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Long noncoding RNA OR3A4 promotes the migration and invasion of melanoma through the PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: Recent studies have revealed the crucial role of long non-coding RNAs (lncRNAs) in tumor progression. This study aims to identify the biological function of lncRNA OR3A4 in the progression of melanoma.

PATIENTS AND METHODS: OR3A4 expression in melanoma cells and tissue samples was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). The regulatory effects of OR3A4 on melanoma cells were identified by performing transwell assay and wound healing assay *in vitro*. The underlying mechanism of OR3A4 in mediating the progression of melanoma was explored by RT-qPCR and western blot.

RESULTS: OR3A4 expression was remarkably upregulated in melanoma tissues compared with normal tissues. Moreover, the migration and invasion of melanoma cells were inhibited after knockdown of OR3A4 *in vitro*, which were promoted after overexpression of OR3A4. Furthermore, the targeted protein kinase/phosphoinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway were downregulated after knockdown of OR3A4 *in vitro*, which were upregulated by overexpressed OR3A4.

CONCLUSIONS: OR3A4 could promote the invasion and migration of melanoma cells by inducing the PI3K/AKT signaling pathway, which may provide a new therapeutic intervention for melanoma patients.

Key Words:
Long non-coding RNA, OR3A4, Melanoma, PI3K/AKT signaling pathway.

Introduction

Melanoma is the most aggressive skin cancer in the world, accounting for nearly 75% of all skin cancer-related deaths. The morbidity of melanoma has steadily increased worldwide every year since 1981[1]. The prognosis of melanoma remains poor due to drug resistance. Therefore, it is urgent to uncover the molecular mechanisms underlying melanoma carcinogenesis and search for novel therapeutic markers.

Several subtypes of non-coding RNA (ncRNA), long non-coding RNAs (lncRNAs) regulate a variety of cellular processes and pathways in tumor development. For example, the expression level of lncRNA CCHE1 is positively related to the malignancy of colorectal carcinoma and it regulates the progression of colorectal carcinoma by the ERK/COX-2 pathway[3]. LncRNA HEIBAS promotes cell growth and migration in hepatocellular carcinoma by acting on the miR-153-5p/ARHGAP18 signaling pathway[4]. LncRNA MEG8 enhances epithelial-mesenchymal transition in pancreatic cancer cells[5]. Moreover, lncRNA HCCL5 accelerates cell viability, migration, epithelial-mesenchymal transition and the malignancy of hepatocellular carcinoma by activating ZEB1[6]. However, the role of lncRNA OR3A4 and its underlying biological mechanisms in melanoma remain unknown.

The present work focused on the potential function of OR3A4 in the progression of melanoma. We evaluated its effects on mediating the migration and invasion of melanoma cells. Moreover, the molecular mechanism of OR3A4 in regulating melanoma was further explored.

Patients and Methods

Clinical Samples

50 malignant melanoma tissues and 33 skin tissues with melanocytic nevus were surgically resected from melanoma patients at the People's Hospital of Gansu Province. All tissues were kept at -80°C. The informed consent was signed by

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every patient before the surgery. This study was approved by the Ethics Committee of the People's Hospital of Gansu Province.

Cell Culture

Melanoma cell lines SK-MEL-28, WM266-4, A375, and SK-MEL-2 (American Type Culture Collection; Manassas, VA, USA), and the human epidermal melanocyte HEMa-LP (Invitrogen, Carlsbad, CA, USA) were used in this study. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Cell Transfection

Short hairpin RNA (shRNA) targeting OR3A4 (sh-OR3A4) or negative control (sh-NC), lentivirus targeting OR3A4 (OR3A4) or scrambled oligonucleotides (NC) were cloned into the pGLVH1/GFP+Puro vector (GenePharma, Shanghai, China). The cells were transfected with sh-OR3A4 or sh-NC, OR3A4 lentivirus (OR3A4) or NC, and those GFP-positive cells were selected for following experiments.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA extracted from samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was reversely transcribed to complementary deoxyribonucleic acids (cDNA) using the PrimeScript Transcription Kit (TaKaRa Biotechnology, Dalian, China). Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) was conducted on the ABI 7500 system (Applied Biosystems, Foster City, CA, USA) with the SYBR Green Real Time PCR kit. Primers used for RT-qPCR were as follows: OR3A4, forward 5′-CCTATCCTTTCTCTAAGAA-3′ and reverse 5′-TGCAAACGTGCTG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-TCAGATGGGGCAATGCTGG and reverse 5′-TGATGGCATGGACTGTGGTCA-3′. The thermal cycle was as follows: 95°C for 5 min, 5 sec at 95°C and 35 sec at 60°C, for a total of 40 cycles.

Wound Healing Assay

Cells seeded into the 6-well plates were cultured in DMEM overnight. A sterile 10 µL pipette tip was used for creating a scratch and then the cells were cultured in serum-free DMEM. Each assay was repeated in triplicate independently. Wound closure was viewed at 0 and 24 h, respectively.

Transwell Assay

5×10^4 cells suspended in 200 µL serum-free DMEM were applied on the top chamber of transwell insert with 8 µm pore size (Millipore, Billerica, MA, USA) pre-coated with Matrigel (50 µg; BD Biosciences, Franklin Lakes, NJ, USA). DMEM containing 10% FBS was applied to the bottom chamber. 24 h later, a cotton swab was used to wipe the top surface of the chamber and cells were fixed with 10% precooled methanol. Subsequently, cells were stained with crystal violet for 30 min. Invasive cells were counted in three fields under a microscope.

Western Blot Analysis

Reagent radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was used to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa Biotechnology, Dalian, China) was chosen for determining the protein concentrations. The target proteins were separated by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and loaded on the polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). Primary antibodies (rabbit anti-PI3K, anti-p-Akt, anti-t-Akt and anti-GAPDH) and secondary antibody (goat anti-rabbit) were provided by Cell Signaling Technology (CST, Danvers, MA, USA). Image J software (NIH, Bethesda, MD, USA) was applied for the assessment of the protein expression.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was utilized to perform statistical analysis. Student's *t*-test was conducted to compare intergroup differences. Data were presented by mean ± SD (standard deviation). $p<0.05$ was considered statistically significant.

Results

Expression Level of OR3A4 in Melanoma

First, RT-qPCR was conducted for detecting OR3A4 expression in 50 malignant melanoma tissues and 33 skin tissues with a melanocytic nevus. As a result, OR3A4 was significantly upregulated in melanoma tissues than that in

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skin tissues with melanocytic nevus (Figure 1A). Identically, OR3A4 level was markedly higher in melanoma cells compared with HEMa-LP cell line (Figure 1B).

OR3A4 Knockdown Inhibited the Migration and Invasion of Melanoma Cells

Transfection efficacy of sh-OR3A4 was first verified in WM266-4 cells, which sufficiently downregulated OR3A4 level (Figure 2A). Wound healing assay indicated that the knockdown of OR3A4 inhibited wound closure percentage of melanoma cells, suggesting the inhibited migratory ability (Figure 2B). The transwell assay showed that the number of invasive cells decreased after OR3A4 knockdown (Figure 2C).

OR3A4 Overexpression Promoted the Migration and Invasion of Melanoma Cells

Transfection efficacy of OR3A4 lentivirus (OR3A4) was verified in SK-MEL-28 cells, which sufficiently upregulated OR3A4 level (Figure 3A). Wound healing assay revealed the accele[illegible] migratory ability after overexpression of [illegible] in SK-MEL-28 cells (Figure 3B). Similar[illegible] transwell assay showed the increased numb[illegible] invasive cells in melanoma cells overexpress[illegible] OR3A4 (Figure 3C).

OR3A4 Activated PI3K[illegible]T Sig[illegible]ng Pathway in Melanom[illegible]

To explore the [illegible] OR3A4 function in [illegible]noma, [illegible]rn blot assay was conducted to detect target gene expressions in the PI3K/AKT signaling pathway. The results showed that the protein levels of PI3K, p-Akt, and t-Akt were downregulated after knock[illegible] of OR3A4 (Figure 4A). Conversely, [illegible] levels of PI3K, p-Akt, and t-Akt [illegible] upregulated by overexpressed OR3A4 ([illegible]e 4B). It is suggested that OR3A4 promoted the [illegible]stasis of melanoma by regulating the [illegible]K/AK[illegible]aling pathway.

Discussion

Accumulati[illegible]esearch ha[illegible]e[illegible] the crucial roles o[illegible]s in the pr[illegible]ion of melanoma. F[illegible]stan[illegible]cRNA FTH1P3 promotes cell growth and migr[illegible]in uveal melanoma *via* targ[illegible]R-224-5p[7]. [illegible]verexpression of lncR[illegible]FALEC promotes cell growth of melanoma [illegible]ownregulati[illegible]p21[8]. Knockdown of lncRNA [illegible]-AS1 depre[illegible]s the proliferation, invasion a[illegible]igration [illegible]melanoma cells by sponging mi[illegible][9]. L[illegible]A HEIH functions as an oncogene i[illegible]ma and enhances cell proliferation and invasion[10]. In addition, PVT1 facilitates cell [illegible]ation and metastasis of melanoma cells, [illegible]ay provide a potential diagnostic marker and therapeutic target for melanoma[11].

Olfactory receptor family 3 subfamily A member 4 (OR3A4) (Accession Number: NR_024128.1) is a novel lncRNA, which is abnormally expressed and related to tumor progression. For example, OR3A4 promotes cell proliferation of gastric cancer and may act as a potential thera-

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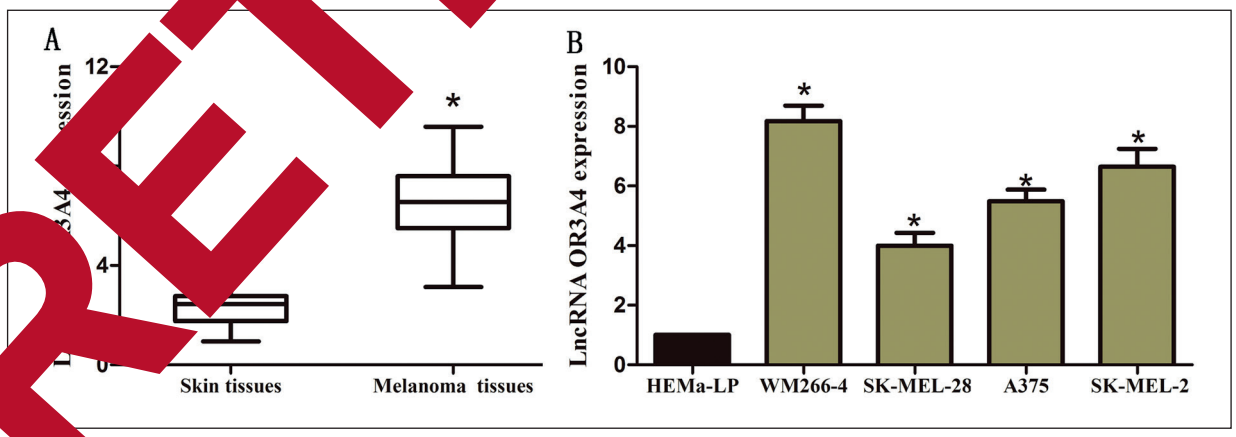


Fig[illegible]e 1. The expression levels of OR3A4 were up-regulated in melanoma tissues and cell lines. ***A,*** OR3A4 expression significantly increased in 50 malignant melanoma tissues compared with 33 skin tissues with melanocytic nevus. ***B,*** The expression levels of OR3A4 relative to GAPDH were determined in the human melanoma cell lines and human epidermal melanocyte (HEMa-LP) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

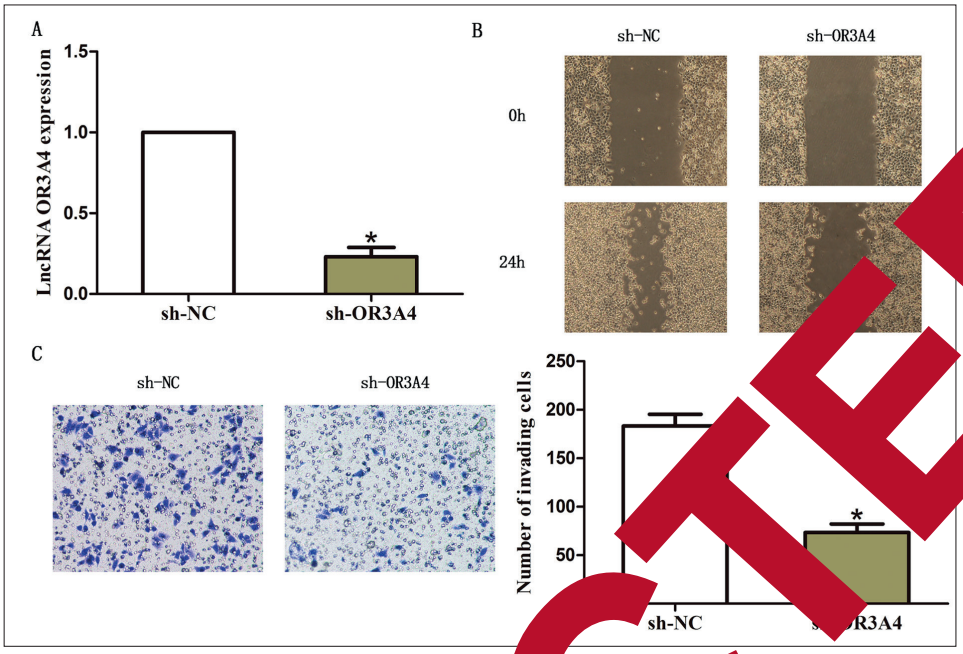
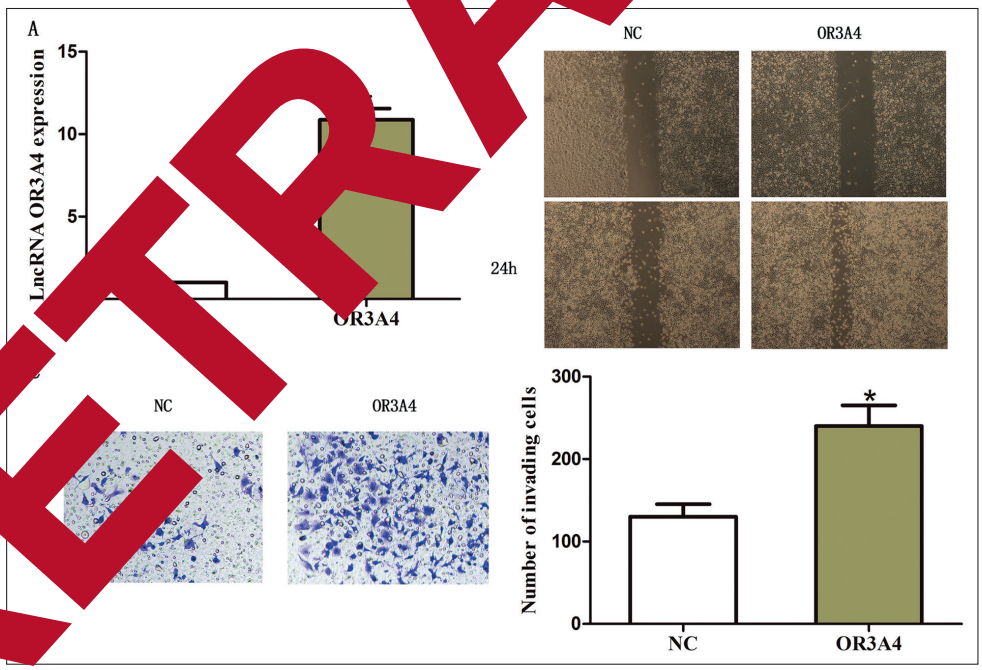


Figure 2. Knockdown of OR3A4 repressed migration and invasion [illegible] melanoma cells. [illegible]R3A4 expression in melanoma cells transfected with control vector (sh-NC) or OR3A4 shRNA (sh-OR3A4 [illegible] detected by R[illegible]CR. GAPDH was used as an internal control. ***B,*** Wound healing assay showed that migrated ability of mela[illegible]ells transf[illegible] with sh-OR3A4 markedly decreased compared with empty control (sh-NC) group (magnification: 40×). ***C,*** The t[illegible]owed that the number of invasive cells in OR3A4 transfected with sh-OR3A4 remarkably decrea[illegible]pared with the [illegible]trol group (magnification: 40×). The results represent the average of three independent experimen[illegible]dard error of the mean). *p<0.05.



[illegible]re 3. Overexpression of OR3A4 promoted migration and invasion of melanoma cells. ***A,*** OR3A4 expression in melanoma c[illegible]nsfected with control vector (NC) or OR3A4 lentivirus (OR3A4) was detected by RT-qPCR. GAPDH was used as an inte[illegible]l control. ***B,*** Wound healing assay showed that migrated ability of melanoma cells overexpressing OR3A4 significantly increased compared with the NC group (magnification: 40×). ***C,*** The transwell assay showed that the number of invasive cells in OR3A4 overexpression group markedly increased compared with the NC group (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

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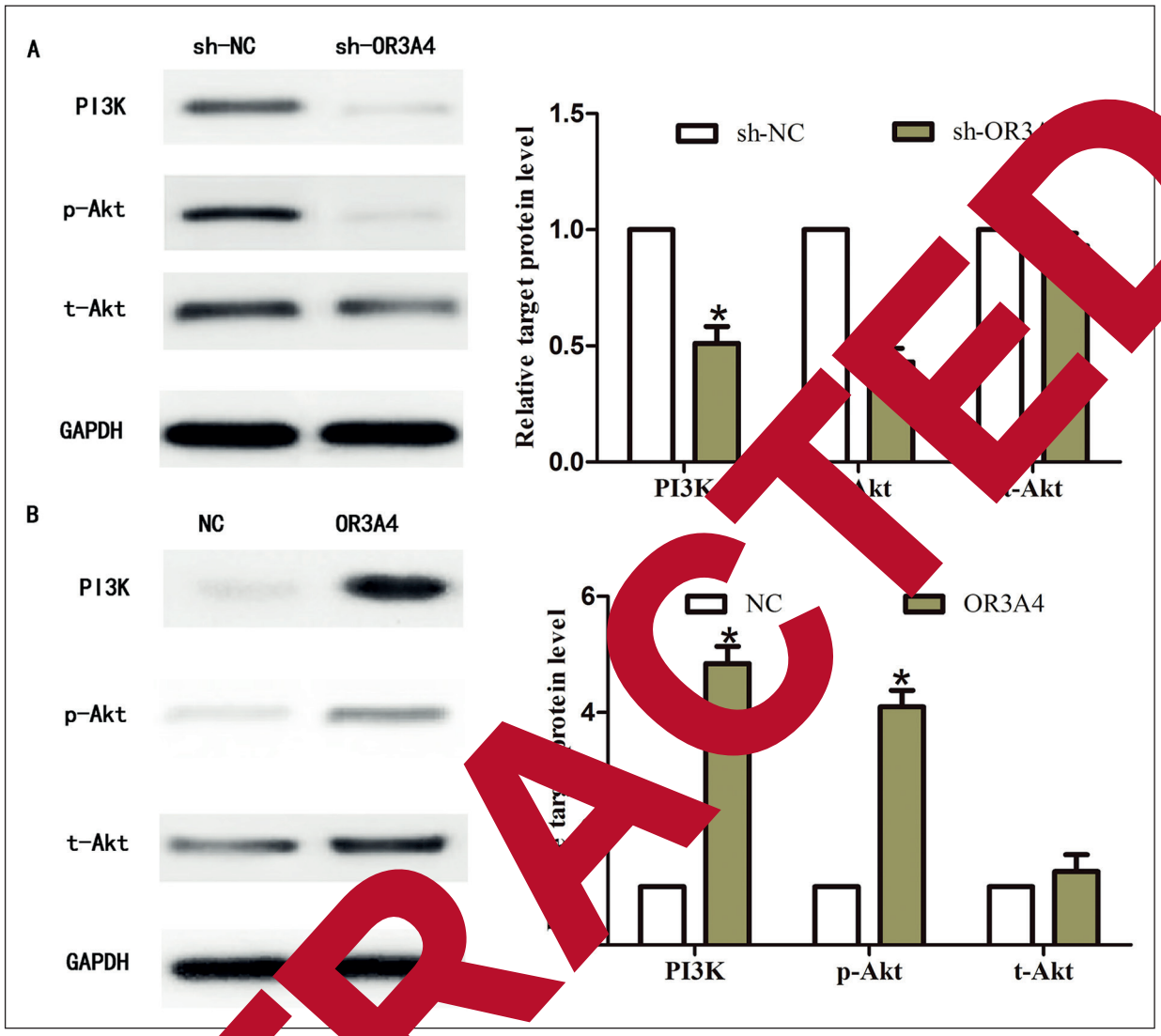

Figure 4. OR3A4 r ed the PI3K/A naling pathway. ***A,*** Western blot assay revealed that the expressions of target proteins in the PI3 naling pathw e downregulated in sh-OR3A4 group compared with the sh-NC group. ***B,*** Western blot as veal the expressio s of target proteins in the PI3K/AKT signaling pathway were upregulated in the OR3A4 g up compared e NC group. The results represent the average of three independent experiments. *$p<0.05$.

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py t[12]. A4 enhances cell proliferation and i *via* ind g epithelial-mesenchy- tran in b cancer[13]. In the present s OR3 ound to be upregulated in bot elanom ssues and cells. Furthermore, th down of OR3A4 inhibited migration in of melanoma cells. Conversely, the erexpression of OR3A4 obtained the opposite ds. These data indicated that OR3A4 served as ncogene in melanoma.

The PI3K/AKT signaling pathway participates in diverse pathological processes. PI3K, p-Akt, and t-Akt are the key genes in the PI3K/AKT signaling pathway. Previous studies[14-16] have suggested that aberrant activation of the PI3K/AKT signaling pathway exerts an important role in regulating tumor development, including melanoma. For instance, lncRNA MALAT1 facilitates cell growth ability and invasion of ovarian cancer by activating the PI3K/AKT pathway[17]. LncRNA BC087858 enhances resistance to EGFR-TKIs in non-small cell lung cancer by activating the PI3K/AKT pathway[18]. A recent study[19] also demonstrated that miR-194 regulates melanoma progression through the PI3K/AKT signaling pathway. In our work, target proteins in the PI3K/AKT signaling

pathway were downregulated after knockdown of OR3A4, which were upregulated by overexpressed OR3A4. It is suggested that OR3A4 might promote tumorigenesis of melanoma *via* regulating the PI3K/AKT signaling pathway.

Conclusions

We observed that lncRNA OR3A4 is vital in the carcinogenesis of melanoma and can be served as a promising hallmark for melanoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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