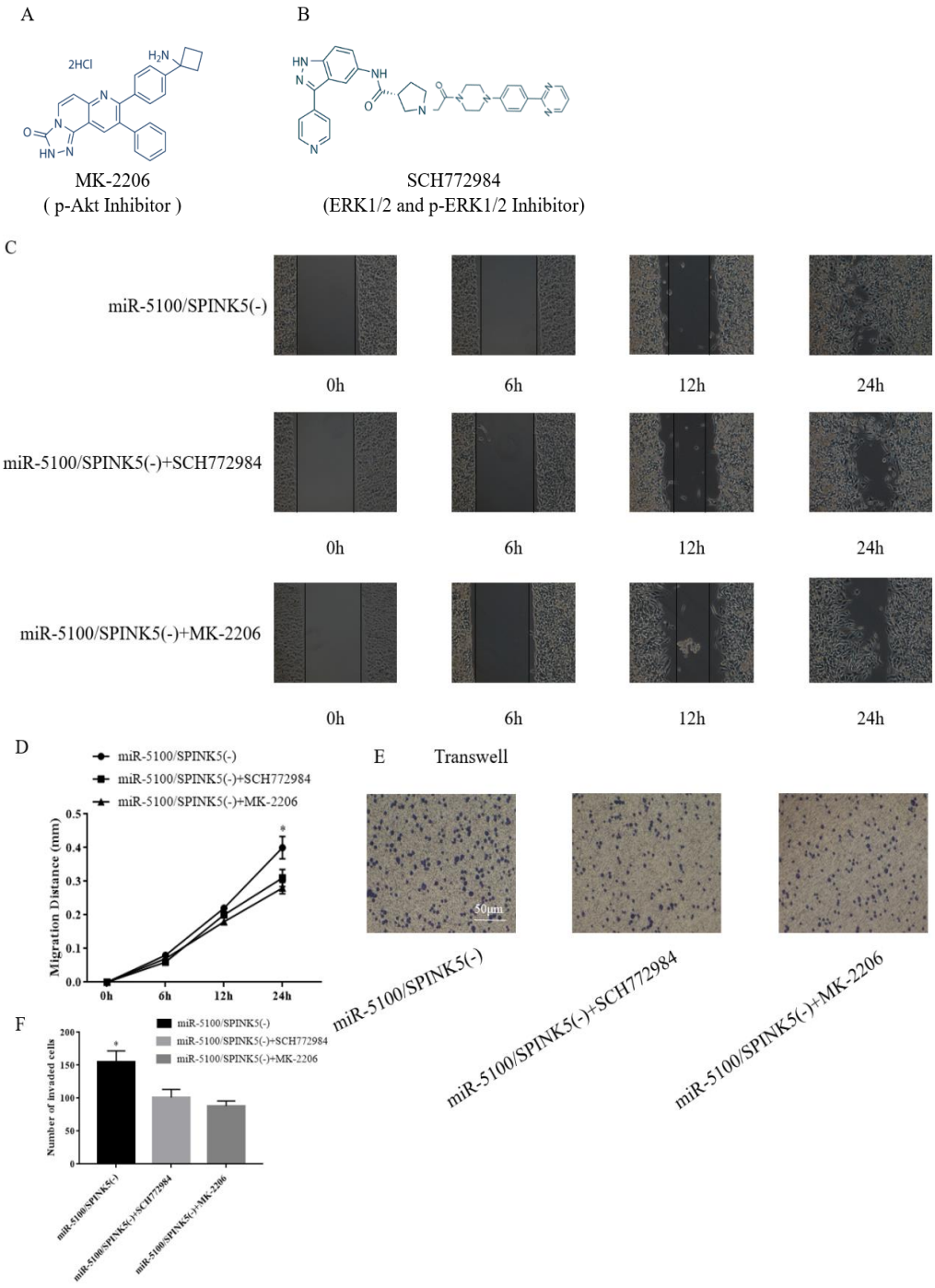

Figure5 A) Chemical structure of MK-2206 (p-Akt Inhibitor); B) Chemical structure of SCH772984 (ERK1/2 and p-ERK1/2 Inhibitor). C), D) Wound assays in groups of miR-5100/SPINK5(-), miR-5100/SPINK5(-) +SCH772984, and miR-5100/SPINK5(-) +MK-2206 at 0, 6, 12, and 24 h. ($^{*}P < 0.05$ miR-5100/SPINK5(-) vs. miR-5100/SPINK5(-) +SCH772984 and miR-5100/SPINK5(-) +MK-2206) Data represent means ± SD (n=10). E), F) Transwell assays in groups of miR-5100/SPINK5(-), miR-5100/SPINK5(-) +SCH772984, and miR-5100/SPINK5(-) +MK-2206 ($^{*}P < 0.01$ miR-5100/SPINK5(-) vs. miR-5100/SPINK5(-) +SCH772984 and miR-5100/SPINK5(-) +MK-2206) Data represent means ± SD (n=10).

inhibit translation through the microRNA discrimination of 3′-UTR of target mRNAs, and play an essential role in regulating gene expression[18]. How microRNA participates in tumorigenesis and development in the role of oncogene and tumor suppressor gene is a hot spot in the field of tumor research[19]. Reports are suggesting that miR-5100 expression is significantly increased in patients with oral squamous cell carcinoma and that miR-5100 levels are inversely related to patient prognosis[12]. The appearance of miR-5100 is increasing in non-small cell lung cancer[20]. However, the mechanism of miR-5100 in malignant melanoma is rarely reported.

The SPINK5 gene is located on human chromosome 5q31-32 and is 61kb in size[21]. SPINK5 is mainly expressed in the granular layer of normal skin epidermis and plays an essential role in maintaining skin barrier function, morphological hair development, anti-inflammatory, and anti-microbial invasion of mucosal epithelium. During tumorigenesis and development, there are specific differences in gene expression between tumor tissue and normal tissue[22]. The difference in expression of SPINK5 in different types of squamous cell carcinoma is related to its role[23]. The expression level of SPINK5 in bladder squamous cell carcinoma is higher than that in normal urothelial tissues, suggesting that it may play an oncogene role in bladder cancer[24]. In head and neck squamous cell carcinoma and oral squamous cell carcinoma, its expression is reduced, which suggests that SPINK5 may act as a tumor suppressor gene[25]. Skin benign pigmented nevus and malignant melanoma originate from melanocytes, and malignant changes of pigmented nevus may progress to melanoma.

According to the prediction of Targetscan database, we verified the regulatory relationship between miR-5100 and SPINK5 in luciferase experiments. SPINK5 was found to significantly inhibit the migration and invasion of A375 cells in wound healing and transwell assays. Mounting evidence supported that the migration and invasion of malignant melanoma are strictly related to the phosphorylation of ERK1/2, AKT, and the data from this study also confirmed this. Our data show that inhibition of spink5 can significantly promote the phosphorylation of ERK1/2 and AKT, and the use of specific inhibitors to inhibit the phosphorylation of ERK1/2 and AKT can dramatically impede the enhancement of migration and invasion activity caused by knocking out SPINK5.Overall, these observations suggested that miR-5100 mediates migration and invasion of melanomatous cells in vitro via targeting SPINK5.

Conclusion

In this study, we used the human malignant melanoma cell line A375 to verify the regulatory relationship between miR-5100 and SPINK5 for the first time and verified that the miR-5100/SPINK5 axis interacts with ERK1/2 and AKT during the migration and invasion of malignant melanoma. The relationship between ERK1/2 and AKT phosphorylation, it was found that the miR-5100/SPINK5 axis promotes the phosphorylation of ERK1/2 and AKT, thereby mediating the migration and invasion of malignant melanoma. These findings suggest that miR-5100 may be a potential biomarker and therapeutic target for MM therapy.

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Disclosure statement

The author declares that there are no competing interests.

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