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Original Article

The expression of miR-211 in cutaneous melanoma and the effect of its overexpression on the proliferation and migration of A375 melanoma cells and on VEGF expression

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Abstract: Objectives: To explore the role and potential mechanisms of miR-211 in human cutaneous melanoma. Methods: The expressions of miR-211 in the human A375 cutaneous melanoma cells line and human cutaneous melanoma tissues were quantified using qPCR. The effects of miR-211 mimics on the proliferation and migration of the A375 cells was determined using CCK-8 and Transwell assays. We examined the influence of the miR-211 mimics on the VEGF expression in the A375 cells using Western blot. Results: The qPCR results showed that the miR-211 expressions in the A375 cells and the human cutaneous melanoma tissues were significantly lower than they were in the control group ($P < 0.001$). The miR-211 mimics significantly inhibited the proliferation and migration abilities of the A375 cell line ($P < 0.05$), and at the same time, the miR-211 mimics significantly decreased the VEGF expression. Conclusions: The expression of miR-211 is down-regulated in human cutaneous melanoma, and miR-211 overexpression significantly inhibits the proliferation and migration abilities of cutaneous melanoma cells, which may be related to the down-regulation of VEGF.

Keywords: MiR-211, proliferation, migration, cutaneous melanoma, VEGF, A375 cell line

Introduction

Cutaneous melanoma is a common type of skin and mucosa cancer. According to the epidemiological studies, the incidence of human cutaneous melanoma is increasing at an average annual rate of 3%-5% [1, 2]. In recent years, with the development of science and technology, our ability to diagnose and treat cutaneous melanoma has made great progress. However, studies have reported that the therapeutic prognosis is still unsatisfactory, and the mortality rate remains high [3] due to the cancer's rapid onset and high rate of metastasis [4]. Cutaneous melanoma seriously threatens patients' physical and psychological health.

Angiogenesis plays an important role in the development of cutaneous melanoma [5, 6]. It is reported that the expression of vascular endothelial growth factor (VEGF) is closely associated with the metastasis and prognosis of cutaneous melanoma [7, 8]. Recently, microRNAs have attracted much attention in tumor research. Many studies have reported that microRNAs are closely linked with tumors in their proliferation and metastasis, and in the creation of new blood vessels and play a very critical role in the occurrence and development of tumors such as cutaneous melanoma [9, 10]. Some studies indicate that the down-regulation of miR-211 expression in ovarian carcinoma tissues is related to the tumors' growth and progression [11]. Moreover, miR-211 also has a great effect on tumor angiogenesis in breast cancer [12]. Thus, it is clear that miR-211 could play a significant role in tumor suppression.

In order to confirm the effects of miR-211 on the cells' proliferation and migration and on the VEGF expression in cutaneous melanoma, we measured the expressions of miR-211 in the

A375 cutaneous melanoma cell line and in cutaneous melanoma tissues. Then we transfected the miR-211 mimics into the A375 cutaneous melanoma cell line using liposome to investigate the possible mechanisms of miR-211 in the development of human cutaneous melanoma. The results of this research may provide an experimental foundation for the targeted therapy of cutaneous melanoma.

Materials and methods

Cell lines

The human melanocytes line HEMn and the A375 human cutaneous melanoma cell line were prepared for this experiment. The cell lines were obtained from the American Type Culture Collection and cultured in DMEM with 10% fetal bovine serum. The A375 human cutaneous melanoma cell line was divided into the control group and the miR-211 mimics group. The cells in the miR-211 mimics group were transfected with miR-211 mimics using liposome2000 according to the operational instructions. The cells in the control group were transfected with an miRNA mimics negative control.

Experimental specimens

Among the specimens we used, the fresh human cutaneous melanoma tissues were from patients who underwent cutaneous melanoma operations. Patients who underwent cutaneous nevus operations were selected as the control group. All the diagnoses were confirmed through pathological examinations. The patients gave their informed consent to our use of the clinical samples before the experiment began, and this study was approved by our hospital's ethics committee.

Reagent and instrument

The high glucose medium DMEM, trypsin, and fetal bovine serum were obtained from the American company Gibco. The rabbit-anti-human VEGF and GAPDH antibodies were purchased from the American company Santa Cruz. The goat anti-rabbit HRP-labeled secondary antibody was obtained from the American company Abcam. The CCK-8 and the crystal violet were obtained from the American company Sigma. The Transwell chambers were obtained from American Corning Costar Company. The qRT-PCR kits were obtained from the American company Roche. The Lipofectamine TM 2000 transfection reagents and the Trizol Regent were obtained from the American company Invitrogen. The RNase A was purchased from the American company Boehringer Mannheim. The RNA extraction kits were purchased from Thermo Fisher Scientific, and the miR-211 was purchased from the American Applied Biosystems Company. The gel imaging system was obtained from Bio-Rad. The inverted microscope was obtained from Nikon. The real-time PCR instrument was obtained from ABI.

qPCR analysis of the miR-211 expressions in the human cutaneous melanoma cell lines and the human cutaneous melanoma tissues

The total miRNAs of the human cutaneous melanoma cell lines and the human cutaneous melanoma tissues were extracted according to the instructions of the one-step method miRNAs kit. The above miRNAs were reversely transcribed into cDNA using TaqMan MicroRNA reverse transcription test kits, and we added miR-211 or internal reference U6 primers for amplification. The primers were designed as follows: miR-211 upstream primer: ATCGTCCAGTTTTCCCAGG-3', downstream primer: 5'-CGC CTCCACACACTCACC-3'. Internal reference U6 upstream primer: ATTGGAACGATACAGAGAAGATT-3', downstream primer: 5'-GGAACGCTTCACGAATTTG-3', The reaction system: upstream and downstream primers were 0.3 μL, SYBR Green Real-time PCR Master Mix 10 μL, cDNA 1.8 μL, ddH_2O 7.6 μL. The reaction requirement: pre-denaturation, 10 min at 95°C, denaturation, 15 s at 95°C and annealing/extension 55 s at 60°C, for a total of 35 cycles. After the completion of PCR, the corresponding Ct value was obtained using ABI 7300 System software by analyzing the gene amplification conditions, and the copy number of the PCR template was corrected using U6 as an internal reference, and finally the relative gene expression amounts were calculated using the 2-ΔΔCt method.

The protein expression of VEGF determined using Western blot

When the rate of cell fusion reached 75%, the miR-211 mimics were transfected into the cells

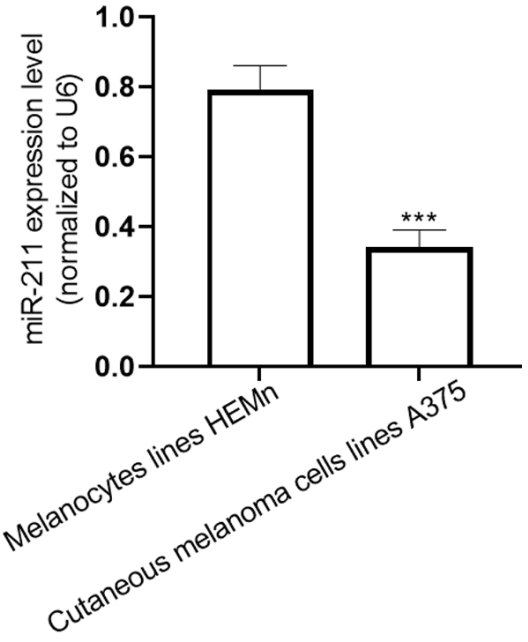

Figure 1. The expression of miR-211 in the A375 cutaneous melanoma cell line, compared with the melanocytes line HEMn, ***P < 0.001.

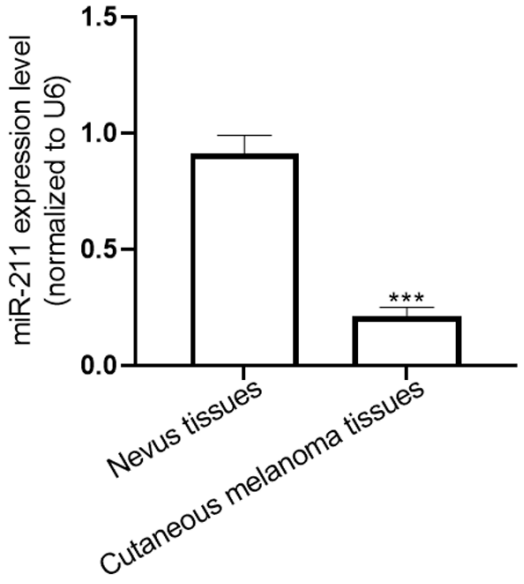

Figure 2. The expression of miR-211 in human cutaneous melanoma tissues. Compared with nevus tissues, ***P < 0.001.

using the Lipo2000 transfection reagent. The protein lysis buffer RIPA was added after the transfection, and the cell total protein was extracted. The BCA method was used to determine the protein concentration. Then, SDS-

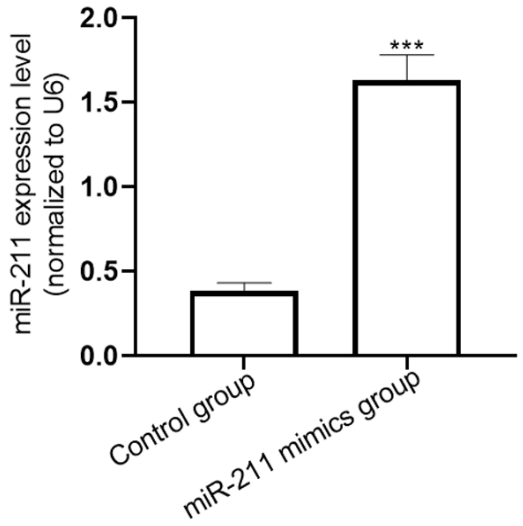

Figure 3. miR-211 expression in A375 cells transfected with miR-211 mimics. Compared with the control group, ***P < 0.001.

PAGE gel electrophoresis was performed. The protein samples were transferred to the PVDF membrane. Next, the membrane was placed in the TBST of 5% non-fat milk powder, and blocked at room temperature for 1 hour. The VEGF antibodies (1:500) were incubated overnight in the shaker at 4°C. After they were rinsed three times, the PVDF membranes were placed into goat anti-rabbit HRP-labeled secondary antibody (1:1000) to incubate at room temperature for an hour. Finally, ECL reagent was added to develop the color and the gel imaging system was used to image in the chemical exposure mode. The integrated optical density (IOD value) of the target brand was determined using the Bio-Rad imaging software. GAPDH was selected as the internal reference.

Measuring the proliferation of the A375 cells using CCK8

The cells in the exponential phase were selected for this experiment, and all the cells were made into a single cell suspension. The concentration of cells was $5*10^4$/ml and the cells were inoculated in 96-well plates, 200 μL per hole, 5 holes in each group. The culture medium was replaced every other day, and we used the CCK-8 method every 24 hours to measure the absorbance value of each

Table 1. A comparison of the proliferation of the A375 cells in the two groups

Group	24 h	48 h	72 h
Control group	0.40±0.03	0.82±0.07	1.51±0.10
miR-211 mimics group	0.37±0.02	0.76±0.06	1.35±0.08*
t value	1.861	1.455	2.794
P value	0.100	0.184	0.023

Note: Compared with Control group, *P<0.05.

group at the wavelength of 450 nm for 3 days.

Measuring the migration of the A375 cells using transwell assays

The A375 cells were inoculated into a 96-well culture plate. When the rate of cell fusion reached 70%, the cells were transfected by the miR-211 mimics through the Lipo2000 transfection reagent. The miR-211 transfected cells were selected as the observation group. Then the cells were digested using trypsin and were placed into a single cell suspension.

Next, these cells were inoculated into Transwell chambers, with 3 complex wells in each group. 100 μL serum-free DMEM culture medium was placed in the upper chamber. 500 μL DMEM culture medium containing 10% fetal bovine serum was placed in the lower chamber. After culturing for 24 hours in a 37°C incubator, the Transwell chambers were taken out and placed into a 0.1% crystal violet staining liquid for 6 min, and then they were washed three times using a PBS buffer solution. After that, the upper unmigrated cells were wiped off using cotton swabs. Under a lighted microscope, the observation fields were randomly photographed. The crystal violet was completely eluted using 33% acetic acid dehydrating, and then the OD value of the elution liquid was examined in a microplate reader at 570 nm.

Statistical methods

The statistical analysis of the data in this study was conducted using SPSS 21.0 software. The measurement data were presented as the mean ± standard deviation (SD). The comparisons between two groups were examined using t-tests. One-way ANOVA was used for the comparisons among three groups. The enumeration data were presented as percentages. The comparisons among groups were examined using chi-square tests. When $P < 0.05$, the difference was considered statistically significant.

Results

The expression of miR-211 in human cutaneous melanoma cells

The qPCR tests showed that the expression level of miR-211 in the A375 human cutaneous melanoma cell lines (0.34±0.05) was much lower than it was in the human melanocytes lines HEMn (0.79±0.07), and the difference was significant ($t=11.700$, $P < 0.001$), as shown in **Figure 1**.

The expression of miR-211 in human cutaneous melanoma tissues

The expression of miR-211 in the human cutaneous melanoma tissues was 0.21±0.04, which was lower than it was in the human nevus tissues (0.91±0.08). Significant differences were found ($t=17.500$, $P < 0.001$), as seen in **Figure 2**.

The measurement of the miR-211 expression after the miR-211 mimic transfection in the A375 cells

As shown in **Figure 3**, after the A375 cells were transfected with the miR-211 mimics, the expression of miR-211 was significantly increased, and significant differences were found (0.38±0.05 vs 1.63±0.15, $t=17.680$, $P < 0.001$).

The effects of the miR-211 mimics on the proliferation of the A375 cells

As shown in **Table 1**, the CCK-8 assays showed that the OD value of A375 in the miR-211 mimic group at 72 h after culturing was 1.35±0.08, which was lower than it was in the control group, and there was a significant difference between the two groups ($P < 0.05$). No significant differences were found between the two groups at 24 h and 48 h after the culturing.

The effects of the miR-211 mimics on the migration of the A375 cells

The Transwell assays showed that the OD value for the migrating cells in the miR-211 mimics group was 0.69±0.09, but in control

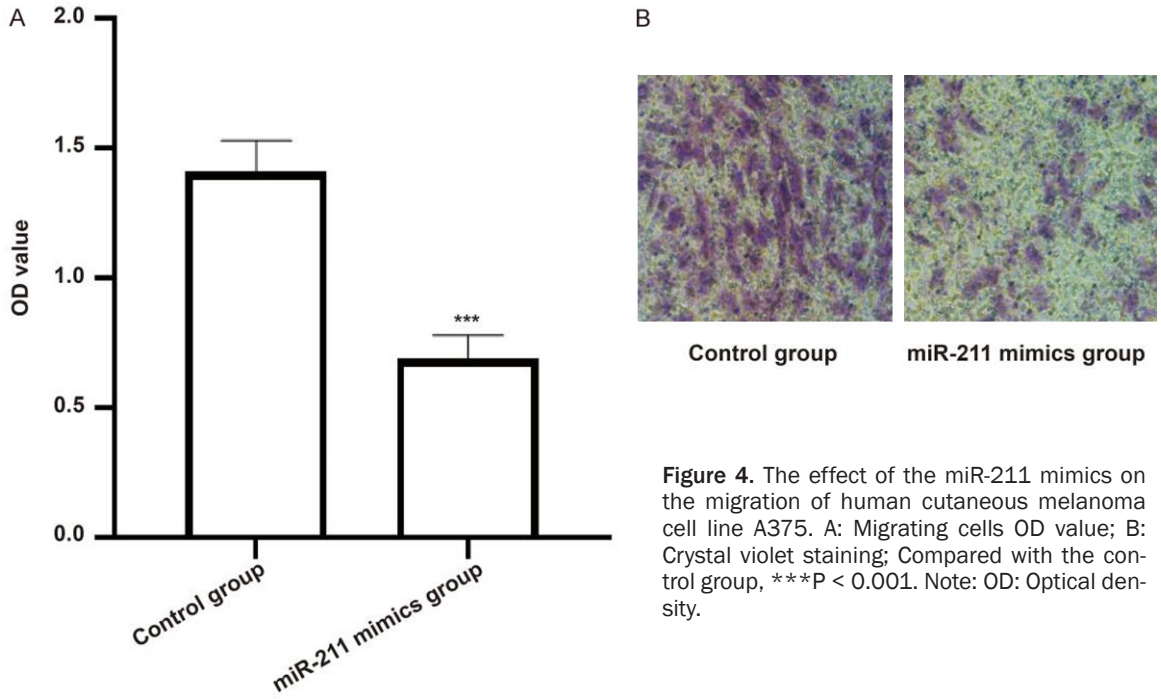

Figure 4. The effect of the miR-211 mimics on the migration of human cutaneous melanoma cell line A375. A: Migrating cells OD value; B: Crystal violet staining; Compared with the control group, ***P < 0.001. Note: OD: Optical density.

group it was 1.41±0.12, and there was a significant statistical difference (t=10.730, P < 0.001), as seen in **Figure 4**.

The effects of the miR-211 mimics on the VEGF expressions in the A375 cells

As shown in **Figure 5**, the miR-211 mimics significantly decreased the VEGF expressions in the A375 human cutaneous melanoma cell lines compared with the control group (0.65±0.07 vs 0.16±0.02, t=15.050, P < 0.001).

Discussion

In recent years, the morbidity of cutaneous melanoma has increased worldwide. The growth and metastasis of the cancer are the principal causes of death in patients diagnosed with cutaneous melanoma. Numerous studies show that tumor growth and metastasis are a complex biological process in which multi-factors interact with each other and influence one another, and this process might involve angiogenesis and the proliferation and migration of tumor cells [8, 13].

miRNAs have been considered a kind of highly conserved, endogenous non-coding small molecule RNA [14, 15]. miRNAs are involved in tumor angiogenesis and influence tumor growth and metastasis through the post-transcriptional inhibition of target gene expressions. In recent years, more and more miRNAs have been shown to be participating in tumorigenesis and tumor development [16]. It is suggested that miRNAs might act as a tumor suppressor gene or an oncogene in tumors.

Previous studies showed that miR-211 expressions in a variety of tumors is remarkably decreased [17, 18], but the role of miR-211 in cutaneous melanoma still remains unclear. To investigate the effects of miR-211 on the proliferation and migration of cutaneous melanoma cells, the expression of miR-211 was examined in the A375 cutaneous melanoma cell line and in cutaneous melanoma tissues using qPCR in this study. The effects of the miR-211 mimics on the proliferation and migration of the A375 cutaneous melanoma cell line were observed using CCK-8 and Transwell migration assays. The results showed that the expression of miR-211 in the A375 human cutaneous melanoma cell line was remarkably lower than it was in the melanocytes line HEMn (P < 0.001), but the miR-211 expression in the human cutaneous melanoma tissues was significantly lower than it was in human cutaneous nevi (P < 0.001). And the miR-211 overexpression significantly inhibited the proliferation and migration of the A375 cutaneous melanoma cell line (P < 0.05), indicating that the miR-211 expression negatively regulated

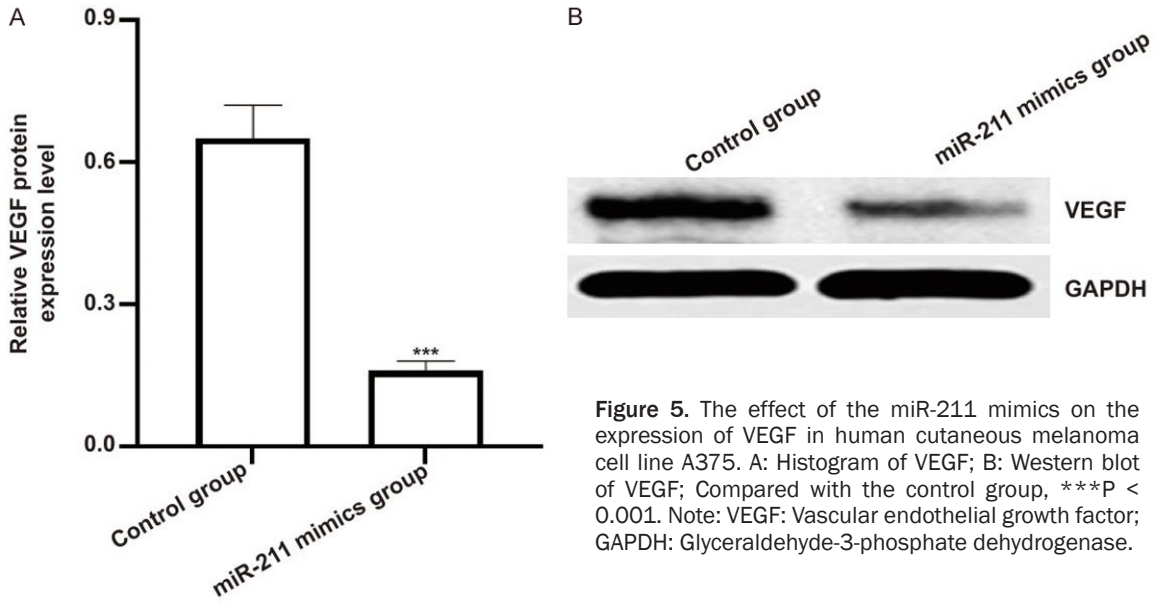

Figure 5. The effect of the miR-211 mimics on the expression of VEGF in human cutaneous melanoma cell line A375. A: Histogram of VEGF; B: Western blot of VEGF; Compared with the control group, ***P < 0.001. Note: VEGF: Vascular endothelial growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

the proliferation and migration of the A375 cutaneous melanoma cell line. This is consistent with the role of miR-211 in other types of tumors [19].

Tumor angiogenesis plays an important role in the growth and metastasis of human cutaneous melanoma [20]. VEGF is considered an important signal pathway molecule that regulates pathological and physiologic angiogenesis. It was reported that VEGF is expressed in about 32%-42% of primary melanoma cases and in about 91% of metastatic melanoma cases [21]. Simonetti et al. [22] reported that the five-year survival rate of tumor patients with high expressions of VEGF was only about 15%, and a high expression of VEGF indicates metastasis and poor prognosis. This shows that VEGF expression is related to the prognosis of tumor patients. Also, the results from the pathological sections of the human cutaneous melanoma tissues showed that the density and distribution of VEGF expression were positively associated with tumor involvement [23]. Previous studies have shown that miRNAs work in the development of human cutaneous melanoma by regulating VEGF expression [24]. In this study, it was shown using Western blot that miR-211 mimics can significantly down-regulate the expression of VEGF in human cutaneous melanoma cell lines. This suggests that miR-211 might affect the growth and metastasis of tumors through the expression of VEGF in human cutaneous melanoma, thus affecting patient prognosis.

In summary, on the one hand, our research showed that low expressions of miR-211 were found in the A375 human cutaneous melanoma cell line and in human cutaneous melanoma tissues. On the other hand, we confirmed the role of miR-211 expression in cutaneous carcinoma. MiR-211 overexpression significantly inhibits the proliferation and migration of human cutaneous melanoma cells and down-regulates the VEGF expression. We will undertake further investigations of the miR-211-regulated target genes and the related signaling pathways, so as to provide new ideas for the targeted treatment of human cutaneous melanoma.

Disclosure of conflict of interest

None.

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