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The miR-31-SOX10 axis regulates tumor growth and chemotherapy resistance of melanoma via PI3K/AKT pathway

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ABSTRACT

MicroRNAs were thought to play a regulatory role through complementarity to target messenger RNA (mRNA). Our previous study revealed a miR-31-SOX10 axis that regulated tumor growth and resistance to chemotherapy of melanoma. Up-regulation of SOX10 and down-regulation of miR-31 were found in melanoma tissues. SOX10 was further identified as a target of miR-31. Overexpression of SOX10 dramatically promoted melanoma cell proliferation and chemotherapy resistance both *in vitro* and *in vivo*. While enforced miR-31 expression suppressed cell growth and enhanced the chemosensitivity of melanoma cells, the re-expression of SOX10 rescued these effects by activating PI3K/AKT signaling pathway. In conclusion, our results demonstrated that SOX10 acted as an oncogene and was negatively regulated by miR-31, which supports the potential therapeutic strategy against melanoma by targeting the miR-31-SOX10 axis.

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1. Introduction

Malignant melanoma (MM) is a type of malignant tumor that originates from ectodermal neural crest [1]. It is formed by the malignant transformation of melanocytes located at the basement of the epidermis, deriving from blemishes or pigmented spots. At present, there are no effective measures to improve the survival rate of patients with metastatic melanoma, and the average survival of patients with advanced malignant melanoma is only 6–9 months [2]. This poor prognosis mainly due to its lower sensitivity to conventional treatment methods such as chemotherapy, radiotherapy and immunotherapy [3]. In recent years, no breakthrough has been made in the chemotherapy drugs for malignant melanoma. Drug resistance and toxic and side effects are two major problems in their clinical application [4]. The most effective drug in clinical chemotherapies is still dacarbazine (DTIC), which is the only drug recommended by the US FDA for the treatment of metastatic melanoma, but its effective rate is approximately 20% [5]. In general, malignant melanoma is still a malignant tumor that is insensitive to traditional treatment methods such as chemotherapy drugs. Therefore, people need to explore new treatments. In general, melanoma is still a malignant tumor that is insensitive to traditional treatment methods such as chemotherapy drugs, so it is urgent to explore new treatments [6].

The mechanism of melanoma development remains unclear. In recent years, it has been found that abnormal gene expression plays an important role in the formation and metastasis of melanoma. Targeted treatment for these changes may be a potential treatment. SOX10 is a type of transcription factor belonging to the SOX (SRY-related HMG-box) family and also contains highly conserved DNA binding sequences [7]. A growing number of studies have shown that SOX10 is highly expressed in a variety of tumors [8–10]. Recent studies have shown that transcription factor SOX10 is closely related to melanoma development, making it a very promising target for the treatment of melanoma [11–13]. Since we found high SOX10 levels in melanoma tissues, the roles and regulatory mechanism of SOX10 in melanoma require further examination.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs that play important roles in cancer progression [14] through mediating mRNA degradation or translational repression [15]. Previous studies demonstrated that miRNAs can function as onco-miRNAs or tumor-suppressive miRNAs [16], affecting cancer development and response to radiotherapy [17]. Emerging evidence indicated that miR-31 was aberrantly expressed in multiple cancer types, such as head and neck carcinoma [18], colorectal

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cancer [19], adult T cell leukemia [20] and breast cancer [21]. Some studies revealed that miR-31 was down-regulated in melanoma and could modulate cell migration and invasion. [22], yet exploring the exact roles of miR-31 and its target genes in melanoma still needs further investigation.

In this study, we investigated the roles of SOX10 and miR-31 in the development of melanoma. We hypothesized and verified that SOX10 was a target gene of miR-31. Thus, we highlighted an axis consisted of SOX10 and miR-31 which regulated tumor growth and chemosensitivity of melanoma.

2. Material and methods

2.1. Patients and tissue specimens, cell lines

94 pairs of melanoma and adjacent normal tissues (males, n = 55; females, n = 39; average age, 52) were obtain from The Affiliated Huaian No.1 People's Hospital of Nanjing Medical University, which was approved by Institutional Review Board (IRB). After resection, tumor tissues were flushed with 0.9% saline, and the region with no necrosis and good vitality were chosen for experiments. Meanwhile, normal adrenal gland tissues that 5 cm away from tumor tissue were collected, flushed with 0.9% saline, and removed nerves, blood vessels, and connective tissues. After marking, all tissues were rapidly frozen in liquid nitrogen and stored at −80 °C. The melanoma cell lines (A375, A2058, SKMEL13, HT144, SKMEL5, A875, SKMEL1, M21) and 293T cells were obtained from the American Type Culture Collection (ATCC), culturing in DMEM/RPMI 1640 (Hyclone) medium with 10% FBS, 100U/ml penicillin at 37 °C.

2.2. RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

We utilized TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) for total RNA isolation. Complementary DNA was synthesized from SuperScript first strand synthesis system (Invitrogen). Primer information: SOX10 forward, 5′-CCT CAC AGA TCG CCT ACA CC-3′ and reverse primer, 5′-CAT ATA GGA GAA GGC CGA GTA GA-3′; GAPDH forward, 5′-TTG GCA TCG TTG AGG GTC T-3′ and reverse primer, 5′-CAG TGG GAA CAC GGA AAG C-3′. Quantitative PCR analysis was performed on the Applied Biosystems 7300 using IQTMSYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

2.3. Immunohistochemistry staining (IHC) and Western blot

For IHC staining, samples were cut into 3 μm-thick sections, and the expression of SOX10 was examined using an anti-SOX10 antibody (ab108408; Abcam). IHC was carried out as described [23]. For Western blot, cells and tissues were lysed using RIPA buffer to obtain total cellular protein, which were then separated with 10% SDS-PAGE electrophoresis, and finally transferred onto nitrocellulose membrane (Sigma). The SOX10 (D5V9L) Rabbit mAb (#89356) and GAPDH (D16H11) XP® Rabbit mAb (#5174) were applied for Western blot assays as described previously [24]. Goat Anti-Rabbit HRP (IgG H&L) (1:2000, ab205718; Abcam) was used as secondary antibody. The expression of proteins was assessed using enhanced chemiluminescence (melanomaL) reagent (Thermo Scientific, USA).

2.4. Transfections and plasmid construction

The cells were transfected with hsa-miR-31 miRNA Mimic (abmGood, Richmond, BC, Canada), hsa-miR-31 miRNA inhibitor (anti-miR-31, Biomics Biotech, China), or their corresponding controls using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA). The SOX10 CRISPR guide RNA sequences (5′-ACG TTG CCG AAG TCG ATG TG-3′) were cloned into the lentiviral expression vector pLentiCRISPR v2 (GenScript, Guangzhou, China) to create knock-out cell lines using the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated 9) system [25]. SOX10 knockout (KO-SOX10) HT144 and SKMEL1 cell lines were selected using 5 μg/mL puromycin (InvivoGen). For stable cell line generation, SOX10 gene sequences (Gene ID 6663) were subcloned into the lentiviral expression vector pCDH-puro (pCDH-SOX10-puro) at EcoRI and BamHI, and packaged into lentiviral particles for high efficiency transduction. Stable expression of SOX10 gene in HT144 and SKMEL1 cells was achieved by puromycin resistant screening.

2.5. Proliferation assays

Dacarbazine (DITC) was obtained from Sigma-Aldrich (Gillingham Dorset, UK), and dissolved in PBS (pH = 7.4). CellTiter 96 AQueous One Solution Cell Proliferation assay (MTS) (Promega Corp., Madison, Wisconsin, USA) was performed to determine proliferation ability. Briefly, 3000 control cells, SOX10-overexpressing cells (SOX10) or SOX10 knock out cells (KO-SOX10) were seeded into 96-well plates, and then treatment with dacarbazine (DTIC; 250 μM) for another 4 days. An equal volume of PBS was used as control. The OD value at 592 nm was detected. The assays were performed three independent times.

2.6. Dual-luciferase reporter assay

SOX10 3′UTR (ENST00000396884.2; 1195 bp) with or without mutation were subcloned into pGL3 promoter plasmid. 30,000 cells were seed into 24-well culture plates in phenol red-free medium. After transfection with miRNA precusor control pre-miRNA (pre-miR-co) or pre-miR-31 (Biomics Biotech) for 48 h, the luciferase activity was determined by the Dual Luciferase Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.7. Lentiviral overexpression of miR-31

Lentiviral vectors for overexpression of miR-31 (pLenti-III-mir-31 Vector, Cat.No.: mh42766) and its empty pLenti-III vector were purchased from Applied Biological Materials Inc. (ABM, MC, Canada). Lentiviral particles were produced via co-transfection of the lentiviral vector with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into 293T cells per manufacturer's protocol. To establish stable miR-31-expressing HT144 cell lines, HT144 cells were infected with lentivirus and selected using 5 μg/mL puromycin.

2.8. In vivo study

6 week-old female mice (Weitonglihua Biotechnology, Beijing, China) were inoculated subcutaneously with either 2×10^6 control cells, SOX10-overexpressing cells (SOX10) or SOX10 knock out HT144 cells (KO-SOX10). In some experiments, 2×10^6 control cells and stable miR-31-expressing HT144 cells were injected on the back of mice. When the tumors reached a mean size of 50–100 mm^3, mice received vehicle (an equal volume of PBS) or dacarbazine (DTIC; 35 mg/kg) every 3 days by intraperitoneal injections. Tumor volume was calculated by the formula π (length $\times$ $width^2$)/6. All animals were kept in a pathogen-free environment. The procedures for care and use of animals were approved by the Ethics Committee.

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