

Original Article

LncRNA UBE2R2-AS1 promotes melanoma cell progression by targeting the TLR4/MyD88/NF- κ B axis

Yujie Ding, Xin Shi, Jiang Ji, Yuhua Su

Department of Dermatology, The Second Affiliated Hospital of Soochow University, Suzhou 215123, Jiangsu, China

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Abstract: Objective: The purpose of this study was to explain the effects and mechanisms of lncRNA UBE2R2-AS1 in melanoma development. Methods: We collected 30 samples of melanoma tumors and the adjacent normal tissues and measured the UBE2R2-AS1 mRNA expression using an RT-qPCR assay. We then transfected the UBE2R2-AS1 in B16 and SK-Mel-2, observed the cell proliferation, apoptosis, invasion and migration using MTT, flow cytometry, Transwell, and wound healing assays. Next, we discuss the relative mechanisms using WB and Cell immunofluorescence assays. Results: Compared with the adjacent normal tissues, the UBE2R2-AS1 mRNA expression was significantly depressed in the cancer tissues. With UBE2R2-AS1 overexpression, the cell proliferation, invasion, and migration abilities were significantly depressed, and the cell apoptosis was significantly increased. Meanwhile, the TLR4, MyD88 and NF- κ B (p65) protein expressions were significantly suppressed, and the NF- κ B (p65) nuclear volume was also significantly down-regulated. Conclusion: lncRNA UBE2R2-AS1 overexpression had anti-tumor effects on melanoma by regulating the TLR4/MyD88/NF- κ B (p65) pathway in this in vitro study.

Keywords: UBE2R2-AS1, melanoma, B16, SK-Mel-2, TLR4

Introduction

The most common and fatal skin tumor, there are 160,000 new cases of melanoma and 48,000 deaths worldwide every year, according to a survey conducted by *The Lancet* in 2015 [1]. Melanoma progresses rapidly, and its metastasis to distant lymph nodes or organs occurs in most patients within a short time, thereby leading to a high mortality [2]. Therefore, it is of great significance to study the mechanism of melanoma cell proliferation and metastasis and find new molecular targets, so as to improve the prognosis. Long non-coding RNA (lncRNA) has been a hot research topic in recent years. It regulates gene transcription or post-transcriptional expression mainly through epigenetic silencing, RNA splicing, RNA metabolism, chromatin remodeling and other mechanisms with more than 200 nucleotides [3, 4]. The latest studies have found that lncRNA is closely related to the occurrence and progression of tumors, for example, the up-regulated expression of lncRNA FALEC in melanoma tissues and cells is related to the

poor prognosis of melanoma [5], and the up-regulated expression of lncRNA-HEIH in melanoma cells can promote cell proliferation, invasion and metastasis [6]. lncRNA UBE2R2-AS1 is a newly discovered lncRNA [7, 8]. The current research results show that UBE2R2-AS1 is underexpressed in glioma, and it can effectively inhibit the biological activity of glioma cells after its expression is up-regulated [7]. So far, the expression and role of UBE2R2-AS1 in melanoma and its related mechanism have not been reported. In this study, the expression level of UBE2R2-AS1 in melanoma tissues and cells was measured to explore the effect of UBE2R2-AS1 on the proliferation and invasion of melanoma cells and its regulatory mechanism, so as to provide a reference for the diagnosis and treatment of melanoma.

Materials and methods

Tissue sample

30 samples of tumor tissues excised in our hospital between January 2015 and January 2019

were collected, with the melanoma confirmed by histopathology. A tissue sample ≥ 5 cm from the tumor in each patient was also collected as a normal adjacent tissue sample. The excised tissue samples were immediately frozen in liquid nitrogen and stored in a refrigerator kept at -80°C until the RNA extraction was performed. This study was approved by the ethics committee of the hospital, and all the patients had complete clinical data, and they signed an informed consent before the surgery, which were kept as a record.

Cell source and treatment

Human melanocyte HEM was purchased from ATCC, and the melanoma cell lines B16, SK-Mel-2, A375, C32 and SK-37 were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The HEM cells were inoculated on a melanocyte culture medium (MGM), and the B16, SK-Mel-2, A375, C32 and SK-37 cells were inoculated on a RPMI1640 culture medium. Both the MGM and RPMI1640 media contained 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, and they were cultured in the cell culture box at 37°C and 5% CO_2 .

Experiment reagents

HEM culture solution (PromoCell, Germany), RPMI1640 culture solution, pcDNA3.1, Trizol reagent (Invitrogen), UBE2R2-AS1 (Shanghai GenePharma Co., Ltd.), RevertAid First Strand cDNA Synthesis Kit (ThermoFisher), SYBR Green PCR Master Mix Kit (TaKaRa, Japan), MTT (Sigma), Annexin V Apoptosis Detection Kit, Matrigel (BD), Transwell Chamber [Corning (China) Co., Ltd.], RIPA cracking fluid, protease inhibitors (ThermoScientific), GAPDH antibodies, TLR4, MyD88, and NF- κB (p65) antibody (Abcam).

Cell transfection

The expression of UBE2R2-AS1 in the cells was up-regulated using PcDNA3.1, and the cells were inoculated on a 6-well plate 24 h before the transfection. When the cell fusion was about 30%-50% complete, UBE2R2-AS1 and pcDNA3.1 were added to the 250 μL DMEM culture solution respectively in accordance with the instructions of pcDNA3.1, and gently

mixed after quiescence at room temperature for 5 min, and then the 500 μL of the mixture was added to the 6-well plate for the subsequent experiments after 48 h of transfection.

RNA extraction, reverse transcription, and real-time PCR

RNA was extracted from the tissues and cells using Trizol reagent, and the concentration and purity of the RNA were determined using a UV spectrophotometer and stored at -80°C . The amplification primers were designed by Shanghai GenePharma Co., Ltd. The RNA was reverted to cDNA according to the specification of the RevertAid First Strand cDNA Synthesis Kit, and the reaction conditions were as follows: 20 min at 37°C , and 5 min at 95°C . Then the PCR reaction was conducted in accordance with the instructions of the SYBR Green PCR Master Mix Kit, and the total reaction system was 25 μL . The reaction conditions were: Initial denaturation at 95°C for 15 s, denaturation at 95°C for 5 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s, 35 cycles in total. The relative expression of each was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Cell proliferation assay

The cells were inoculated on 96-well plates and incubated at 37°C and 5% CO_2 for 24 h, 48 h, 72 h, and 96 h respectively. 10 μL MTT solution (5 mg/mL) was added to each well 2 h before each of the above time points, and 200 μL DMSO solution was added after further incubation for 4 h. The absorbance (A) value of each well was measured at 490 nm, and the cell proliferation rate of each group was calculated.

Transwell assay

The treated cells were washed with a serum-free medium three times, and then they were counted and prepared into a cell suspension. The Matrigel was washed in a serum-free medium once, and 200 μL of the cell suspension was added to each well. 500 μL of the conditioned medium containing 20% FBS was added to the lower chamber. After incubation in 37°C incubator for 24 h, the Transwell was removed and washed with PBS twice, and then it was fixed using 5% glutaraldehyde at 4°C . 0.1% crystal violet staining was added for 10 min at room temperature, and the cells on the upper

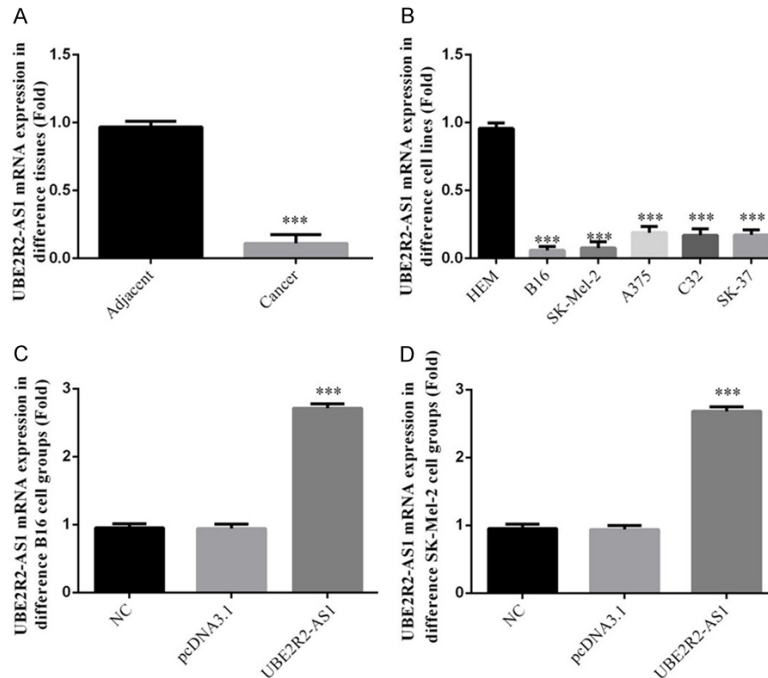


Figure 1. The UBE2R2-AS1 mRNA expressions in the melanoma tissues and cells. A. The UBE2R2-AS1 mRNA levels in the different tissues, ***: $P < 0.001$, compared with the adjacent normal tissues. B. The UBE2R2-AS1 mRNA expressions in the different cell lines, ***: $P < 0.001$, compared with the HEM cell line. C. The UBE2R2-AS1 mRNA levels of the different B16 cell groups, ***: $P < 0.001$, compared with the NC group. D. The UBE2R2-AS1 mRNA levels of the different SK-Mel-2 cell groups, ***: $P < 0.001$, compared with the NC group. NC group, the normal control group.

surface were washed with PBS twice. The cells on the upper surface were wiped off with a cotton ball, and the mean value of 9 random vision fields under a microscope at a magnification of 200 \times was recorded as the number of cells passing through the filter membrane, and the statistical data were recorded.

Wound healing

The wound healing insert was placed on a 24-well plate, and the transfected melanoma cells in the logarithmic growth stage were taken and adjusted to a cell quantity of 5×10^5 . The cells were cultured overnight (about 16 h) at 37°C and 5% CO₂. After being carefully removed, the wound healing insert was rinsed with a complete medium three times, and 10 μ mol/L mitomycin was added to continue the culture for 2 hours, and then it was replaced with a fresh complete medium, and photos were taken using a microscope at 0 h, and after continuing the culture for 24 h, and photos

were also taken at 48 h to analyze the cell migration.

Flow cytometry

The cells were digested with 0.25% pancreatic enzyme at 37°C for 5 min, and then mixed with PBS to prepare the cell suspension, which was centrifuged at 2,000 r/min for 5 min, and then the cells were collected, re-suspended, and combined with a buffer solution (cell density of 1×10^6 /mL), then 5 μ L Annexin V was added to the cell suspension and incubated in the dark for 15 min, and then 10 μ L PI was added to continue the incubation for 5 min, and the quantification was completed within 1 hour.

Western blotting

The cells were lysed using a RIPA solution, the total protein was extracted, and the protein concentration was determined using the BCA method. The 30 μ g total protein was isolated using 8% SDS-PAGE gel and then transferred to a PVDF membrane, and 5% skim milk was added and it was sealed for 2 h at room temperature; then, TLR4, MyD88, and NF- κ B antibodies (1:1,000) were added and incubated overnight at 4°C. The next day, horseradish peroxidase (HRP)-labeled goat anti-rat secondary antibody (1:1,000) was added and incubated at room temperature for 1 h. All bands were colored using ECL, and the gray value of the protein bands was determined using ImageJ scanning.

Cell immunofluorescence detection

After being inoculated on a confocal culture dish and being fixed with 4% paraformaldehyde at room temperature for 30 min, the melanoma cells in their logarithmic growth phase were subject to 0.2% Triton X-100 cytomembrane breaking for 15 min, and then they were

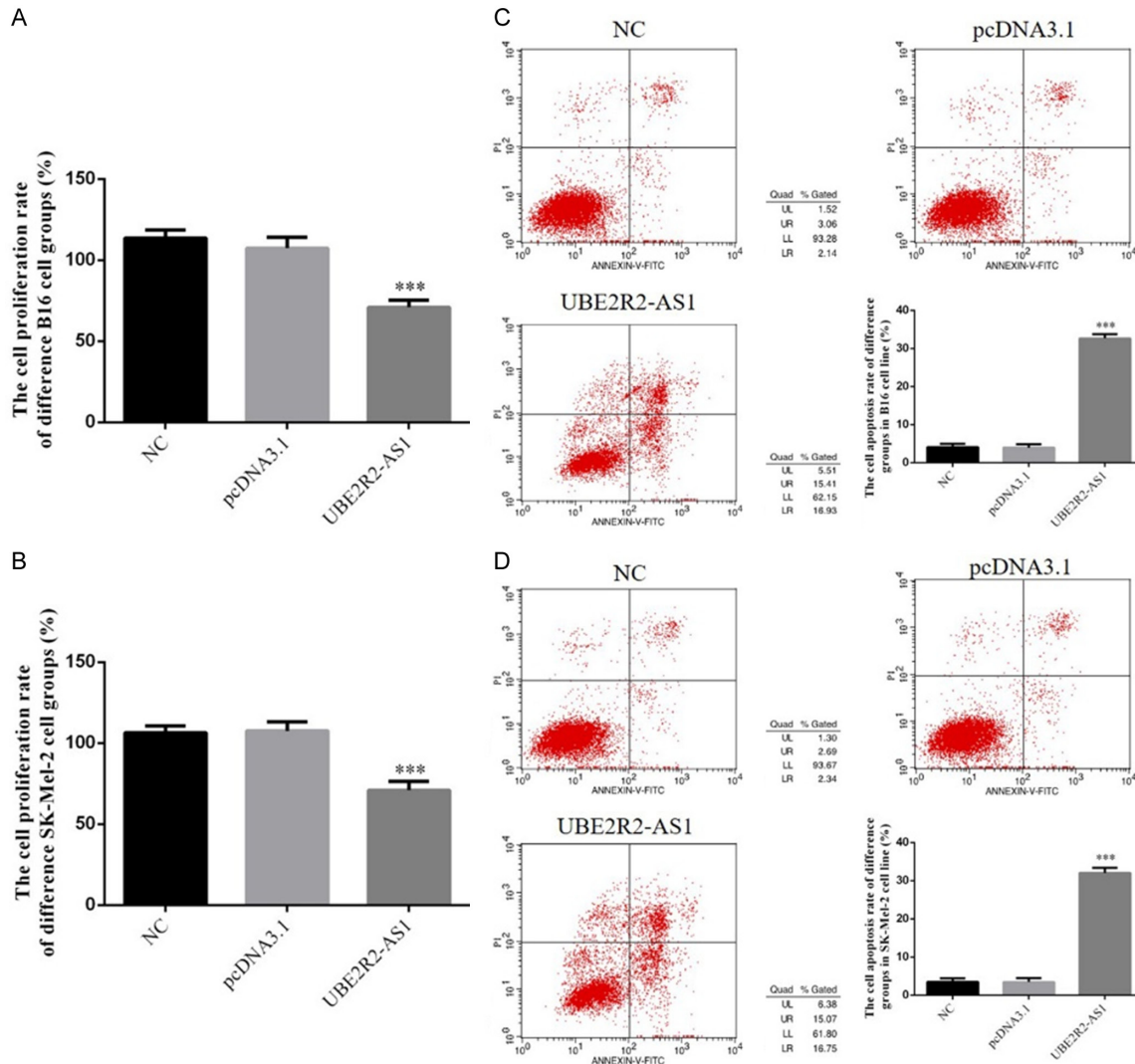


Figure 2. The effect of the overexpression of UBE2R2-AS1 on the proliferation and apoptosis of melanoma cells. A. The B16 cell proliferation rate of the different groups using an MTT assay, ***: $P < 0.001$, compared with the NC group. B. The SK-Mel-2 cell proliferation rate of the different groups using an MTT assay, ***: $P < 0.001$, compared with the NC group. C. The apoptosis rate of the different B16 cell groups, ***: $P < 0.001$, compared with the NC group. D. The apoptosis rate of the different SK-Mel-2 cell groups, ***: $P < 0.001$, compared with the NC group. NC group, the normal control group.

sealed with 5% newborn bovine serum for 1 h, and cultured at 37°C for 18 h after being mixed with NF- κ B (1:100). On the next day, the corresponding fluorescent second antibody (1:200) was added for incubation in a dark environment at 37°C for 3 h. After the cells were washed with PBST, the cell nuclei were stained with 5% 4', 6 amidine-2-phenyl indole. Finally, the anti-fluorescence quenching agent was added to observe and collect the images under a confocal microscope. Image J Image analysis software was used to analyze the nuclear uptake of NF- κ B in each group.

Statistical analysis

SPSS 19.0 and GraphPad Prism 7.0 software were used for the data processing. The measurement data were expressed as the mean \pm SD, and the comparisons of the means among multiple groups were made by means of one-way ANOVA, and the comparisons between two groups were done using Student's *t* test. A Pearson correlation analysis was employed to test the correlations between UCA1 and p21 in the melanoma tissues. $P < 0.05$ indicated that the difference was statistically significant.

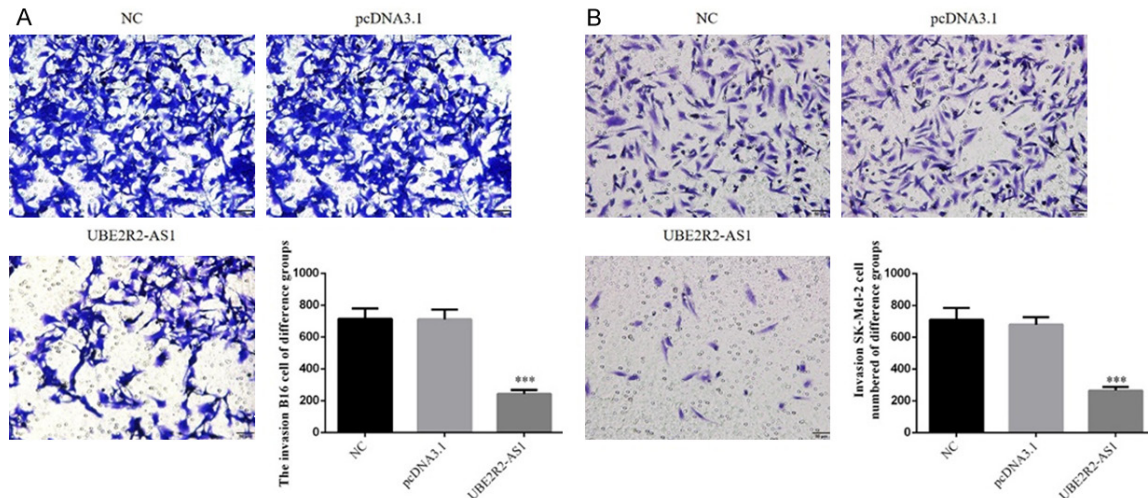


Figure 3. The effect of the overexpression of UBE2R2-AS1 on the invasion of the melanoma cells. A. The invasion B16 cell number of the different groups using a transwell assay (200×), ***: P<0.001, compared with the NC group. B. The invasion SK-Mel-2 cell number of the different groups using a transwell assay (200×), ***: P<0.001, compared with NC group. NC group, normal control group.

Results

The expression of UBE2R2-AS1 in the melanoma tissues and cells

The expression of UBE2R2-AS1 in the melanoma tissues was significantly lower than it was in the adjacent normal tissues (P<0.001, **Figure 1A**). Thus, the expression of UBE2R2-AS1 in the melanoma cell lines was significantly lower than it was in normal melanocyte HEM (P<0.001, respectively, **Figure 1B**). Among them, its expressions in B16 and SK-Mel-2 were the lowest; therefore, the B16 and SK-Mel-2 cells were selected as the research objects in this study. In the subsequent cell experiment, after being transfected into the B16 and SK-Mel-2 cells, the expression level of the UBE2R2-AS1 gene was significantly increased in the UBE2R2-AS1 group compared with in the NC group (P<0.001, respectively, **Figure 1C** and **1D**).

The effect of the overexpression of UBE2R2-AS1 on the proliferation and apoptosis of the melanoma cells

After UBE2R2-AS1 was transfected into the B16 and SK-Mel-2 cells using pcDNA3.1, the results showed that the proliferation activity in the UBE2R2-AS1 group with the overexpressed UBE2R2-AS1 was significantly lower than it was in the pcDNA3.1 group (P<0.001, respec-

tively, **Figure 2A** and **2B**). The results of the flow cytometry showed that the apoptosis rate in the UBE2R2-AS1 group was significantly higher than it was in the pcDNA3.1 group (P<0.001, respectively, **Figure 2C** and **2D**).

The effect of the overexpression of UBE2R2-AS1 on the invasion of melanoma cells

The Transwell experiment showed that, compared with the pcDNA3.1 group, the UBE2R2-AS1 group with over-expressed UBE2R2-AS1 had a significantly reduced number of transmembral cells, and the difference was statistically significant (P<0.001, respectively, **Figure 3A** and **3B**).

The effect of the overexpression of UBE2R2-AS1 on the migration of the melanoma cells

The results of the wound healing assay showed that, compared with the pcDNA3.1 group, the wound healing rate of the UBE2R2-AS1 group with the overexpressed UBE2R2-AS1 was significantly inhibited at 24 h and 48 h, and the difference was statistically significant (P<0.001, respectively, **Figure 4A** and **4B**).

The effect of the overexpression of UBE2R2-AS1 on the associated proteins

The Western blots showed that the expression levels of TLR4, MyD88, and NF-κB (p65) were

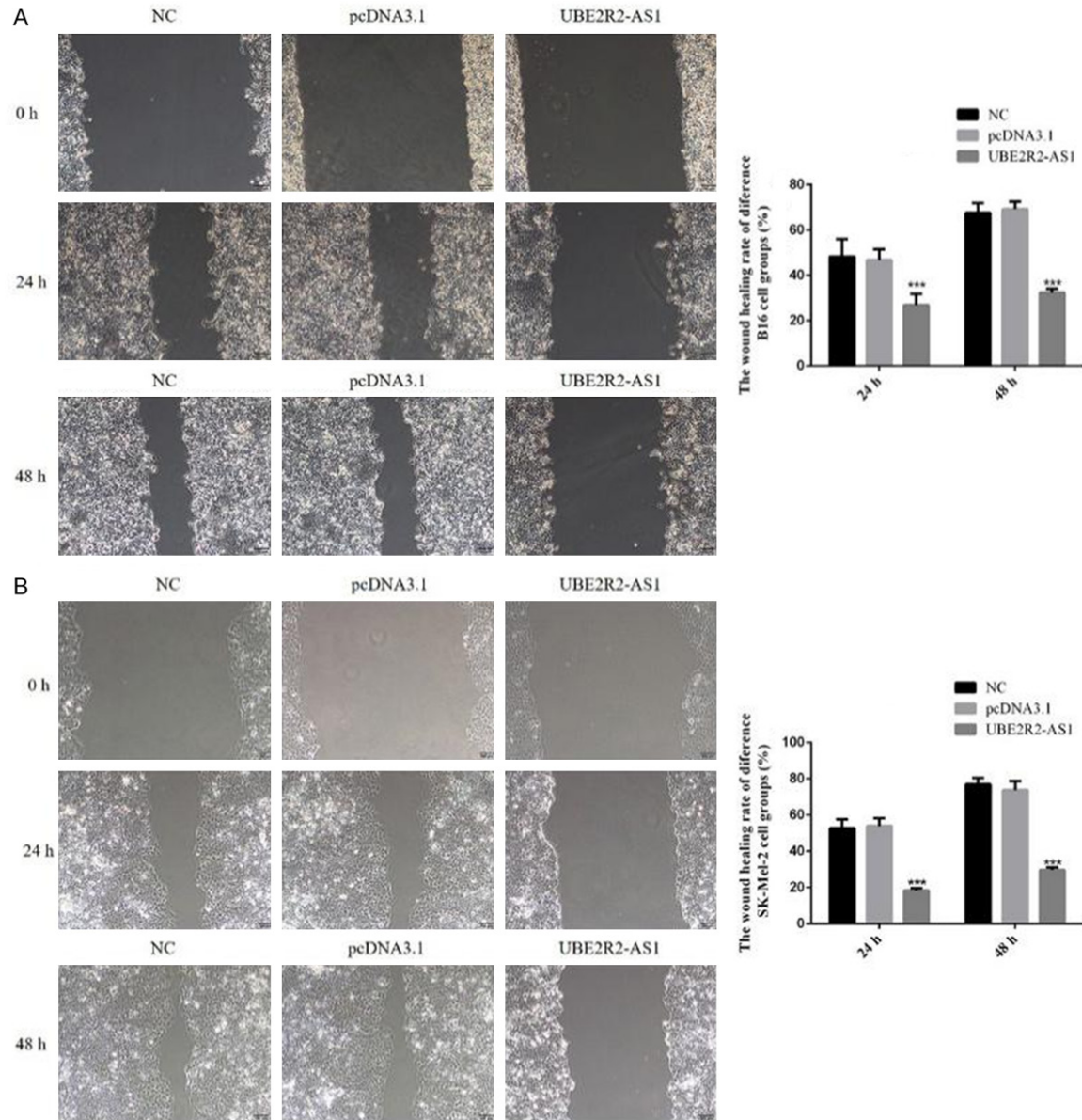


Figure 4. The effect of the overexpression of UBE2R2-AS1 on the migration of the melanoma cells. A. The wound healing rate of the different B16 cell groups (100 \times), ***, $P < 0.001$, compared with the NC group. B. The wound healing rate of the different SK-Mel-2 cell groups (100 \times), ***, $P < 0.001$, compared with the NC group. NC group, the normal control group.

significantly inhibited in the UBE2R2-AS1 group compared with the pcDNA3.1 group ($P < 0.001$, respectively, **Figure 5A** and **5B**).

The effect of the overexpression of UBE2R2-AS1 on the nuclear uptake of NF- κ B (p65)

The results of the cell immunofluorescence assay showed that, compared with the pcDNA3.1 group, the nuclear uptake of NF- κ B (p65) in the UBE2R2-AS1 group was significantly lower, and

the difference was statistically significant ($P < 0.001$, **Figure 6A** and **6B**).

Discussion

LncRNAs, a type of non-coding RNA, play a key role in regulating epigenetics, the cell cycle, cell differentiation, and other life activities. Recent studies have found that, as they are closely related to the occurrence and development of tumors, lncRNAs can regulate the invasion,

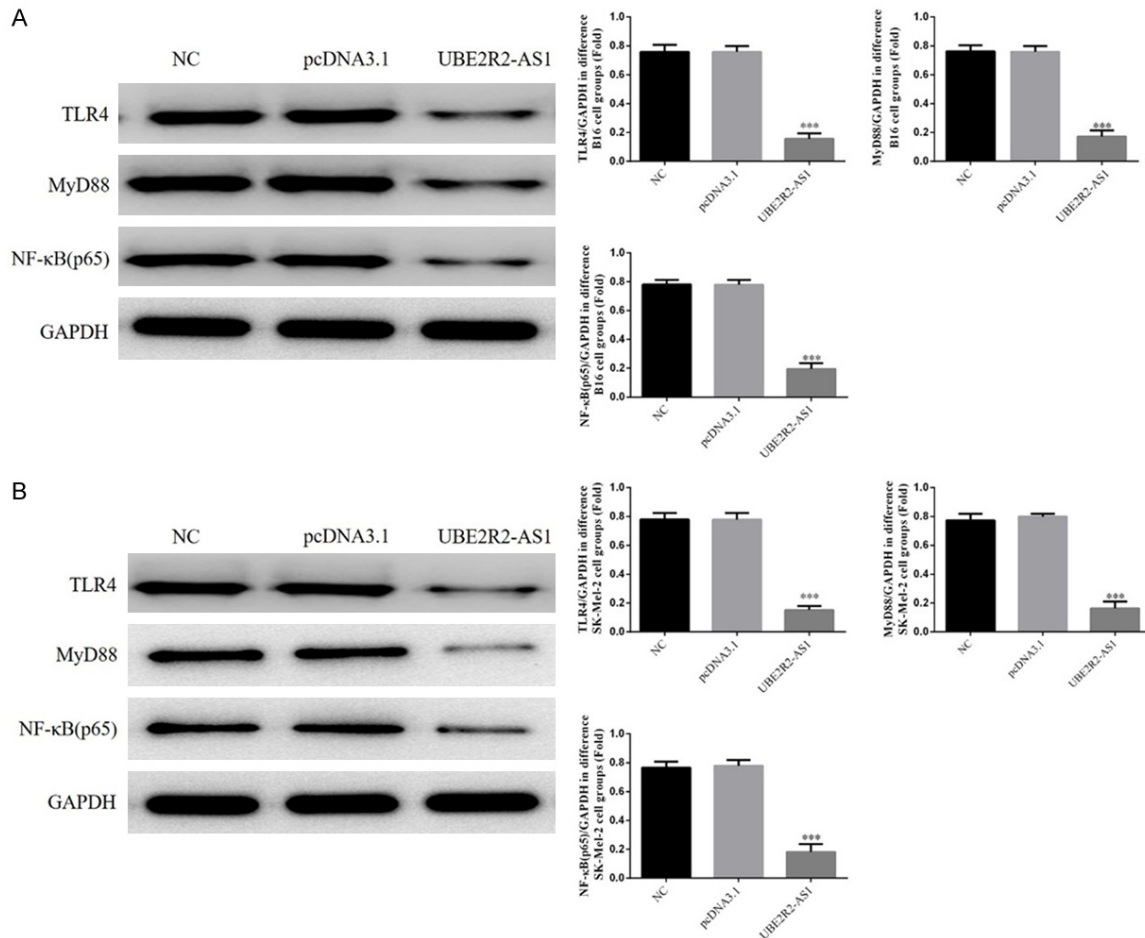


Figure 5. The effect of the overexpression of UBE2R2-AS1 on the associated proteins. A. The relative protein expressions of the different B16 cell groups using a WB assay, ***: $P < 0.001$, compared with the NC group. B. The relative protein expressions of the different SK-Mel-2 cell groups using a WB assay, ***: $P < 0.001$, compared with the NC group. NC group, the normal control group.

migration and chemoradiotherapy resistance of tumor cells through a series of signaling pathways [9-12]. UBE2R2-AS1 is a newly discovered lncRNA which is underexpressed in glioma tissues [7, 8], suggesting that UBE2R2-AS1 may be a potential targets for the diagnosis and treatment of malignant tumors. In this study, the expression of UBE2R2-AS1 was low in the melanoma tissues and cells, especially in the B16 and SK-Mel-2 melanoma cells, indicating that UBE2R2-AS1 may be related to melanoma. Since the follow-up data of the patients were not sorted out in this study, the relationship between UBE2R2-AS1 and the prognosis of melanoma could not be determined. In later studies, we will focus on analyzing the relationship between UBE2R2-AS1 and the prognosis of melanoma.

In this study, after being transfected with UBE2R2-AS1, the biological activities (proliferation, invasion, and migration) of the B16 and SK-Mel-2 melanoma cells were significantly inhibited, and the TLR4 signaling pathway was also significantly inhibited simultaneously. TLR4 is a transmembrane protein that plays an important role in the body's natural immune recognition. Previous research has shown that the TLR4-mediated MyD88 signaling pathway is mainly involved in tissue injury and inflammation [13]. In response to external stimuli, the activation of TLR4/MyD88 signal transduction will bring the nuclear migration of NF-κB (p65) by up-regulating the expressions of the inflammatory cytokines, chemokines, and inflammatory mediators, so as to activate gene transcription to release more inflammatory fac-

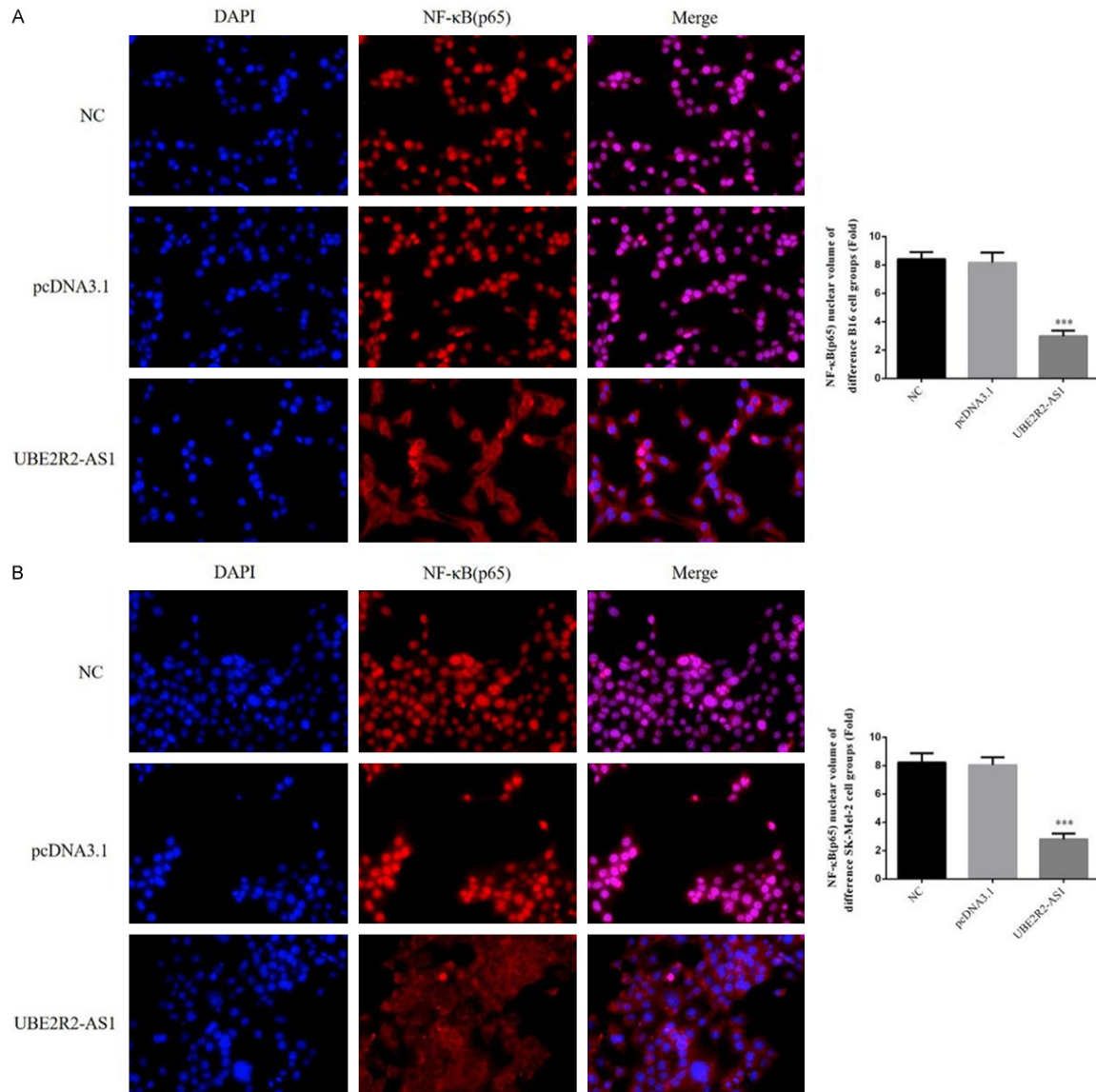


Figure 6. The effect of the overexpression of UBE2R2-AS1 on the nuclear uptake of NF-κB (p65). A. The NF-κB (p65) protein nuclear volume of the different B16 cell groups (200×), ***: $P < 0.001$, compared with the NC group. B. The NF-κB (p65) protein nuclear volume of the different SK-Mel-2 cell groups (200×), ***: $P < 0.001$, compared with the NC group. NC group, the normal control group.

tors, thereby causing further deterioration of the inflammation and even canceration [14-16], and this process is closely related to the TLR4/MyD88/NF-κB (p65) signaling pathway [17, 18]. Recent studies have found that the TLR4 signaling pathway is involved in the occurrence and development of pancreatic cancer [19, 20], colon cancer [21], cervical cancer [22], breast cancer [23], etc. In this study, we found that the overexpression of UBE2R2-AS1 can significantly reduce the protein expression levels of TLR4, MyD88, and endonuclear NF-κB

(p65), suggesting that the inhibition of the proliferation and metastasis of melanoma cells by the overexpression of UBE2R2-AS1 might be related to the TLR4/MyD88/NF-κB (p65) signaling pathway.

The results of this study show that the overexpression of UBE2R2-AS1 can effectively inhibit the proliferation, invasion, and migration of melanoma cells, which might be related to the inhibition of the protein expression levels of TLR4, MyD88, and NF-κB (p65). The above ex-

perimental results indicate that UBE2R2-AS1 is closely related to the proliferation and metastasis of melanoma, in which the TLR4/MyD88/NF- κ B (p65) signaling pathway is involved, so UBE2R2-AS1 may become an important target in the pathogenesis and treatment of melanoma.

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Disclosure of conflict of interest

None.

Address correspondence to: Yujie Ding, Department of Dermatology, The Second Affiliated Hospital of Soochow University, No. 1055, Sanxiang Road, Gusu District, Suzhou 215123, Jiangsu, China. Tel: +86-0512-82911893; E-mail: dingyuejie123@sina.com

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