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Full paper

Morin inhibits proliferation and self-renewal of CD133$^+$ melanoma cells by upregulating miR-216a

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ABSTRACT

Melanoma is one of the most malignant skin tumors with high mortality rate. Morin has been reported to treat several cancers. However, whether or how Morin affects melanoma progression is still poorly understood. Either Morin treatment or miR-216a overexpression reduced cell viability, sphere formation ability and expressions of stem cell marker genes CD20, CD44, CD133 and Wnt-3A. MiR-216a was induced by Morin treatment in CD133$^+$ melanoma cells. Melanoma xenograft model treated by Morin showed reduced tumor size, weight as well as expressions of stemness markers and Wnt-3A. Inhibition of the stemness marker gene expressions in CD133$^+$ melanoma cells is mediated by downregulating Wnt-3A through miR-216a. MiR-216a and Wnt-3A may potentially serve as clinical biomarkers of melanoma, and Morin may contribute to the treatment of melanoma.

1. Introduction

Melanoma is a class of highly malignant skin tumors, and the mortality rate accounts for 80% of all skin tumors.[1] Therefore, improving the clinical efficacy of melanoma treatment has become a research focus. Melanoma tumor stem cells (MTSCs) are the leading player in the development and drug-resistance. Tumor stem cells (TSCs) were first reported in acute myeloid leukemia, and the following massive tumor stem cell-like cells, including melanoma, breast cancer, lung cancer and other solid tumors have been identified.[2–5] TSCs are a small group of heterogeneous cells with stem cell characteristics in tumor tissues. TSCs exhibit characteristics of self-renewal, multi-directional differentiation, high tumorigenicity and multidrug resistance. Moreover, TSCs can be detected by specific surface markers for sorting and identification, thus are widely used in tumorigenesis, invasion and metastasis, drug resistance, targeted immunotherapy and other related research.

Morin (3,5,7,2′,4′-pentahydroxyflavone), a polyphenol compound originally isolated from woods of *Moraceae* family plants, has been shown to suppress the proliferation of many kinds of tumor cells, including oral squamous cell carcinoma, leukemia and colorectal cancer cells in SCID mice.[6–8] However, the effect of Morin in melanoma, especially the stemness of melanoma, is not clear.

MicroRNAs (miRs) have become a hotspot in molecular biology research. MiRs play a critical role in development and various cellular processes, including proliferation, differentiation, cell growth and apoptosis. MiRs play an important role in tumorigenesis and progression by regulating expressions of target tumor genes and/or tumor suppressor genes as well as related signal pathways in multiple cancer types, such as oral cancer, endocrine cancer, prostate cancer, esophageal cancer and hepatocellular carcinoma.[9–12] For example, miR-218 was significantly decreased in cisplatin-resistant esophageal cancer cell line, which was associated with cisplatin resistance in these cells via targeting survivin expression.[13] MiR-302d promotes hepatocellular carcinoma cell growth and migration, suppresses cell apoptosis and affects cell cycle distribution *in vitro*, and promotes tumorigenicity *in vivo*.[12] Studies have shown that miR-216a was downregulated in breast cancer, liver cancer, prostate cancer and other tumors.[13] However, the expression and functional mechanism of miR-216a in cancer stem-like cells (CSCs) remain largely unknown.

Wnt signaling pathway plays important roles in TSCs,[11–13] and is a key signaling pathway that regulates the self-renewal and differentiation of CSCs. The classical Wnt signaling pathway refers to

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the Wnt/β-catenin signaling pathway with β-catenin as the core protein factor. Wnt proteins can be secreted, which then bind to their membrane receptors, as well as membrane receptors on adjacent cells, to participate in autocrine regulation. Among the 19 Wnt proteins that have been found, only Wnt1, Wnt3A, Wnt8, Wnt10B and other proteins can lead to the accumulation of intracellular β-catenin signaling molecules. Chronic metabolic stress in tumor microenvironment drives a CSC-like pheno-conversion of non-stem cancer cells dependent on the Wnt pathway.[14] It was also reported that, for CSC property-related signaling pathways, six miRs were markedly enriched in Wnt, Hedgehog and Notch signaling pathways through regulating 108 target genes, including WNT3A and WNT5A.[15] However, natural compounds that regulate Wnt3A expression haven't been well studied.

In this study, we found that Morin inhibited the expressions of stemness marker genes in $CD133^+$ melanoma cells. MiR-216a was induced by Morin treatment, and miR-216a was able to inhibit Wnt3A. Finally, we confirmed these findings by *in vivo* tumorigenesis model. Our study provides a new theoretical basis and treatment therapy for melanoma.

2. Materials and methods

2.1. Isolation of $CD133^+$ melanoma cells and cell culture

Human melanoma cell lines MV3 and M14 were bought from American Type Culture Collection (ATCC, Rockville, MD, USA). MV3 $CD133^+$ melanoma cells were isolated from cell culture suspensions with magnetically labeled CD133 micro beads followed by a MACS® column (Miltenyi Biotech, Moenchengladbach, Germany) as described.[1] Then $CD133^+$ melanoma subpopulation was cultured in Quantum 263 medium with 5% CO_2 at 37 °C.

2.2. Cell viability assay by cell counting kit-8 (CCK-8)

To test the effects of Morin on $CD133^+$ melanoma cells, cells were seeded into 96-well plates at 3, 000 cells in 100 μl medium per well with treatment of 50 μM Morin or with DMSO as the negative control. After 72 h inoculation, 10 μl of CCK-8 reagent was added to each well and incubated for 2 h in the incubator. The absorbance was then measured at 450 nm using a microplate reader. In order to explore the function of miR-216a on the viability of $CD133^+$ melanoma cells, miR-216a and miR-negative control were used to transfect the cells. Next day, cells were seeded into 96-well plates and 72 h later the cell viability was measured.

2.3. Western blot

Standard Western blot was performed. Briefly, 20 μg protein samples from $CD133^+$ melanoma cells were loaded on the SDS-PAGE gels, and then transferred electronically to PVDF membranes (Life technologies, Pleasanton, CA, USA). After incubation with primary antibody and corresponding secondary antibody, SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) were used to show the blotted target protein signal. The antibodies used were as follows: anti-CD20 antibody (ab88247, Abcam, Cambridge, MA, USA), anti-CD44 antibody (ab157107, Abcam, Cambridge, MA, USA), anti-CD133 antibody (MAB4399-I, Millipore, Billerica, MA, USA), anti-Wnt3A antibody (ab28472, Abcam, USA), anti-GAPDH (MAB374, Millipore, USA), HRP-conjugated anti-mouse secondary antibody (A8924, Sigma–Aldrich, St. Louis, MO, USA) and HRP-conjugated anti-rabbit secondary antibody (A0545, Sigma–Aldrich, USA).

2.4. Sphere formation assay

1 ml of single cells at 1000 cells/ml were seeded into a 24-well plate with ultra-low attachment surfaces (Corning, NY, USA) in sphere medium, which was serum-free DMEM/F-12 (1:1) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1XB27 (Invitrogen, USA), 20 ng/ml EGF, 10 ng/ml basic fibroblast growth factor and 5 mg/ml insulin. Sphere medium were refreshed in sphere culture every three days. Numbers of round shaped spheres between 50 and 250 μm were counted manually using a microscope after 7–10 days. Average sphere numbers from 5 microscopic fields with 200 × magnification were used to calculate the sphere formation efficiency.

2.5. MiR profiling

MiR profiling was conducted comparing Morin-treated and DMSO-treated $CD133^+$ melanoma cells by the reported protocol.[16] In brief, the following steps were included: microarray fabrication, microarray processing, image analysis and data validation by real-time PCR. GenePix Pro was utilized for the analysis of raw data of microarray images, and post-processing was performed as reported.[17]

2.6. QRT-PCR

Total RNA of the cells was isolated using the Trizol reagent according to the manufacturer's instructions. Then reverse transcriptase reactions were performed as described by using stem–loop RT primer. Next, real-time PCR was conducted by a standard TaqMan PCR kit with Applied Biosystems 7900HT Detection System. MiR expression was detected by the corresponding miR probe with β-actin as the internal control and the relative expression of miR copy number was calculated by $2^{-\triangle\triangle CT}$ method. The following primers were used: miR-216a (Thermofisher Scientific #4427012), miR-137 (Thermofisher Scientific #4426961), miR-7 (Thermofisher Scientific #4426961), miR-199a (Thermofisher Scientific #4426961), WNT3A (Thermofisher Scientific #4351372), and β-actin (Thermofisher Scientific #4331182).

2.7. Dual luciferase assay

Wild type and mutated sequences of WNT3A 3′-UTR mRNA were separately cloned into the downstream of the firefly luciferase gene. MV3 cells transfected with miR-216a and miR negative control, respectively, were plated into 12-well plates. Then cells were transfected with wild type WNT3A and mutated WNT3A vectors, and co-transfected with pTK-Renilla vector as internal control with Lipofectamine LTX (Invitrogen). 24 hr post transfection, cells were harvested and the luciferase activity was analyzed with normalization to the Renilla activity by Dual-luciferase reporter assay kit (Promega, USA) according to manufacturer's instructions.

2.8. Mouse melanoma xenograft model and Morin treatment

The experiment involving mice was performed according to the protocols approved by Wuxi No.2 People's Hospital. Severe combined immuno-deficient (SCID) mice of 6 weeks were bought from Charles River. 1.0×10^6 cells suspended in 100 μl serum-free 1640 medium were subcutaneously injected into each side of posterior flank of the SCID mice (n = 7/group). Morin (50 mg/kg) or the vehicle, DMSO was administrated to mice by peritoneal injection. Starting from day 8 till day 24, tumors were measured every 4 days with the volumes being calculated by the formula of