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Original Article

The negative regulation of miR-149-5p in melanoma cell survival and apoptosis by targeting LRIG2

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Abstract: MicroRNAs (miRNAs) are key regulators of diverse biological processes in tumor progression including melanoma. LRIG2 is reported as an oncogene in cancer, however, little is known regarding the molecular and functions of LRIG2 in melanoma. In this study, we reported that LRIG2 expression was higher in melanoma tissues and cell lines and was regulated by miR-149-5p. Furthermore, a luciferase reporter assay and rescue experiment indicated that miR-149-5p directly targeted LRIG2 by binding its 3'UTR. The overexpression of miR-149-5p significantly suppressed melanoma cell proliferation, colony formation, and promoted cell apoptosis. These results suggest that miR-149-5p acts as a suppressing molecule and may be a good method for melanoma therapy.

Keywords: miR-149-5p, LRIG2, melanoma, cell proliferation, apoptosis

Introduction

Malignant melanoma is a skin cancer from the transformation of melanocytes. The prognosis of the melanoma patients is poor and the 5-year survival rate is very low [1, 2]. Recent studies have identified some potential target molecules in melanoma including oncogenes and tumor suppressors, which show promise to serve as therapeutic targets for the treatment of it [1, 2]. Leucine-rich repeats and immunoglobulin-like domains (LRIG) proteins are a family of integral membrane proteins [3-5]. The mammalian LRIG gene family is composed of three paralogues, LRIG1, LRIG2, and LRIG3 [5]. The best-studied family member, LRIG1, antagonizes growth factor signaling mediated and is suggested to be a tumor suppressor [6-8]. LRIG1 expression is associated with a favorable prognosis in many cancer types [6-9]. LRIG3 acts as a tumor suppressor in cancers [10, 11]. LRIG2 is reported as an oncogene in cancer [12-16]. However, little is known regarding the molecular and developmental functions of LRIG2 in melanoma.

Emerging evidence has indicated that microRNAs (miRNAs) are involved in gene regulation in various biological processes. MiRNAs are a class of short noncoding RNAs which negatively regulate the gene expression through binding to the 3' untranslational region (UTR) of their target mRNA, thus resulting in mRNA degradation or translational repression [17]. MiRNAs play regulatory roles in cell survival and proliferation, cell cycle progression, apoptosis, angiogenesis and tumorigenesis [17-19]. Moreover, aberrant upregulation or downregulation of miRNAs has been observed in melanoma, some of which have been confirmed to play key roles in the development and progression of this disease [17, 18]. However, the regulation of LRIG2 by miRNAs is not known.

In this study, we focused on the regulation and roles of LRIG2 by miRNAs in melanoma. The data showed that miR-149-5p regulated LRIG2 expression and promoted melanoma cell apoptosis by targeting LRIG2. The study supplied valuable epigenetic data on miR-149-5p in melanoma tissues and cells, which supports the use of miR-149-5p as a therapeutic molecule against melanoma.

Materials and methods

Melanoma samples

All samples including melanoma and their adjacent tissues were collected from the patients at

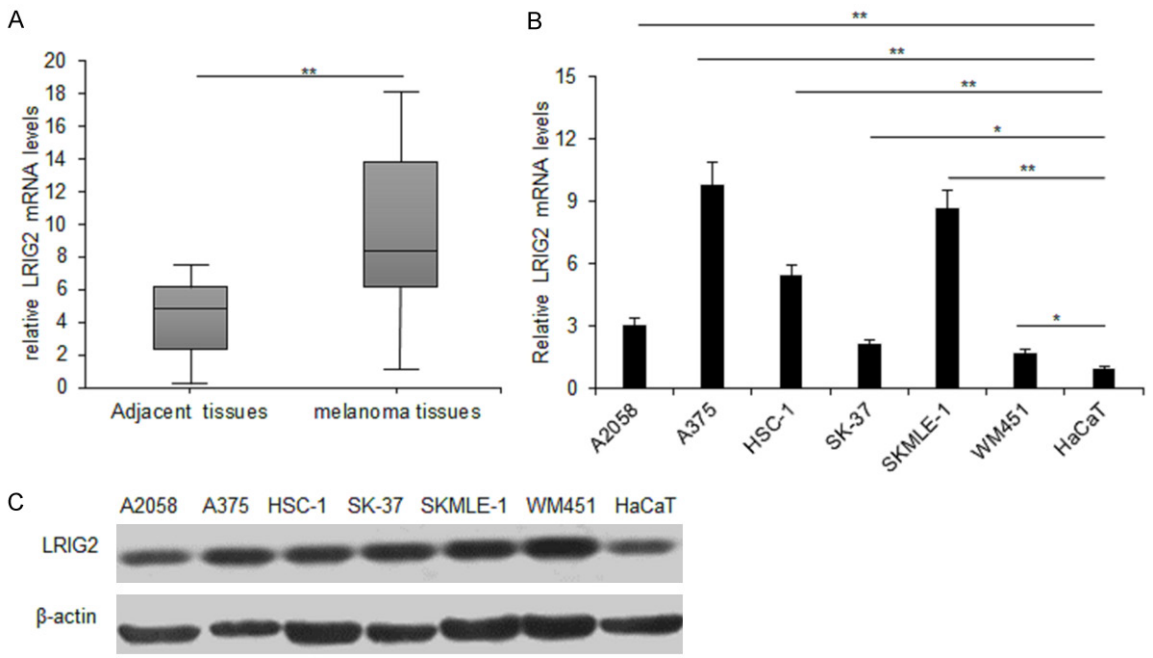


Figure 1. High levels of LRIG2 expression was shown in melanoma tissues and cell lines. A. Quantitative RT-PCR (QRT-PCR) was used to examine the mRNA levels of LRIG2 in melanoma tissues. B. Quantitative RT-PCR (QRT-PCR) was used to examine the mRNA levels of LRIG2 in melanoma cells. C. Western blotting was used to examine the protein levels of LRIG2 in melanoma cell lines. All the experiments were repeated for 3 times. $^{**}p < 0.01$ vs. normal controls and $^{*}p < 0.05$ vs. normal controls.

Nanjing First Hospital (Nanjing Medical University, Nanjing, China) after the patients provided written informed consent from the Ethics Boards of the hospital. The diagnosis was based on pathological evidence.

Cell culture

All melanoma cell lines used in the study were primarily obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured according to the standard protocols. The cells were incubated in a humidified incubator at 37°C with 5% CO_2.

MiRNA and siRNAs

MiR-149-5p mimics (miR-149-5p) and its negative control (miR-control), a miR-149-5p inhibitor and its control. LRIG2 siRNAs and its control were obtained from RiboBio (Guangzhou, China). Lipofectamine RNAiMAX or Lipofectamine 2000 (Invitrogen) was used for miRNAs or siRNA transfection.

Colony formation assay

Melanoma cells were transfected miR-149-5p or LRIG2 siRNAs and cultured in the normal condition. 200 cells were seeded in 6-well plates. The cells were cultured for 10 days, washed with 1 × PBS, fixed with 70% ethanol for 5 min and stained with 0.5% crystal violet for 3 min at room temperature. The colonies (>50 cells) were counted.

Cell proliferation

Melanoma cells were seeded in 6-well plates and transfected with miR-149-5p or miR-149-5p inhibitors or LRIG2 siRNAs and cultured in the normal condition. Cell survival ability was tested by the method of MTT (Sigma) assay.

Dual luciferase reporter assay

LRIG2 promoter activity was examined using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Cells were seeded in 24-well plates and transfected the LRIG2 3'UTR luciferase reporter, wild type or mutant reporter constructs and Renilla plasmid by using lipofectamine 2000 (Invitrogen). Luciferase activity were performed 48 hours post-transfection using the Dual luciferase assay system (Promega, WI).

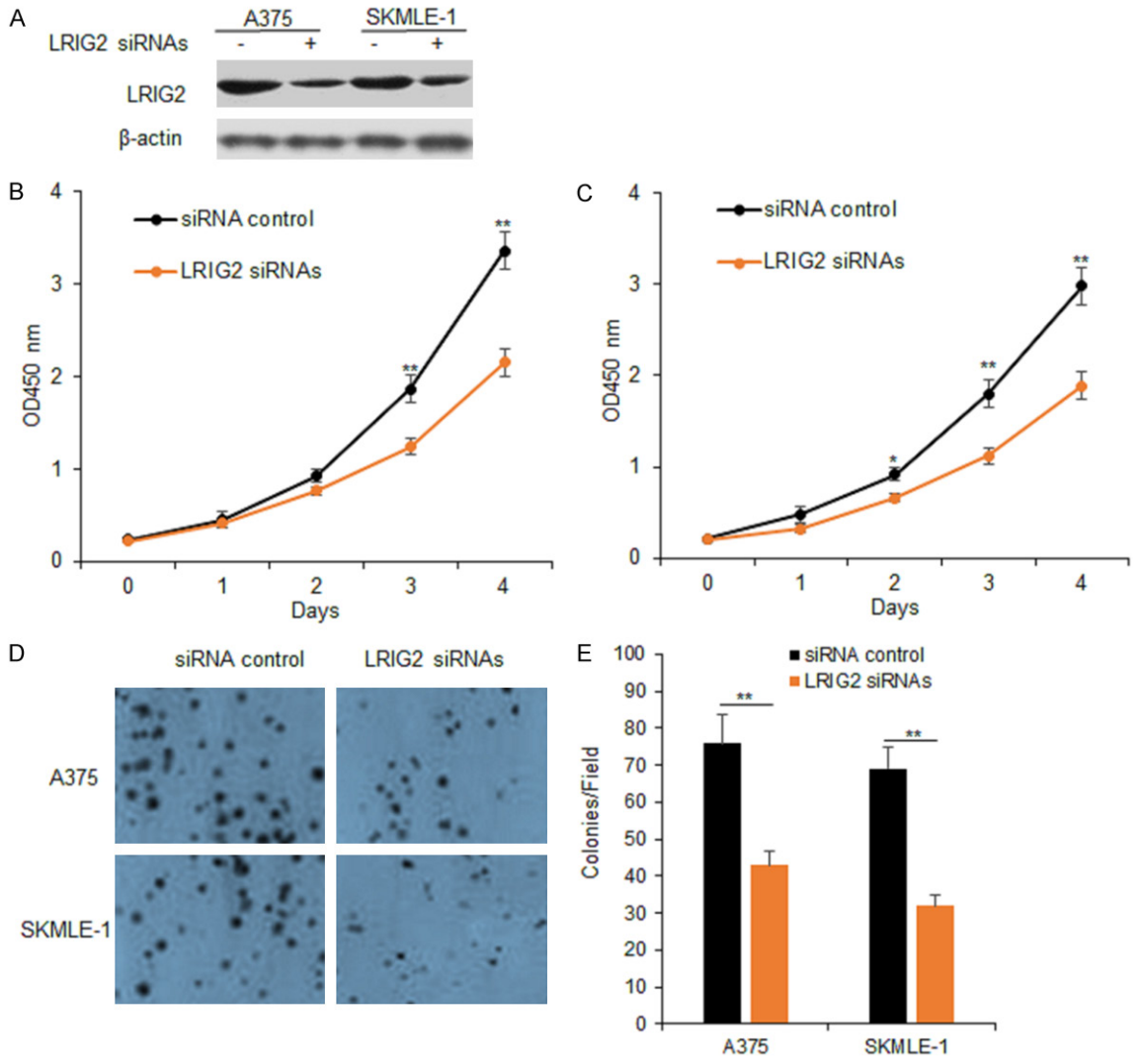


Figure 2. LRIG2 promoted melanoma cell growth. A. LRIG2 expression in melanoma cells. A375 and SKMLE-1 cells were transfected with LRIG2 siRNAs for 48 h and LRIG2 protein was detected by western blotting. B, C. Knocking down LRIG2 decreased A375 and SKMLE-1 cell proliferation. A375 and SKMLE-1 cells were transfected with LRIG2 siRNAs for 1 day and then cells were seeded in 96-well plates for MTT assay at 1, 2, 3 and 4 day. D, E. Knocking down LRIG2 decreased melanoma cell proliferation. A375 and SKMLE-1 cells were transfected with LRIG2 siRNAs for 1 day and then cells were seeded in 6-well plates (200 cells/well) for colony formation assay. **p < 0.01 vs. normal controls and *p < 0.05 vs. normal controls.

RNA extraction and real-time PCR analysis

Gastric cancer cells were transfected with miR-149-5p or LRIG2 siRNA or the controls for 48 h and then total RNA was isolated for Real time RT-PCR analysis. The expression level of miRNAs was defined based on the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, using the expression level of the U6 snRNA as a reference gene.

Sodium bisulfite modification and methylation-specific PCR (MS-PCR)

Total DNA from cell or tissues was isolated using Wizard DNA Purification Kit (QIAGEN, Valencia, CA) according to instruction. 10 ng DNA was used to PCR amplification.

Western blotting

Cultured cells were harvested and lysed with RIPA buffer containing the protease inhibitors

on ice for 30 mins. Protein was separated by SDS-PAGE. Protein was transferred onto nitrocellulose membrane using and probed with primary antibodies including LRIG2 or GAPDH and then horseradish peroxidase–labeled secondary antibodies. The protein band signals were visualized using an ECL.

Statistical analysis

All analysis were performed using the SPSS 15.0 or Excel. Every experiment was completed independently at least three times. A p value < 0.05 was considered significant.

Results

High levels of LRIG2 expression was shown in melanoma tissues and cell lines

To explore the biological relevance of LRIG2 in melanoma, LRIG2 mRNA levels in melanoma samples was examined using qRT-PCR. QRT-PCR results showed that LRIG2 mRNA levels were significantly higher in melanoma tissues compared to them in adjacent non-tumor tissues (**Figure 1A**). To confirm the results in melanoma cells, six melanoma cell lines including A2058, A375, HSC-1, SK-37, SKMLE-1 and WM451 were used to examine LRIG2 mRNA and it was shown that LRIG2 mRNA levels were higher in melanoma cell lines than HaCaT normal skin cells (**Figure 1B**). LRIG2 protein levels increased in most of melanoma cell lines, especially in A375 and SKMLE-1 cells (**Figure 1C** and **1D**).

LRIG2 promoted melanoma cell growth

To investigate the role of LRIG2 in melanoma cell proliferation, A375 and SKMLE-1 cells were transfected LRIG2 siRNAs. LRIG2 protein was down-regulated in A375 and SKMLE-1 cells (**Figure 2A**). MTT assay results showed that cell proliferation decreased in A375 and SKMLE-1 cells with LRIG2 down-regulation (**Figure 2B** and **2C**). The colony formation assay was used to verify the results in melanoma cells (**Figure 2D** and **2E**).

LRIG2 was a target gene of miR-149-5p in melanoma

The predicted miRNAs regulating LRIG2 expression were from Targetscan and miRbase databases. LRIG2 may be a target gene of miR-149-5p (**Figure 3A**). The detailed sequences of miR-149-5p targeting LRIG2 were shown in **Figure 3B**. The expression of miR-149-5p was down-regulated in all the melanoma cell lines comparing with HaCaT normal skin cells (**Figure 3C**). Consistently, miR-149-5p was lack of expression in melanoma tissues comparing with adjacent normal tissues (**Figure 3D**). These data indicated that miR-149-5p might function as an inhibitor of melanoma.

MiR-149-5p expression was methylated in melanoma

A375 and SKMLE-1 cells were treated with the histone deacetylase inhibitor trichostatin A (TSA) or DNA methylation inhibitor 5-aza-CdR (AZA). MiR-149-5p expression was increased in both A375 and SKMLE-1 cells after having treated with TSA or AZA (**Figure 4A** and **4B**). We also measured the methylation levels of melanoma tissues and adjacent non-tumor tissues by MS-PCR. The methylation level of GC tissues was higher than that of adjacent non-tumor tissues (**Figure 4C**).

MiR-149-5p inhibited cell growth by down-regulation of LRIG2 expression in melanoma cells

To elucidate whether miR-149-5p regulates LRIG2 expression in melanoma cells, miR-149-5p was introduced into the melanoma cells and miR-149-5p expression increased (**Figure 5A**). A wide typed LRIG2 3'-UTR and mutated LRIG2 3'-UTR vectors (including Mut1-Mut4) were constructed. A375 cells were co-transfected with above vectors and miR-149-5p and the result showed that the luciferase activity of the wide typed LRIG2 3'-UTR significantly decreased compared with the luciferase activity of the mutated LRIG2 3'-UTRs (**Figure 5B**). The qRT-PCR results showed that LRIG2 mRNA levels were decreased in the melanoma cells with miR-149-5p transfection (**Figure 5C**). The western blotting results showed that LRIG2 protein levels were decreased in the melanoma cells with miR-149-5p transfection (**Figure 5D**). These results strongly indicate that the LRIG2 was a direct target of miR-149-5p. Next, the melanoma cells were transfected with miR-149-5p and LRIG2, cell survival ability was assayed by colony formation. It was shown that miR-149-5p overexpression could inhibit colony

A

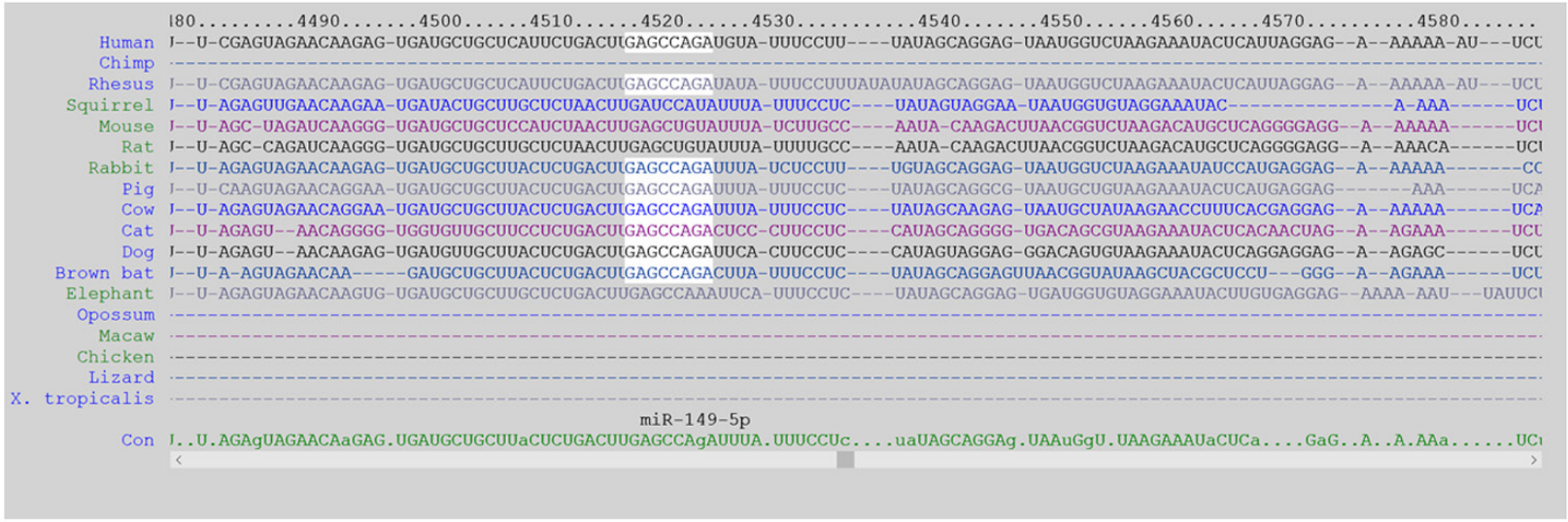
Conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 4519-4526 of LRIG2 3' UTR	5' ...UGCUCAUUCUGACUUGAGCCAGA...	8mer	-0.34	97	-0.31	3.248	N/A
hsa-miR-149-5p	3' CCCUCACUUCUGUGCCUCGGUCU						

Poorly conserved

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 1543-1550 of LRIG2 3' UTR	5' ...CUGUGACAUGAAUUGGAGCCAGA...	8mer	-0.37	98	-0.34	0.236	N/A
hsa-miR-149-5p	3' CCCUCACUUCUGUGCCUCGGUCU						
Position 4907-4913 of LRIG2 3' UTR	5' ...CAGAAGAUAGCCUGUGAGCCAGU...	7mer-m8	-0.19	91	-0.18	1.044	N/A
hsa-miR-149-5p	3' CCCUCACUUCUGUGC----CUCGGUCU						
Position 5305-5311 of LRIG2 3' UTR	5' ...GUCUGGUCUCCUAAAGAGCCAGG...	7mer-m8	-0.11	83	-0.10	0.236	N/A
hsa-miR-149-5p	3' CCCUCACUUCUGUGCCUCGGUCU						

B



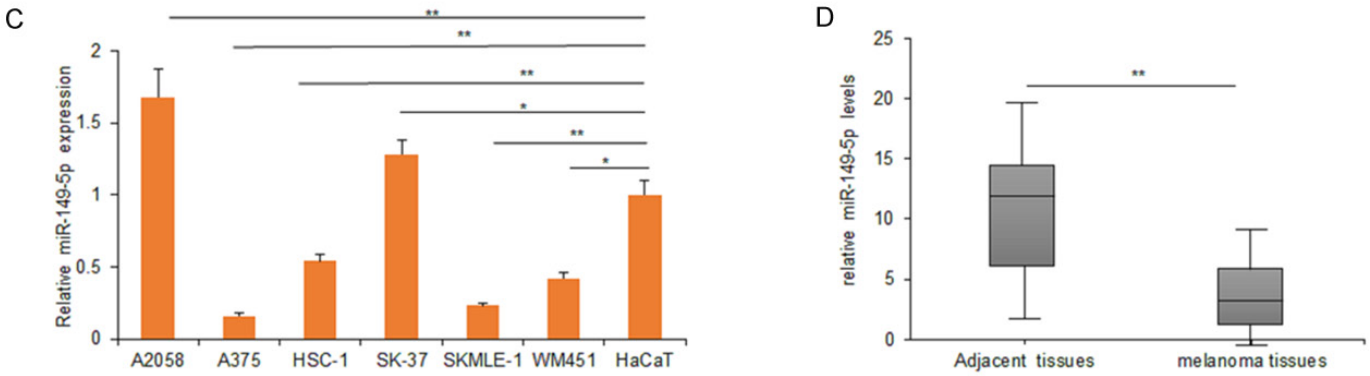


Figure 3. LRIG2 was regulated by miRNAs in melanoma cells. A. The predicted miRNAs targeting LRIG2 from the databases of Targetscan and miRbase. B. The detailed sequences of miR-149-5p targeting LRIG2. C. Real time RT-PCR was used to examine the expression of miR-149-5p in melanoma cells. D. Low levels of miR-149-5p expression in melanoma tissues. Real-time PCR was used to examine the levels of miR-149-5p in melanoma tissues and their adjacent tissues. **$p < 0.01$ vs. normal controls and *$p < 0.05$ vs. normal controls.

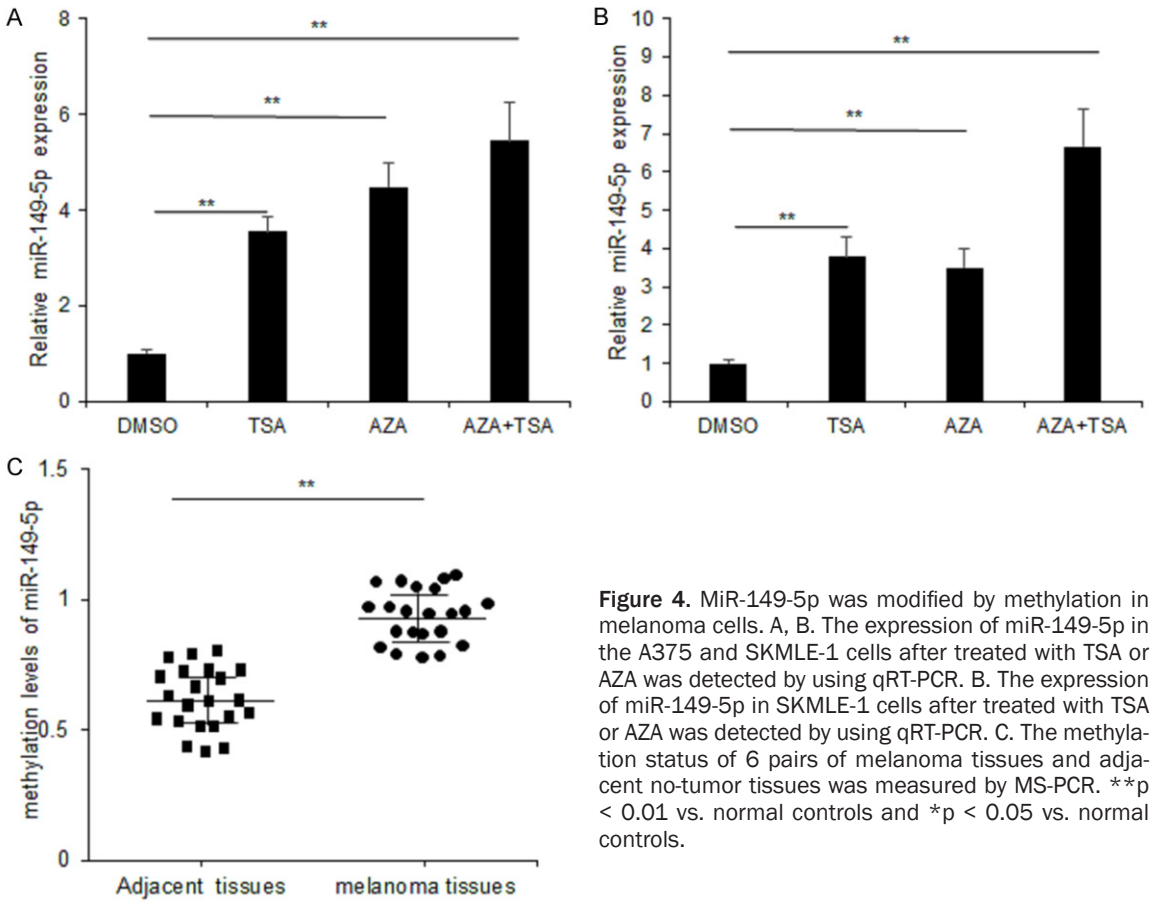


Figure 4. MiR-149-5p was modified by methylation in melanoma cells. A, B. The expression of miR-149-5p in the A375 and SKMLE-1 cells after treated with TSA or AZA was detected by using qRT-PCR. B. The expression of miR-149-5p in SKMLE-1 cells after treated with TSA or AZA was detected by using qRT-PCR. C. The methylation status of 6 pairs of melanoma tissues and adjacent no-tumor tissues was measured by MS-PCR. **p < 0.01 vs. normal controls and *p < 0.05 vs. normal controls.

formation in melanoma cells, also inhibited LRIG2 enhanced colony formation rates (**Figure 5E** and **5F**). Cell apoptosis was also texted by flow cytometry. The data indicated that miR-149-5p overexpression could promoted apoptosis of melanoma cells, also promoted LRIG2 decreased cellular apoptosis stimulated by drug (**Figure 5G** and **5H**). The results further verified that miR-149-5p suppressed melanoma cell growth via targeting LRIG2 expression.

Discussion

In the present work, it was demonstrated that LRIG2 promoted melanoma cell proliferation. Moreover, bioinformatics analysis and luciferase activity assay demonstrated that LRIG2 was a direct target genes of miR-149-5p in melanoma cells. The miR-149-5p mimics can significantly decrease protein expression level of LRIG2 in melanoma. Our results showed that miR-149-5p functioned as a negative regulator by targeting LRIG2 in melanoma.

LRIG2 could function as an oncogene in glioblastoma [13, 14], oligodendoglioma [15] and non-small cell lung cancer [16]. In glioblastoma, LRIG2 was overexpressed and promoted cell proliferation, metastasis and induced apoptosis resistance [13, 14]. It was found that LRIG2 expression levels were positively correlated with the grade of glioma and soluble LRIG2 was capable of being released from glioblastoma cells and exerted a pro-proliferative effect by interacting with EGFR and activating its downstream PI3K/Akt pathway. Furthermore, the patients with high LRIG2 cytoplasmic expression showed short survival times, and the five-year survival rate for patients with high LRIG2 expression was lower than the survival rates in patients with low LRIG2 expression, indicating that LRIG2 expression levels may have a potential role in the pathogenesis of NSCLC, and also a significant prognostic value [16]. Our study showed that LRIG2 expression was higher in melanoma cells and tissues than their controls.

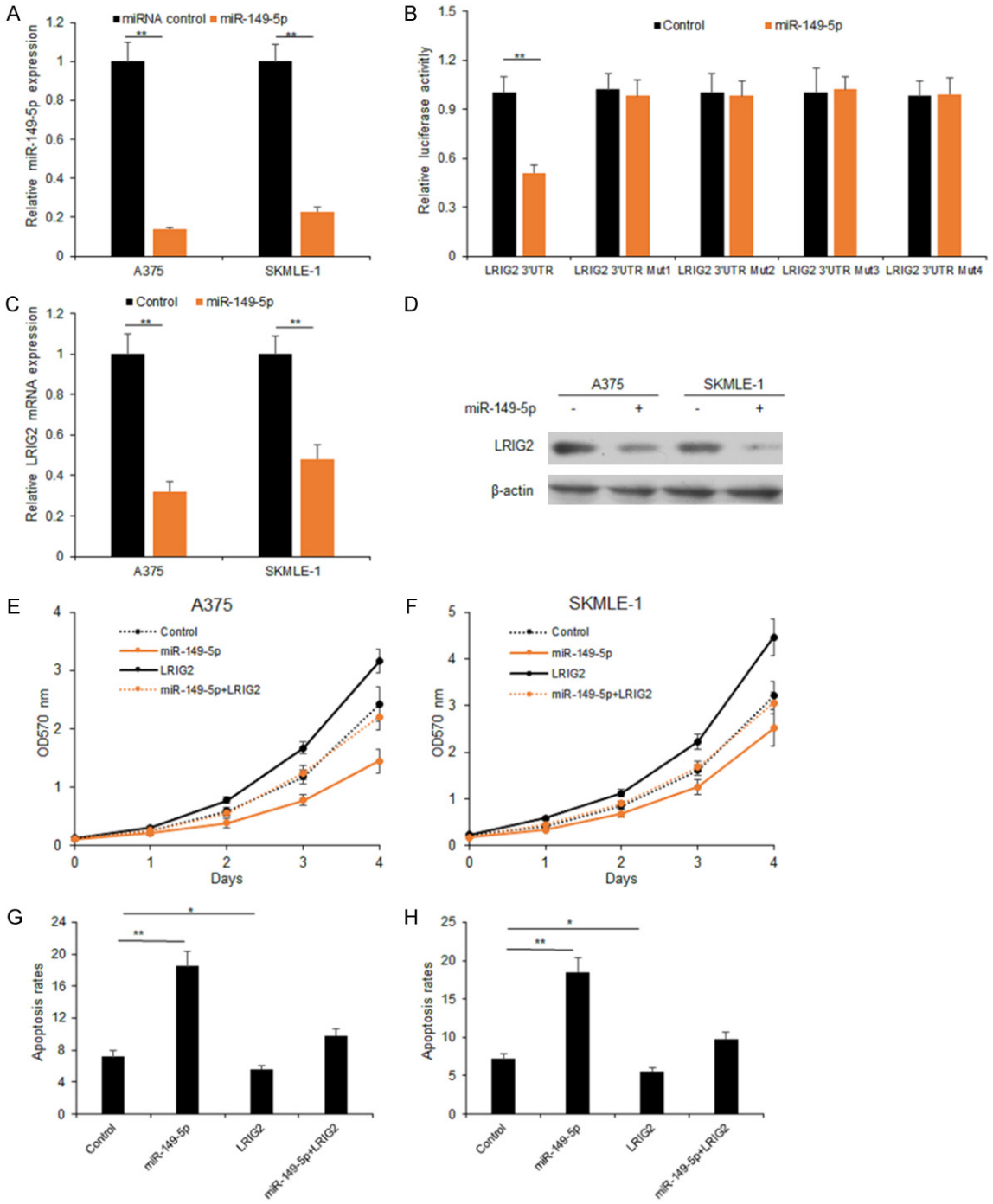


Figure 5. MiR-149-5p inhibited cell growth by down-regulation of LRIG2 expression in melanoma cells. A. miR-149-5p expression in the melanoma cells with miR-149-5p transfection. MiR-149-5p expression was examined by qRT-PCR. B. The influence of miR-149-5p on the luciferase activity of LRIG2 3'UTR in melanoma cells. A375 cell were co-transfected with miR-149-5p and the wide type or mutated type of LRIG2 3'UTR luciferase vectors for 36 h. The luciferase activity was analyzed using the dual-luciferase assay kit. C. LRIG2 mRNA decreased in the melanoma cells with miR-149-5p transfection. mRNA levels were examined by qRT-PCR. D. LRIG2 protein decreased in the A375 and SKMLE-1 cells with miR-149-5p overexpression. Protein levels were examined by western blotting. E, F. The effect of miR-149-5p on melanoma cell proliferation. A375 and SKMLE-1 cells were transfected with miR-149-5p or LRIG2 for 24 h, and then the cells were seeded in the 6-well plates for MTT assay. G, H. The effect of miR-149-5p on the apoptosis of melanoma cells. A375 and SKMLE-1 cells were transfected with miR-149-5p or LRIG2 for 24 h, and then the cells were seeded in the 6-well plates for apoptosis assay. **p < 0.01 vs. normal controls and *p < 0.05 vs. normal controls.

LRIG2 could promote melanoma cell proliferation, which was regulated by miR-149-5p.

Some dysregulated miRNAs are reported in melanoma [17, 18]. For example, miR-29b promotes melanoma invasiveness [19]. MiR-320 cluster acts as a tumor suppressor in melanoma [20]. MiR-7 reverses the resistance to BRAF inhibitor in melanoma by targeting EGFR/IGF-1R/CRAF and inhibiting the MAPK and PI3K/AKT signaling pathways [21]. MiR-330-5p regulates PDIA3 expression and suppresses cell proliferation and invasion in cutaneous malignant melanoma [22]. MiR-339-3p acts as a tumor suppressor in melanoma [23]. MiRNA-211 functions as a metabolic switch in human melanoma cells [25]. In our present study, functional analyses revealed that miR-149-5p suppressed cell growth and metastasis in melanoma, indicating that miR-149-5p could function as a suppressor in melanoma through LRIG2. Previous studies indicate that miR-149-5p regulates the DP5 and PUMA expression in human pancreatic beta cells [26] and miR-149-5p is associated with cellular migration, proliferation and apoptosis in renal cell carcinoma [27]. Here, we presented that miR-149-5p expression was lower in melanoma cells and suppressed cell survival ability,

In summary, our data demonstrated that LRIG2 was overexpressed in melanoma cells and tissues. Down-regulation of LRIG2 inhibited melanoma cell proliferation and metastasis. LRIG2 was regulated by miR-149-5p, which was modified by methylation in melanoma cells. We propose that miR-149-5p may be beneficial for melanoma therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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