

MiR-431 is a prognostic marker and suppresses cell growth, migration and invasion by targeting NOTCH2 in melanoma

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Abstract. – OBJECTIVE: Aberrantly expressed microRNAs (miRNAs) are comprehensively involved in oncogenesis. A tumor-associated miRNA, miR-431, has been shown to play a functional effect in several tumors. However, the studies on the effects of miR-431 in melanoma were limited. The present study aimed to determine the levels of miR-431 in melanoma and to explore its clinical significance and potential function in melanoma carcinogenesis.

PATIENTS AND METHODS: Aberrant miRNAs in melanoma tissues were studied via miRNA microarray. MiR-431 expression in melanoma cell lines and carcinomas tissues were detected using RT-PCR. The clinical data were interpreted by the Chi-square test, Kaplan-Meier analysis, and multivariate analysis. The cell count kit (CCK-8) assay, flow cytometry wound healing, and transwell assays were used to assess the possible influence of miR-431 on tumor ability. The potential targets of miRNA-431 were predicted using an online tool and demonstrated by the use of dual luciferase assay and Western blot analysis.

RESULTS: We observed that miR-431 expression was down-regulated in melanoma cells and tumor tissues, and reduced miR-431 levels were related to ulceration and tumor stage. The survival data revealed that melanoma patients with lower miR-431 suffered poorer overall survival. Multivariate analysis confirmed that miR-431 may be an independent prognostic marker for melanoma patients. Functional studies showed that miR-431 down-regulation inhibited melanoma growth and metastasis *in vitro*, while its overexpression has the opposite effects. Furthermore, we identified NOTCH2 as a direct target gene of miR-431 in melanoma cells. Besides, the restoration of NOTCH2 significantly reversed the inhibitory effects of miR-431 on melanoma cells growth and metastasis.

CONCLUSIONS: Our observation suggested that miR-431 could be a new therapeutic target and prognostic marker of melanoma.

Key Words:

MiR-431, NOTCH2, Melanoma, Growth, Metastasis, Biomarker.

Introduction

Melanoma is one of the most common tumors from skin cancer and one of the most aggressive forms of skin cancer with high patient mortality¹. The annual incidence of melanoma is five to seven cases per million populations². Despite recent advances in molecularly targeted therapy and immunotherapy, people with distant metastasis receive poor prognosis, with a 5-year survival rate of only 16%^{3,4}. Up to date, as the dissemination and initiation mechanisms of melanoma remain unclear, there have not been any effective therapeutic approaches to metastatic diseases^{5,6}. Hence, the discovery and application of new biomarkers and targets are of great importance to provide melanoma patients with an earlier and more accurate diagnosis, as well as a more effective treatment.

MicroRNAs (miRNAs) are a kind of endogenous small non-coding RNAs with a total length of about 19-25 nucleotides⁷. It has been confirmed that miRNAs play a negatively regulating role in the expression of genes at post-transcription level, which binds to mRNA transcripts' 3'-untranslated region (3'-UTR) and targets these transcripts for degradation⁸. Growing evidence indicates that miRNAs acts as potential tumor inhibitors or oncogenes, which may well be applied to the regulation of tumor cell metastasis, invasion, and proliferation, thus suppressing angiogenesis and cell apoptosis⁹⁻¹¹. In addition, many studies¹²⁻¹⁴ reported that these small non-coding RNAs can also play a role to help tumor patients screen, discover

and treat their tumors in an early stage. Up to date, the deregulated miRNAs and their roles in several tumor developments have also attracted much attention, and a set of miRNAs has been implicated in tumor carcinogenesis and progression^{15,16}. However, the dysregulated miRNAs and their biological function in melanoma remain largely unclear.

As a newly studied miRNA, miR-431 was located on 14q32.2 and acted as a tumor regulator in several common tumors, such as lung cancer and hepatocellular carcinoma^{17,18}. Current evidence revealed miR-431 as a tumor suppressor due to its frequent down-regulation in studied tumors. In addition, functional investigations with *in vitro* assays confirmed the tumor suppressive roles of miR-431 in papillary thyroid carcinoma, lung cancer, and colorectal cancer¹⁹⁻²¹. However, the evidence about whether miR-431 was abnormally expressed in melanoma was limited, and its biological function in this skin tumor has not been investigated. In this study, we provided more evidence that miR-431 expression was also down-regulated in melanoma. Then, the clinical value of miR-431 and its possible regulatory effects in melanoma was also functionally clarified.

Patients and Methods

Specimens Collection

Tissue samples were collected from patients with melanoma at the People's Hospital of Rizhao from March 2010 to August 2013. After signing written informed consent, a total of 113 paired melanoma tissue specimens from patients were collected. The protocols were approved by the Ethics Committee of People's Hospital of Rizhao. None of the patients received anti-cancer therapy before surgery. Table I summarized the detailed patient demographic information. The tissues were immediately preserved at -80°C after resection.

Cell Culture and Transfection

A875, HBL, 1205Lu, A375, SK-MEL-1, HE-Ma-LP (served as control cells), and CHL-1 cells were brought from Hitogene Biological Company (Qingdao, Shandong, China). The cells were maintained at 37°C with 5% CO₂ and cultured in 10% FBS. Lipofectamine 2000 reagent kits were used for cell transfection. The miRNA mimics and inhibitors were all obtained from Yuhon Biotechnology Company (Nanjing, Jiangsu, China). NOTCH2 was constructed into the pcDNA3.1

empty vector to overexpress NOTCH2 by Anjie Biological Company (Yangpu, Shanghai, China).

Quantitative Real Time-PCR Analyses

Total RNAs were extracted using RNA isolation kits (Hongene, Wuhan, Hubei, China). Then, cDNA was prepared with cDNA synthesis kits (Hongene, Wuhan, Hubei, China), followed by conducting qPCR analyses using SYBR Green qPCR kits (YingrunBio, Changsha, Hunan, China). The miRNA extraction and measurement were separately conducted using Qiagen miR-Neasy mini kits (Weicell, Nanjing, Jiangsu, China) and miRNA qPCR assay kits (DiyaoBio, Hefei, Anhui, China), respectively. The calculation of relative mRNA or miRNA expressing changes were performed using the 2^{-ΔΔCt} method. The expression of gene or miRNA was normalized to GAPDH or U6, respectively. The qPCR primes were presented in Table II.

Cell Proliferation Analyses

Melanoma cells were plated into 96-well plates in triplicate (2000 cells/well). Next, the cell count kit (CCK-8) reagent (10 μl/well; JinKeBio, Dalian, Liaoning, China) was added into the plates at 0, 24, 48, and 72 h. After incubation for an additional 2 h, the optical density (OD) was examined using a microplate reader at a wavelength of 450 nm.

Colony Formation Assays

Twenty-four hours post-transfection, 2000 cells were seeded into 6-well plates. RPMI-1640 media (with 10% FBS) was used to culture the cells for 15 days. Until the colonies were visible, the crystal violet (0.1%) was applied to be incubated with the colonies. After being washed with PBS, the cell colonies were observed by a microscope.

Cell Apoptosis Analysis

The melanoma cells were transfected with miRNA mimics or inhibitors. After 48 h, the

Table I. Primers for qPCR-PCR in this study.

Name	Sequence (5'-3')
miR-431(forward)	TGTCTTGCAGGCCGTCATG
miR-431 (reverse)	GCTGTCAACGATACGCTACCTA
NOTCH2(forward)	CCTTCCACTGTGAGTGTCTGA
NOTCH2 (reverse)	AGGTAGCATCATTCTGGCAGG
GAPDH (reverse)	ACCCACTCCTCCACCTTTG
GAPDH (forward)	CTCTTGCTCTTGTCTGGG

Table II. Correlation between miR-431 expression and different clinicopathological features in patients with hepatocellular carcinoma

Variable	Number	miR-431 expression		p-value
		High	Low	
Age				0.390
≤60	59	28	31	
>60	54	30	24	
Sex				0.962
Male	66	32	32	
Female	47	24	23	
Tumor thickness (mm)				0.167
≤2.0	67	38	29	
>2.0	46	20	26	
Ulceration				0.048
-	72	42	30	
+	41	16	25	
Lymph node metastasis				0.061
-	83	47	36	
+	30	11	19	
Tumor stage				0.027
I/II	77	45	32	
III/IV	36	13	23	

cells were collected in 350 µl PBS. The apoptotic rates of the treated cells were then assayed by staining using AnnexinV-FITC/PI double reagent (TianHaoBio, Binhai, Tianjin, China) on a flow cytometer system.

Caspase 3/9 Activity Analysis

Forty-eight hours post-transfection, the melanoma cells were collected and lysed using lysis buffer provided in the Beyotime Caspase 3/9 activity detection kits (LaiboBio, Jinan, Shandong, China). After collected the supernatant (by centrifuging), an appropriate volume of Ac-DEVD-pNA solution was added into the supernatant. After 20 min of incubation, the optical density (OD) was examined using a microplate reader at a wavelength of 405 nm.

Wound Healing Assays

The melanoma cells were treated with miRNA mimics or inhibitors until they reached the 95% confluence in 24-well plates. Then, the cell monolayer was disrupted by scraping with a 200 µl pipette tip. The floating cell debris was washed out, and a microscope was employed to capture the wound closure at 0 h and 48 h post-scraping.

Transwell Assays

The melanoma cells (1×10^5) in 200 µl media without serum were placed in the upper Costar

transwell chambers (8 µm; JeoniBio, Hangzhou, Zhejiang, China) with Matrigel. The media (650 µl) with 15% FBS was then added into the lower chamber. The cells were allowed to invade through the membranes, and crystal violet (0.1%) was applied to be incubated with the membranes. After being washed with PBS, the invaded cells were observed by the use of a microscope.

Luciferase Activity Analyses

The wild-type (WT) or mutant-type (MUT) that predicted miR-431 targeting site in the 3'UTR of NOTCH2 were separately constructed into pGL3 empty vector as luciferase reporter plasmids by Dongao Biological company (Xi'an, Shanxi, China). Then, HEK293T cells were co-transfected with WT or MUT luciferase reporter plasmids and pRL-TK Renilla luciferase plasmids, along with miR-431 mimics or NC mimics. After 48 h, the luciferase activity of each group was assayed using Promega Dual-Luciferase reporter kits (Kaicheng, Jinan, Shandong).

Statistical Analysis

The statistical analyses were performed using SPSS 20.0 (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA). The differences between the values were statistically analyzed using the Student's *t*-test. The overall survival was determined by the Kaplan-Meier method and

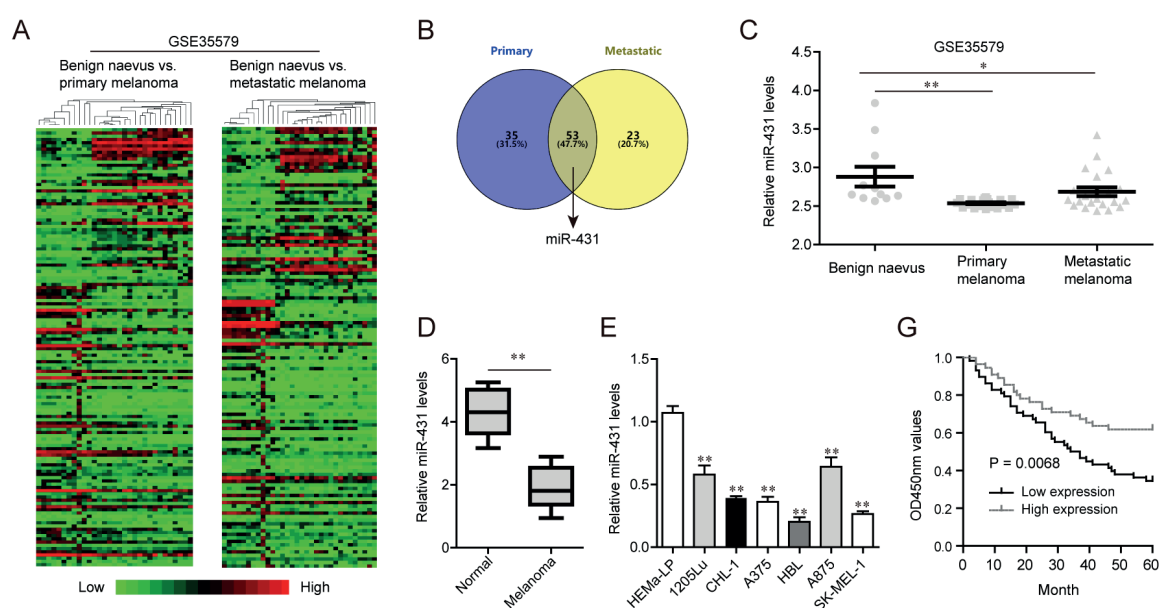


Figure 1. Decreased expression of miR-431 predicted poor prognosis in melanoma. **A**, The significantly differentially expressed miRNAs in melanoma tissues reflected by heat map. **B**, **C**, miR-431 was highly expressed in both two primary melanoma tissues and metastasis tissues. **D**, Measurement of miR-431 expression in 113 melanoma specimens and adjacent normal skin tissues using RT-PCR. **E**, Comparing differences in the expression levels of miR-431 between melanoma cell lines and the normal HEMA-LP cells. **G**, Melanoma patients with high miR-431 expression had better 5-year overall survival. * $p < 0.05$, ** $p < 0.01$.

log-rank test. The relationships between miR-431 and clinicopathologic characteristics were determined using the Chi-square test. The factors with a value of $p < 0.05$ in univariate assays were used in the subsequent multivariate assays. The p -values of less than 0.05 were considered statistically significant.

Results

Decreased MiR-431 Expression in Melanoma Tissues and Cell Lines

Firstly, differentially expressed miRNAs were identified in melanoma biopsies and normal skin tissues using microarray data from GSE35579. As shown in Figure 1A, Heat map demonstrated differently expressed miRNAs, and 352 of them were identified. Among these miRNAs, miR-431 was confirmed to be one of the most significantly downregulated miRNAs (Figures 1B and 1C). Then, the RT-PCR was performed to determine the levels of miR-431 in 113 pairs of melanoma tissues and matched normal tissues to further demonstrate the results of microarray data. The result showed that miR-431 expression levels were significantly lower in melanoma tissues compared with non-cancerous skin tissues ($p <$

0.01). Moreover, it was also observed that, compared with the normal skin cells HEMA-LP, all six melanoma cell lines which were determined using RT-PCR expressed a low level of miR-431. Overall, our findings revealed that miR-431 was downregulated in melanoma.

Clinical Significance of MiR-431 Expression in Melanoma

For a better understanding of the potential clinical utility of miR-431 low expression, our group divided the 113 melanoma patients into a high expression group ($n=58$) and a low expression group ($n=53$) based on the median levels of miR-431 in all tumor tissues. Then, the Chi-square test was used for exploring the associations between miR-431 levels and clinicopathological features and the results showed that high miR-431 levels were distinctly associated with ulceration ($p = 0.048$) and tumor stage ($p = 0.027$), indicating that the clinical progress of melanoma patients may be influenced by miR-431. Moreover, the Kaplan-Meier analysis and log-rank test were used for the validation of the prognostic value of miR-431 in melanoma patients, and the data suggested that patients with a high expression level of miR-431 had shorter overall survival than those with a low expression level of miR-431 ($p = 0.0068$). Finally,

by using univariate and multivariate analyses, we confirmed that miR-431 (HR=3.019, 95% CI: 1.201-3.792, $p = 0.008$) was a potential independent prognosis factor in melanoma.

MiR-431 Depressed Proliferation and Accelerated Apoptosis of Melanoma Cells

To figure out the biological roles of miR-431 in modulating melanoma cell proliferation and apoptosis, miR-431 mimics or inhibitors were transfected into HBL and SK-MEL-1 cells. Real Time-PCR analyses demonstrated that miR-431 mimics transfection could elevate the miR-431 levels and that the transfection of miR-431 inhibitors was able to suppress the expression of miR-431 (Figure 2A). CCK-8 assays revealed that cell proliferation was remarkably impeded

in melanoma cells after treated with miR-431 mimics, while silencing miR-431 could restore the proliferative abilities of HBL and SK-MEL-1 cells (Figures 2B and C). Similar results were also observed by using the colony formation assays that forced the expression of miR-431 which significantly reduced the colony number, while the transfection of miR-431 inhibitors could markedly increase the number of cell colonies (Figure 2D). Subsequently, the influence of miR-431 on cellular apoptosis was determined by flow cytometry. The data suggested that cell apoptosis was notably increased in HBL and SK-MEL-1 cells after transfection with miR-431 mimics, whereas the apoptotic rates significantly decreased in miR-431 inhibitors-transfected-melanoma cells (Figure 2E). In addition, caspase 3/9 activity

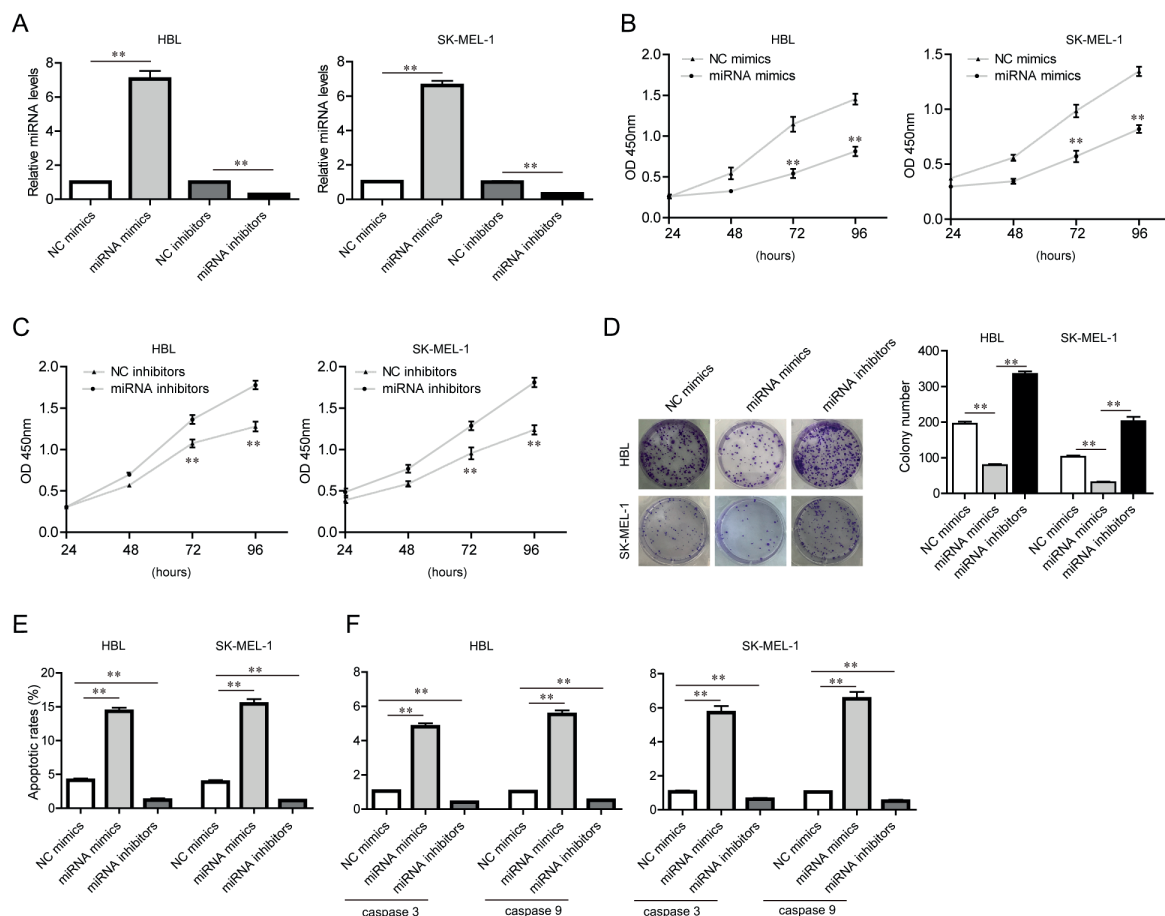


Figure 2. Upregulation of miR-431 expression depressed cell proliferation ability and promoted cell apoptosis. **A**, Real-time PCR analysis showed that miR-431 levels were effectively up-regulated after transfection with miR-431 mimics, and down-regulated after transfection with miR-431 inhibitors. **B**, **C**, The cellular growth of HBL and SK-MEL-1 cells after transfection with miR-431 mimics or inhibitors was evaluated by CCK-8 assays. **D**, Clonogenic assay illustrated the colony number in HBL and SK-MEL-1 cells of miR-431 overexpression or knockdown (magnification: 10 ×). **E**, Flow cytometry analyzed the apoptotic rates of HBL and SK-MEL-1 cells. **F**, Caspase 3/9 activity determination. * $p < 0.05$, ** $p < 0.01$.

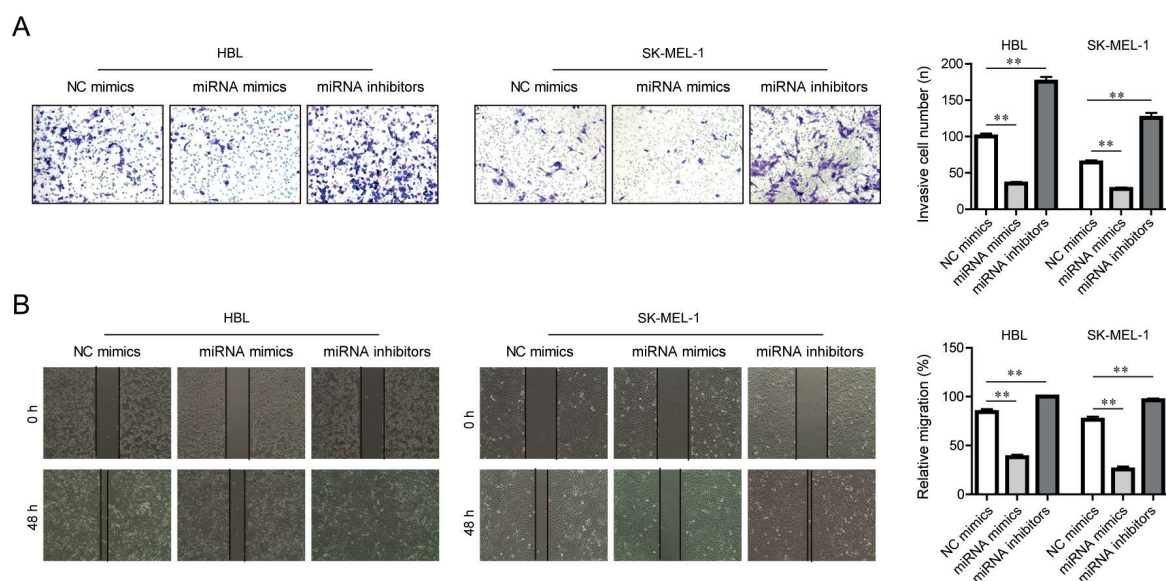


Figure 3. MiR-431 affected the metastasis of melanoma cells. **A**, The invasion capabilities of HBL and SK-MEL-1 cells were examined by the transwell assays (magnification: 40 ×). **B**, Migratory abilities were determined in melanoma cells via wound healing assays (magnification: 10 ×). * $p < 0.05$, ** $p < 0.01$.

Table III. Univariate and multivariate analyses of prognostic factors in melanoma patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Age	1.472	0.782-2.173	0.372	-	-	-
Sex	1.782	0.829-2.472	0.137	-	-	-
Tumor thickness	2.147	0.923-2.562	0.119	-	-	-
Ulceration	2.237	1.173-2.751	0.086	-	-	-
Lymph node metastasis	2.852	1.372-3.775	0.015	2.658	1.183-3.472	0.023
Tumor stage	3.127	1.472-4.194	0.005	2.825	1.288-3.738	0.009
miR-431 expression	3.452	1.484-4.379	0.003	3.019	1.201-3.792	0.008

detection assays indicated that the activity of caspase 3/9 was remarkably elevated in melanoma cells after the transfection with miR-431 mimics, while the transfection with miR-431 inhibitors repressed the caspase 3/9 activity (Figure 2F). Therefore, these data suggested that miR-431 inhibited melanoma cells proliferation and induced cell apoptosis.

The Mobility of Melanoma Cells was Attenuated by MiR-431 Overexpression

To assess whether miR-431 was able to regulate the mobility of melanoma cells, we next carried out wound-healing and transwell assays using HBL and SK-MEL-1 cells. As the results from transwell assays, the cells with increased levels of miR-431 markedly impaired the in-

vative ability of melanoma cells, whereas the down-regulation of miR-431 significantly promoted the cell invasion (Figure 3A). Moreover, the wound-healing assays confirmed that the cells with miR-431 mimics transfection had notably lower migration capacity, and the miR-431 inhibitors-transfected-cells showed a significantly higher migratory ability (Figure 3B). Hence, these data demonstrated that miR-431 could attenuate the metastatic potentials of melanoma cells.

NOTCH2 was a Direct Target of MiR-431 in Melanoma Cells

To further elucidate the molecular mechanism by which miR-431 modulated the malignant phenotypes of melanoma, the possible target genes

of miR-431 were searched using the StarBase program. NOTCH2, which has been reported to be an oncogene in many types of cancers, was found to be a potential downstream target gene of miR-431 (Figure 4A). When we transfected miR-431 mimics into melanoma cells, the levels of NOTCH2 was markedly decreased, while miR-431 inhibitors' transfection led to remarkably increased expression of NOTCH2 (Figure 4B). Therefore, we next constructed the luciferase reporter plasmids containing wild-type (WT) or mutant (MUT) miR-431 targeting site in NOTCH2 3'UTR and conducted the luciferase activity analyses to certify whether NOTCH2 was a target of miR-431. The data demonstrated that miR-431 could notably elevate the luciferase activity in cells transfected with WT luciferase reporter plasmids, which indicated that NOTCH2 was the exact target of miR-431 (Figure 4C). Further CCK-8 assays proved that the ectopic expression of NOTCH2 with pcDNA3.1-NOTCH2 overexpressing plasmids partially rescued miR-431-mediated reduction of melanoma cells proliferation (Figure 4D). Similarly, the suppressing impact of miR-431 on cellular migration was also abrogated by the enhancing expression of NOTCH2 (Figure 4E). Thus, these data indicated that miR-431 regulated the malignant phenotypes of melanoma via directly targeting NOTCH2.

Discussion

Up to date, with the translation of molecular insights into treatment benefit for melanoma patients, great success has been achieved in the clinical therapeutics of melanoma with metastasis²². Of note, immune-therapeutic attracted increasing attention. However, the sensitive cancer biomarkers which can be used for the guidance of a therapeutic method is of great importance^{23,24}. In the last decade, the detection of dysregulated miRNAs became simple due to the advancement of the High Throughput Sequencing Technology. On the other hand, the important regulatory function of miRNAs targeting critical tumor-related genes made them become novel therapeutic targets and cancer biomarkers. In other tumors, such as lung cancer and gastric cancer, a large number of miRNAs have been functional characterized^{25,26}. However, in melanoma, only a few miRNAs were identified. In this study, we focused on a new melanoma-related miRNA miR-431.

In this study, we analyzed microarray data to preliminarily explore the expression trend of miR-431 in melanoma tissues, finding that miR-431 was lowly expressed, which was confirmed by RT-PCR which was used to detect the expression of miR-431 in melanoma tissues and matched normal skin tissues from our hospital.

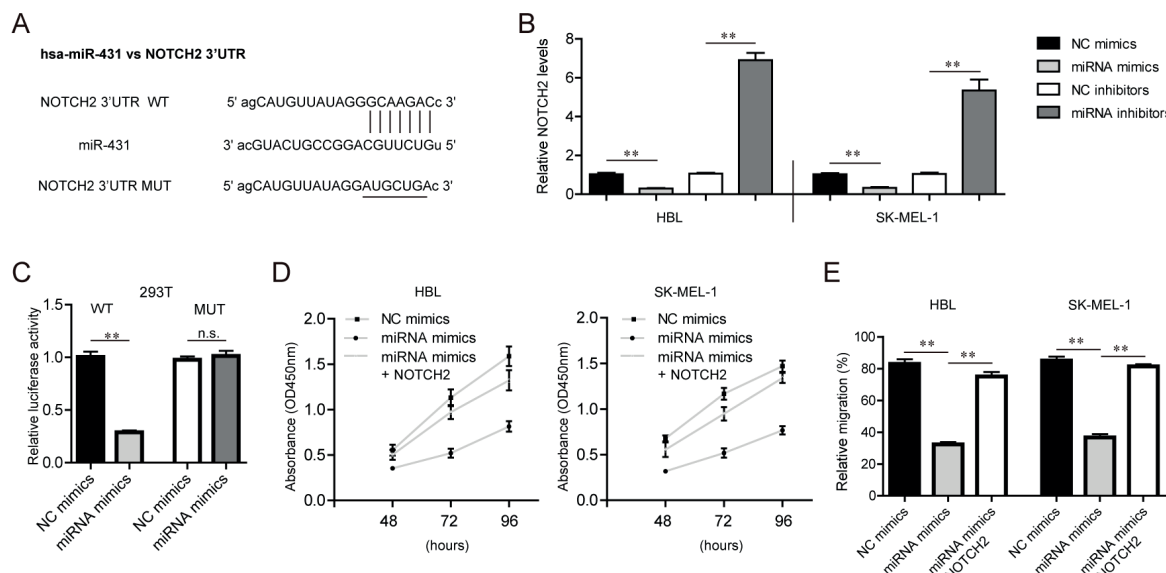


Figure 4. NOTCH2 acted as the target of miR-431. **A**, Complementary binding sites of miR-431 in the 3'-UTR of NOTCH2 mRNA predicted by StarBase. **B**, Real-time PCR analysis illustrated the NOTCH2 mRNA levels. **C**, Luciferase activity detection assay was conducted to confirm the binding of miR-431 with the 3'-UTR of NOTCH2 mRNA. **D**, CCK-8 assays detected the cellular growth. **E**, Wound-healing assays determined the migratory ability. * $p < 0.05$, ** $p < 0.01$.

Then, the clinical investigation revealed that low miR-431 levels were associated with ulceration and tumor stage, suggesting that miR-431 may have the potential to influence the clinical development of melanoma patients. With five years of follow-up, the clinical survival data were collected and analyzed; the results showed that low miR-431 expression had a significant impact on the overall survival of patients. More importantly, in a multivariate Cox model, the high levels of miR-431 were observed by our group to be an independent prognostic factor for melanoma patients. Thus, our findings suggested the possible clinical benefit for melanoma patients.

Previously, functional investigations have found miR-431 played a negative role in reported tumors. For instance, it was reported that miR-431 was lowly expressed in lung cancer and its forced expression inhibited cells proliferation and invasion via targeting DDX5²⁰. In pancreatic cancer, miR-431 expression was also shown to be reduced, and its overexpression resulted in the increased capability of tumor cells²⁷. Here, si-miR-431 was designed and used for the suppression of miR-431 in melanoma cell lines. *In vitro* assays suggested that the up-regulation of miR-431 suppressed cells proliferation, migration and invasion, and promoted apoptosis. Our findings provided important evidence that miR-431 acted as a tumor suppressor in cells behavior of melanoma, suggesting its potential application as a novel therapeutic target against the metastatic melanoma.

Notch signaling is a highly evolutionarily conserved pathway which has been confirmed to exhibit essential roles in embryonic growth and human homeostasis²⁸. The study of tumor biology increased the comprehension that dysregulated Notch signaling is involved in the modulation of tumor progression by affecting tumor angiogenesis and metastasis²⁹. It has been confirmed that this signal consists of four transmembrane receptors (NOTCH1-4). Of note, in melanoma, NOTCH2 has been reported to be up-regulated and promotes proliferation, metastasis, and EMT progress in tumor cells^{30,31}. Besides, previous studies^{32,33} also reported that several miRNAs displayed its tumor-promotive or tumor-suppressive roles by targeting NOTCH2. In this study, bioinformatical assays predicted that NOTCH2 was a putative target of miR-431. Subsequently, our group further demonstrated their targeting association using Luciferase reporter assays. Furthermore, by using *in vitro* assays, the

up-regulation of NOTCH2 distinctly reversed the inhibitory effect of miR-431 on melanoma cell proliferation and migration. Thus, the findings of our present study revealed that NOTCH2 is a downstream target of miR-431 which may inhibit cells proliferation and metastasis by targeting NOTCH2 in melanoma.

Conclusions

We observed that miR-431 expressions are distinctly decreased in melanoma and correlated with malignant clinical parameters of melanoma patients and poor clinical prognosis. Moreover, miR-431 suppressed tumor growth and metastasis of melanoma via modulating NOTCH2. MiR-431 might be a valuable target for melanoma therapy, and it is useful as a novel prognostic biomarker for melanoma.

Conflict of interest

The authors declare no conflicts of interest.

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