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Upregulation of LINC00963 facilitates melanoma progression through miR-608/NACC1 pathway and predicts poor prognosis

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ABSTRACT

Long noncoding RNAs (lncRNAs) are acknowledged as crucial regulators involved in multiple pathological processes, including cancer. Although some lncRNAs are studied in melanoma, the association between lncRNA and melanoma progression still remains vague. And the function of LINC00963 in melanoma is waiting for investigation. In this study, upregulated level of LINC00963 in melanoma tissues was observed. Notably, we found DNA copy-number-gain of LINC00963 contributes to its high expression. And high expression of LINC00963 predicts poor prognosis in patients with melanoma. Functional investigation indicated that LINC00963 knockdown dramatically suppressed melanoma cell proliferation, migration and invasion. Mechanistically, we found that LINC00963 could interact with miR-608 while miR-608 could target NACC1. Upregulated LINC00963 led to elevated expression of NACC1 through inhibiting miR-608, which consequently promoted melanoma malignant progression. Taken together, our results illustrated that LINC00963-miR-608-NACC1 pathway might be a potential target for melanoma therapy.

1. Introduction

Originated from melanocyte pigment cells, melanoma is the most malignant skin cancer worldwide [1]. About 30% melanoma patients are commonly accompanied with metastasis at advanced stage [2], which contributes to increased difficulty for melanoma treatment. Accounting for 75% deaths caused by all types of skin cancers, melanoma has become a great challenge for human's health [3]. Additionally, the five-year survival rate of melanoma patients exhibits extremely low [4]. However, its pathological molecular mechanism still remains largely unknown. Thus, it is urgent to investigate how melanoma occurs and develop novel therapeutic targets.

Long oncoding RNAs (lncRNAs) are critical participants in gene expression regulation. Increasing reports have focused on lncRNA function and demonstrated that lncRNAs participate in various cancers [5,6]. LncRNAs could serve as either oncogenes or tumor-suppressors through regulating biological processes, including survival, division, metastasis and differentiation [7,8]. In melanoma, several lncRNAs are reported. For example, Shi et al. found that lncRNA H19 is upregulated in melanoma and promotes tumor growth and metastasis [9]. Long et al. showed that lncRNA-MEG3 regulates CYLD expression by inhibiting miR-499, leading to suppression of melanoma progression [10]. Long et al. showed that lncRNA TUG1 upregulates AEG-1 level to promote melanoma development and aggression [11]. Additionally, Ni et al. demonstrated that lncRNA ALEC overexpression is associated with poor prognosis and initiates melanoma growth by suppressing P21 [12]. Altogether, the importance of lncRNA in melanoma is widely acknowledged.

Previous studies link LINC00963 with hepatocellular carcinoma and prostate cancer [13,14]. However, its role and mechanism in melanoma remain unclear. In this study, we aimed to investigate the potential function of LINC00963 in melanoma. We showed that LINC00963 is overexpressed in melanoma tissues and predicts poor prognosis. LINC00963 knockdown suppressed melanoma cell proliferation, migration and invasion through LINC00963/miR-608/NACC1 pathway. Our study suggests that LINC00963 might be a promising therapeutic target.

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2. Materials and methods

2.1. Patient tissue samples

A total of 37 melanoma samples and matched non-tumor tissues were collected from The First Hospital of Jilin University. Written informed consent was got from all participants. No patients received radiotherapy or chemotherapy before operation. Tissues were stored in liquid nitrogen. This study was approved by the Ethics Committee at The First Hospital of Jilin University.

2.2. Cell culture

Human melanoma cell lines, including A375 and A2508 cells were bought from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were cultured using DMEM medium supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) and maintained in a humidified atmosphere at 37 °C with 5% CO_2.

2.3. Cells transfection

siRNAs against LINC00963 (siRNA-1, 5′-GGGTCAAAGGAGAGCACAA-3’; siRNA-2, 5′−CCAGACACTGAACTGCCTT-3′) or NACC1 (5′-CGAATGAAATATTGAAGTA-3′), miR-608 mimics, miR-608 inhibitors and negative controls were purchased from RiboBio (Guangzhou, People's Republic of China). Cell transfection was conducted using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h, cells were collected and silencing or overexpressing efficiency was confirmed by qRT-PCR.

2.4. Cell proliferation assay

Cell proliferation was determined by CCK8 assay (Dojindo Molecular Technologies, Rockville, MD, USA) and colony formation assay. For CCK8 assay, 2000 cells were planted into 96-well plates and cultured for indicative times (1, 2 and 3 days), then 10 μl CCK8 solution was added and incubated for 2 h, followed by absorbance (OD 450 nm) measurement using a microplate reader (Thermo Labsystems, Waltham, MA, USA). For colony formation assay, 500 cells were seeded into 6-well plates and cultured for 14 days. Then clones were fixed and stained with 0.1% crystal violet for 30 min. Colony number was counted.

2.5. Transwell assay

Transwell assay was carried out to determine cell migration and invasion according to a previous study [9]. In brief, 1×10^5 cells in 200 μl serum-free medium were seeded into the upper chamber pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA) for invasion. 500 μl complete medium was added into the lower chamber. After incubation for 24 h, the cells in the upper chamber were removed and cells in the lower chamber were fixed with methanol and stained with 0.1% crystal violet for 30 min. Migrated or invaded cell number was counted using a light microscope.

2.6. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as described before [15]. In brief, total RNAs were isolated from tissues and cell lines using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Then RNA was inversely transcribed and relative expression was analyzed according to the $2^{-\Delta\Delta CT}$ method. U6 and GAPDH was serve as internal controls, as appropriate.

2.7. RNA pull-down by MS2-MBP

Maltose-binding protein (MBP)-affinity purification and pull-down assay was performed to identify the interaction between LINC00963 and miR-608 according to a previous study [16].

2.8. Dual luciferase reporter assay

To obtain reporter plasmid, the synthesized LINC00963 or NACC1 3′UTR sequence containing putative binding sequences (wild-type or all sites mutated) was inserted into pMIR-REPORT vector. Then reporter plasmid and miR-608 mimics were co-transfected into A375 cells. After 48 h, the luciferase activity was determined using Dual Luciferase Assay (Promega, WI, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

All data were analyzed using GraphPad Prism software (Version 6.0) or SPSS Version 13.0 software. The results were presented as mean ± SD. Student's t-test or one-way ANOVA was utilized to analyze significant difference. Survival rate was determined using Kaplan−Meier method and analyzed with the log-rank test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. LINC00963 upregulation in melanoma tissues

To identify critical lncRNAs involved in melanoma progression, the GEO dataset (GSE3189) about gene expression patterns in melanoma tissues and normal tissues was analyzed. We found that the expression of LINC00963 was upregulated nearly 3 times in melanoma tissues, compared to normal tissues (Fig. 1A). To confirm it, we further analyzed its expression in 37 pairs of melanoma tissues and non-tumor tissues. Results indicated that LINC00963 level was also increased in melanoma tissues (Fig. 1B). Moreover, higher expression of LINC00963 was observed in metastatic melanoma tissues (Fig. 1C), indicating LINC00963 might be an important factor for melanoma aggression. DNA copy number variation, including amplification also occurs in melanoma and contributes to tumor development by upregulating oncogenic gene expression [17]. Thus, we analyzed the correlation between DNA copy number amplification of LINC00963 and its expression in these 37 samples. Results indicated that LINC00963 DNA copy number was increased in some samples and positively correlated with its expression level (Fig. 1D), further suggesting an important role of LINC00963 in melanoma progression. Finally, we analyzed whether LINC00963 could serve as a prognostic biomarker using Kaplan-Meier curve. We found that higher level (median expression value as the cut-off) of LINC00963 predicted lower survival rate (Fig. 1E). All above imply that LINC00963 is involved in melanoma progression.

3.2. Effects of LINC00963 knockdown on malignant behaviors

Then, we knocked down LINC00963 to investigate its potential roles. qRT-PCR analysis confirmed the silencing efficiency of LINC00963 in A375 and A2058 cells (Fig. 2A). CCK8 and colony formation assays were conducted and showed that knockdown of LINC00963 significantly suppressed the proliferation and reduced colony numbers (Fig. 2B and C). Further, transwell assay was performed. Results showed that LINC00963 silence led to decreased cells of migration or invasion (Fig. 2D and E), which is consistent with our previous observation that LINC00963 expression is associated with melanoma metastasis (Fig. 1C).

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