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Clinical significance of lncRNA MIR31HG in melanoma

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Abstract. – **OBJECTIVE:** **The aim of this study was to explore the expression of long non-coding RNA (lncRNA) MIR31HG in malignant melanoma (MM), and to investigate its clinical significance.**

PATIENTS AND METHODS: **The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of lncRNA MIR31HG in MM tissues and cells. The relationship between lncRNA MIR31HG expression and the clinicopathological characteristics was analyzed. Furthermore, the cell counting kit-8 (CCK-8) and the transwell assays were performed to assess the effect of MIR31HG on cell proliferation and metastasis *in vitro*, respectively.**

RESULTS: **The expression of MIR31HG was significantly upregulated in MM tissues and cells. To explore the relationship between MIR31HG expression and clinical features, the patients were divided into two groups according to the mean expression of MIR31HG, including high expression group and low expression group. The subsequent results indicated that MIR31HG expression was correlated with lymph nodes metastasis, distal metastasis, and TNM stage. The multivariate analysis indicated that a high expression of MIR31HG could be used as an independent prognostic factor for MM. MIR31HG low-expression cells were constructed *in vitro*. Compared with the control cells, the cells with low expression of MIR31HG showed significantly low malignancy, including decreased cell proliferation rate and migration and invasion rates.**

CONCLUSIONS: **LncRNA MIR31HG was a novel factor involved in MM progression, which could be used as a potential biomarker and therapeutic target for MM.**

Key Words:
Malignant melanoma (MM), LncRNA MIR31HG, Proliferation, Invasion and migration.

Introduction

Malignant melanoma (MM) is a common malignant tumor caused by a massive proliferation of mutant melanocytes. The incidence of MM accounts for 4%-5% of all malignant tumors in human body[1,2]. Currently, the clinical treatment of MM includes extended surgical resection and lymph node dissection. However, distant metastasis occurs in a considerable number of MM patients, with a five-year survival rate lower than 20%[3]. Therefore, early diagnosis and treatment of MM are of great significance for patients.

For a long time, lncRNAs have been mistaken for the noise of genomic transcription without being valued[4,5]. With the development of the high-throughput technologies and gene chips in recent years, more and more lncRNAs have been discovered and annotated. It has been confirmed that they are not a dark substance in transcription; on the contrary, they exhibit enormous potential in the process of gene regulation in organisms[6]. The expression pattern of lncRNAs is spatial-temporal specific[7]. Meanwhile, they can participate in multiple cellular biological processes at various levels, such as epigenetic modification, gene transcription, post-transcriptional modification, and protein modification[8-12]. Therefore, exploring the potential molecular mechanisms of lncRNAs in MM may help to understand the pathogenesis of MM.

LncRNA MIR31HG, also known as LOC554202, is located on chromosome 9 of the human genome (9p21.3), with 2166 bp in length[13], and it plays different roles in different malignancies. Yang et al[14] have found that MIR31HG is upregulated in pancreatic cancer. The knockdown of MIR31HG expression can significantly inhibit the malignant behaviors of pancreatic cancer cells. In addition, MIR31HG can be used as an endogenous "sponge" to compete with microRNA-193b[14], thereby promoting cancer development. Sun et al[15] have demonstrated that the high expression of lncRNA MIR31HG in esophageal squamous cell carcinoma (ESCC) is

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associated with high cell growth and metastasis rate. Moreover, lncRNA MIR31HG has exhibited high diagnostic sensitivity and specificity for predicting ESCC occurrence. Otherwise, lncRNA MIR31HG may also act as a tumor suppressor gene in gastric cancer[16]. Meanwhile, its expression is associated with larger tumor size and advanced pathological stage. Furthermore, the prognosis of patients with lower lncRNA MIR31HG expression is remarkably worse. He et al[17] have indicated that lncRNA MIR31HG is low-expressed in bladder cancer and is negatively associated with TNM stage. All these findings reveal that MIR31HG may function as a cancer-suppressor gene.

In this study, we first examined the expression of MIR31HG in MM tissues. The relationship between MIR31HG expression and the clinicopathological data of MM patients was analyzed. After that, we constructed a cell model of MIR31HG knockout *in vitro*. In addition, the effects of MIR31HG on the biological behaviors of the cells were investigated, such as proliferation, migration, and invasion.

Patients and Methods

Tissue Specimens

This study was approved by the Ethics Committee of Jining No. 1 People's Hospital. The signed written informed consents were obtained from all participants before the study. The melanoma tissues and adjacent normal skin tissues were collected from melanoma patients who received treatment in our hospital. No patient received any chemotherapy, radiotherapy, and immunotherapy before the study. The collected specimens were frozen in liquid nitrogen at -80°C immediately after the surgery for RNA extraction. The clinico-pathological features of MM patients were also collected.

Cell Culture

Human Epidermal Melanocytes (HEMn) and MM cells (MEL-RM) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a sterile incubator at 37°C with 5% CO_2 and saturated humidity. Logarithmic phase cells in good condition were used for subsequent *in vitro* experiments.

Cell Transfection

The cells were first transferred into 6-well plates. Then, the cells were transfected with negative control or si-MIR31HG according to the instructions of Lipofectamine™ 2000 and OPTI-MEMI (Invitrogen, Carlsbad, CA, USA). After transfection for 48 h, the cells were harvested for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), cell counting kit-8 (CCK-8), and transwell assays.

QRT-PCR Analysis

The total RNA in cells and tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the extracted RNA was reverse transcribed into complementary deoxyribose nucleic acids (cDNAs) using the PrimeScript™ reverse transcription kit. With cDNA as a template, the quantitative PCR assay was carried out using a quantitative PCR instrument (PRISM7000; Applied Biosystems, Foster City, CA, USA) in accordance with the fluorescent quantitative PCR. The specific reaction conditions were as follows: 94°C for 10 min, and 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s, for a total of 40 cycles, followed by extension at 72°C for 10 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: lncRNA MIR31HG, F: 5'-GCTCGTAACACATATCCGACTG-3', R: 5'-GATCTGCAGGACCATCGTGGAACG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Proliferation

The cells in the logarithmic growth phase were collected and inoculated into 96-well plates at a density of 1×10^4 cells per well. The cell transfection was performed according to relevant instructions. After culture in an incubator for 24, 48, 72, and 96 h, respectively, CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well, followed by incubation for 2 h in the dark. The absorbance at 450 nm was measured by a microplate reader. The proliferation curves were plotted based on the average absorbance.

Cell Invasion and Migration

Cell invasion and migration were performed by the transwell assays (Corning, Corning, NY,

USA) with 8-μm-pore size membranes with Matrigel (for invasion assay) or without Matrigel (for migration assay). Briefly, 2×10^4 cells in serum-free medium were planted into the upper chambers. On the other hand, the lower chamber was added with complete medium containing 10% fetal bovine serum as a chemoattractant. After 24 h of incubation, the cells on the top of the membrane were wiped off. Subsequently, the membranes were fixed with 95% ethanol and stained with 0.2% crystal violet. Migrating or invading cells were observed under an inverted microscope. Five high-power fields (200×) were randomly selected for each sample, and the number of migrating or invading cells was counted.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The *t*-test was used for analyzing the measurement data. The Student's *t*-test was applied to compare the differences between the two groups. One-way ANOVA was used to compare the differences among different groups, followed by the post-hoc test (Least Significant Difference). The Chi-square test was performed for the association between lncRNA MIR31HG and clinicopathological parameters of MM patients. $p<0.05$ was considered statistically significant.

Results

LncRNA MIR31HG Was Highly Expressed in MM

The expression of MIR31HG in MM tissues and adjacent normal tissues was detected by qRT-PCR. The results showed that the expression level of MIR31HG in MM tissues was significantly higher than that of the adjacent normal tissues. Similar results were obtained at the cellular level (Figure 1). Based on the mean expression level of MIR31HG in MM tissues (2.366 ± 0.207), the patients were divided into two groups, including MIR31HG high-expression group (n=29) and low-expression group (n=26).

In addition, the relationship between MIR31HG expression and clinicopathological features of MM patients was analyzed. The statistical analysis showed that MIR31HG level was associated with lymph node metastasis, distal metastasis, and TNM stage ($p<0.05$). However, other parameters, including gender, age, and invasion, were not associated with EMR31HG level in MM ($p>0.05$) (Table I).

Multivariate Analysis of MIR31HG Expression and MM Clinicopathological Data

As shown in Table II, the multivariate analysis showed that high expression of MIR31HG, together with invasion, lymph nodes metastasis, distal metastasis, and TNM stage might function as an independent prognostic factor for MM. The results detected that MIR31HG played an important role in the development of MM, which could be used as a specific biomarker for poor prognosis.

Down-regulation of MIR31HG Limited Proliferation, Invasion, and Migration of MM Cells

To further explore the role of MIR31HG in MM metastasis, we constructed MIR31HG low-expression cell model by transfection of si-MIR31HG into MEL-RM cells (Figure 2A).

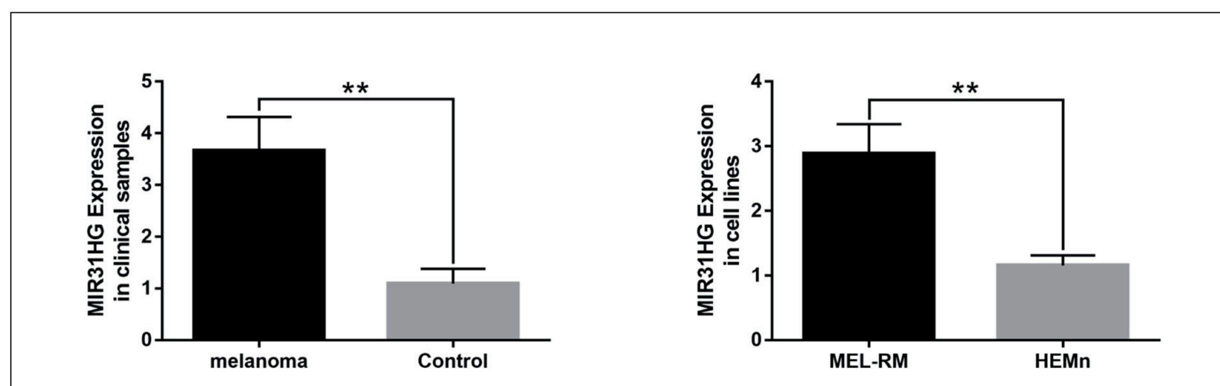

Figure 1. The expression of lncRNA MIR31HG in clinical samples and cell lines was detected by qRT-PCR. (**$p<0.01$).

Table I. Correlation between MIR31HG expression and clinical features.

Variable	No.	LncRNA MIR31HG		*p*-value
		High	Low	
No.	55	29	26	
Gender				0.841
Male	30	16	14	
Female	25	13	12	
Age(years)				0.077
< 50	33	14	19	
≥ 50	22	15	7	
Invasion				0.053
T0-T2	28	12	16	
T3-T4	27	17	10	
Lymph nodes metastasis				0.020
N0-N1	24	9	15	
N2-N3	31	20	11	
Distal metastasis				0.000
M0	43	19	24	
M1	12	10	2	
TNM stage				0.045
I-II	29	11	18	
III-IV	26	18	8	

In vitro results demonstrated that si-MIR31HG transfection could significantly inhibit the malignant behaviors of MM cells. The CCK-8 assay (shown in Figure 2B) indicated that the treatment of si-MIR31HG could significantly reduce the absorbance of MEL-RM cells at 450 nm. This suggested that si-MIR31HG transfection significantly inhibited the proliferation of MEL-RM cells. Subsequent transwell assay showed that the number of migration and invasion cells significantly decreased in the transfection group when compared with the control group (Figure 3).

Discussion

MM is a common malignant skin tumor. The pathogenesis of MM is complex and still remains unclear[18]. The genetic variation may be the main cause of the MM development[19]. Current researches have focused on MM at the genome level. However, few analyses have investigated the relationship between lncRNA and MM. Over the past decade, different roles of lncRNA in cell function have been widely studied. More attention has been paid to the role of lncRNAs in diseases,

Table II. Multivariate analysis of prognostic parameters in patients with melanoma by Cox regression analysis.

Variables	Category	*p*-value
Gender	Male	0.759
	Female	
Age (years)	< 50	0.391
	≥ 50	
Invasion	T0-T2	0.027
	T3-T4	
Lymph nodes metastasis	N0-N1	0.021
	N2-N3	
Distal metastasis	M0	0.008
	M1	
TNM stage	I-II	0.004
	III-IV	
MIR31HG expression	Low	0.027
	High	

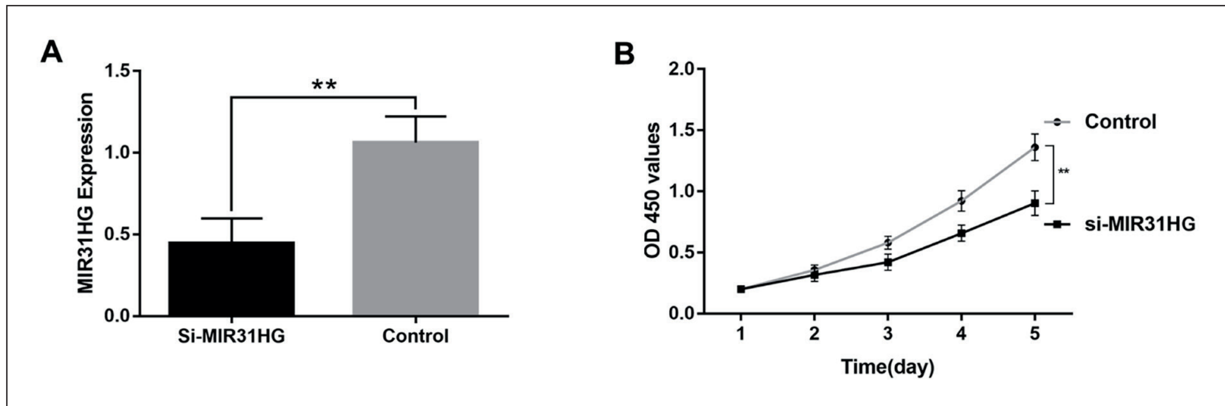

Figure 2. **A,** LncRNA MIR31HG was efficiently knocked-down by transfection of si-MIR31HG. (***p*<0.01).**w**Cell proliferation detected by CCK-8 assay. (***p*<0.01).

especially in the pathogenesis of malignant tumors. LncRNA SLNCR1 (mentioned in Schmidt et al[20]), together with brain-specific homeobox protein 3a (Brn3a) and androgen receptor (AR), was specifically required for the transcriptional activation of MMP9 and increased melanoma invasion. Wei et al[21] have indicated lncRNA UCA1 is significantly upregulated in MM tissues and cells. Inhibiting the expression of lncRNA UCA1 can inhibit the proliferation and invasion of MM cells. Further experiments have suggested that the miR-507-FOXM1 axis is a downstream target of lncRNA UCA1. Chen et al[22] systematically examined the function of lncRNA GAS5 in mediating MM metastasis. They have revealed that lncRNA GAS5 plays an anti-cancer role in MM *via* regulating gelatinase A and B both *in vitro* and *in vivo*. Therefore, screening lncRNA related to the pathogenesis and progress of MM may be helpful to elucidate the mechanism of MM. This is of great significance for the treatment of MM and the development of new drugs.

LncRNA MIR31HG has been previously reported to be deregulated in human cancers[14-17,23,24].

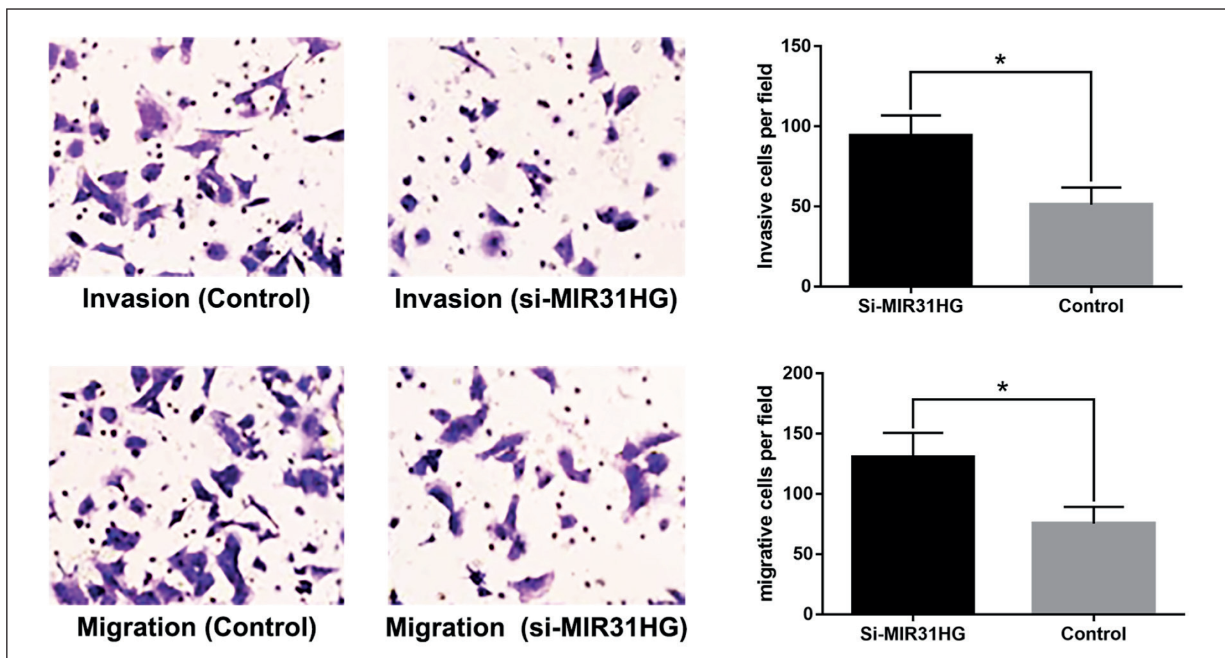

Figure 3. Cell invasion and migration detected by transwell assay and observed under a microscope (magnification × 200). (**p*<0.05).

However, the role of MIR31HG in MM and the relationship between MIR31HG expression and clinical characteristics of MM have not been fully elucidated.

In our study, lncRNA MIR31HG was found highly expressed in MM patients, which was consistent with Montes et al[25]. At the same time, we analyzed the relationship between the expression levels of MIR31HG with the clinical-pathological data of the MM patients. The results indicated that the high level of MIR31HG was positively correlated with lymph nodes metastasis, distal metastasis, and TNM stage. The multivariate analysis indicated that a high expression of MIR31HG might function as an independent prognostic factor for MM. Based on the high expression level of MIR31HG in the cells, we constructed a MIR31HG low-expression cell model *in vitro*. The biological effects of MIR31HG were analyzed *in vitro*. It was demonstrated that the downregulation of the expression of MIR31HG in MM cells could significantly affect cell proliferation, invasion, and migration. However, limited by the small clinical trial sample size, the specific role and molecular mechanism of MIR31HG in MM remained to be further studied.

Conclusions

We first indicated that MIR31HG is involved in the regulation of the occurrence and development of MM, which might be a potential biomarker and therapeutic target for MM patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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