Abstract. – OBJECTIVE: The aim of this study was to analyze the biological effects of circ-0079593 and its potential mechanism in the progression of melanoma.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was carried out to detect circ-0079593 expression in melanoma tissue samples and cell lines, and the relationship between circ-0079593 expression and prognosis of patients with melanoma was analyzed based on collected clinical information. Then, the melanoma cell line stably over-expressing circ-0079593 was constructed using lentiviral stable transfection technique, and then, Cell Counting Kit-8 (CCK-8) and transwell assays were carried out to detect the proliferation rate, migration, as well as invasion abilities of melanoma cells, respectively. In addition, the potential binding targets of circ-0079593 were searched through bioinformatics analysis, and the results were verified by Dual-Luciferase assay.

RESULTS: It was found that, in comparison with the normal control group, circ-0079593 showed a significant high expression in melanoma tissues and cell lines, which predicted a poor prognosis of melanoma patients. In vitro experiments showed that the overexpression of circ-0079593 remarkably enhanced proliferation rate, as well as invasion ability of melanoma cells. Moreover, bioinformatics data analysis revealed that there exist binding sites of microRNA-433 both in circ-0079593 and EGFR. Meanwhile, the results of the Luciferase assay confirmed that circ-0079593 probably bound to microRNA-433, as an endogenous competitive RNA (ceRNA), to regulate EGFR expression. At last, cell reverse experiments demonstrated that the overexpression of microRNA-433 could attenuate the capacity of melanoma cells to proliferate and migrate, while simultaneous overexpression of circ-0079593 partially restored those cell functions.

CONCLUSIONS: In melanoma, circ-0079593 may serve as a cancer-promoting gene to accelerate the rates of cell proliferation and migration, which may exert its effects by elevating EGFR expression by binding to microRNA-433.

Key Words: Melanoma, Circ-0079593, CeRNA, Cell proliferation, Cell invasion.

Introduction

Melanoma, a high-risk skin malignant tumor, is mainly caused by the rapid proliferation of skin cells exposed to ultraviolet radiation. With its rapid development, so far, melanoma has become the most invasive skin malignancy, and its mortality rate has reached up to 80%, with its incidence still on the rise in the past few decades. Studies have shown that the 5-year survival rate of early melanoma is as high as 98%, while that of advanced melanoma is only 16%. Moreover, brain metastasis often occurs in advanced melanoma, which is the leading cause of death in patients with melanoma. However, the resection of the original tumor affects the appearance of the patients and thus lowers the quality of their lives.

Circular RNAs (circRNAs) are a new class of non-coding RNAs that have been discovered in recent years and play a crucial role in various biological processes. A circRNA has a covalent closed loop structure, which was first discovered in human cells in 1991 but not recognized as a functional RNA until 2013. Since the circular RNA has a highly stable structure, it can effectively resist degradation caused by exonuclease. Bian et al. have shown that circular RNA can be localized and function in the cytoplasm, so as to adsorb small RNAs (microRNAs) to affect the translation process.
or directly bind to proteins to regulate protein translation and function. In recent years, many circRNAs have been found to have various biological functions. In particular, circRNA CDR1as can regulate downstream gene expression by combining with microRNA-7, and circRNA 100290 is highly expressed in oral squamous cell carcinoma, which regulates CDK6 expression by binding to the microRNA-29 gene family. Therefore, the study on the molecular mechanism by which circ-0079593 affects the progression of melanoma is urgent and essential.

In 2011, Salmena et al proposed the concept of competing endogenous RNAs (ceRNAs), a class of RNAs with miRNA binding sites that can compete with miRNAs to bind miRNAs. The authors elucidated a complex post-transcriptional regulatory network, including mRNAs, lncRNAs, and other types of RNAs. Cesana et al discovered a muscle-specific lncRNA LINC-MD1 that regulates the expression of myocyte-specific enhancer 2C by adsorbing microRNA-133. In addition, Qu et al observed that LINC ARSR mediates the resistance of tumor cells to sunitinib treatment in renal cell carcinoma by competitively binding to miR-34/miR-449 and promoting the expression of AXL receptor tyrosine kinase and c-MET. However, the study of ceRNA in melanoma is not completely clear and remains to be further explored.

Previously, circ-0079593 has been confirmed to be highly expressed in melanoma, but its potential biological effects and molecular mechanisms remain to be clearly determined. On this basis, the role and potential molecular mechanism of circ-0079593 in melanoma were investigated in this research.

**Patients and Methods**

**Research Objects and Sample Collection**

Tumor tissue specimens and normal controls were collected from 42 patients with melanoma and stored at -80°C. All patients did not receive any treatment such as radiotherapy and chemotherapy before the surgery and all collected tumor samples were confirmed as melanoma by postoperative pathology. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This study was approved by the Ethics Committee of Jining No. 1 People’s Hospital and complied with the Declaration of Helsinki. Signed written informed consents were obtained from all participants before the study.

**Cell Culture**

Melanoma cell lines A375, SK-MEL-2, SK-MEL-28, A2058, and normal human epidermal melanocytes HEKa-LP were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The above cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal calf serum (HyClone, South Logan, UT, USA) and 1% antibiotics (penicillin/streptomycin) in an incubator with 5% CO₂ at 37°C.

**Cell Transfection**

For transfection, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with LV-circ-0079593 and microRNA-443 mimics (Jikai, Shanghai, China), and the transfection efficiency was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) after 48 h.

**RNA Extraction**

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After extracting with chloroform, the aqueous phase was transferred to a new tube, and the RNA in the aqueous phase was precipitated by isopropanol. The RNA precipitate was washed with 75% ethanol, dried at room temperature, dissolved with diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China), and placed in a refrigerator at -20°C.

**QRT-PCR**

The extracted total RNA was reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using a reverse transcription kit (TakaRa, Otsu, Shiga, Japan). Both mRNA and miRNA quantification were performed using the SYBR Green PCR Master Mix (Takara, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal standards for mRNAs and miRNAs, respectively. The primer sequences are as follows: circ-0079593 F: 5′-TTCCCCACCCACTTCGGGAT-3′ R: 5′-GCA-CATGTCTCAACATTGCCT-3′, microRNA-433 F: 5′-TCCTGGAATACCCCATACTTAGC-3′ R: 5′-GCACGACTGGAAAGTTGTAATCC-3′, GAPDH F: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, R: 5′-AGCCTTCTCCATGTGGTGAAGAC-3′.
Circ-0079593 promotes melanoma cell proliferation, migration and invasion

U6 F: 5′-GCTGAGGTGACGCTCAAA-3′, R: 5′-GCCTCCCAGTTTATGGACA-3′, EGFR F: 5′-AGGCACGAGTAACAAGCTCAC-3′, R: 5′-ATGAGGACATAACCACCACC-3′. The relative expression of the genes was calculated using the 2−∆∆Ct method.

Cell Counting Kit-8 (CCK-8) Assay
After 24 to 48 hours of cell transfection, the cells were seeded in 96-well plates (4×10^4 cells/well) in 100 μL of culture medium. CCK-8 assay was performed according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA).

Transwell Assay
24 h after transfection, the cells were prepared into cell suspensions and seeded in the upper chamber (50,000 cells/well) in serum-free medium, and then, 10% fetal bovine serum (FBS) medium (Gibco, Rockville, MD, USA) was added to the lower compartment of the chamber. The migrated cells were counted after staining with crystal violet (Beyotime, Shanghai, China) after washing.

Luciferase Assay
The Luciferase reporter vector containing circ-0079593 or EGFR was constructed by GenePharma (Shanghai, China), and the relative fluorescence value after plasmid transfection was measured by a standardized method. The same experiment was repeated 3 times, and the average value was taken for statistical analysis.

Western Blot
The proteins were extracted from the cells using a radioimmunoprecipitation assay (RIPA) kit (Beyotime, Shanghai, China), and protein content determination was carried out using a protein detection kit (Beyotime, Shanghai, China). Next, the protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), and incubated with 5% skim milk at room temperature. Finally, immunoprecipitation was subsequently conducted based on instructions.

Statistical Analysis
Statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA). Student’s t-test was used to compare the differences between the samples analyzed. Data are expressed as mean ± standard deviation. p<0.05 was considered statistically significant.

Results

Circ-0079593 Has an Abnormal High Expression in Melanoma
As shown in Figure 1A, qRT-PCR results indicated a significant increase in circ-0079593 expression in melanoma tissue specimens in comparison to that in normal control tissues, which was consistent with previous studies. Meanwhile, a same tendency of circ-0079593 expression was found in melanoma cell lines and normal ones (Figure 1B). Subsequently, in order to explore the correlation between circ-0079593 and patients' prognosis, the subjects were divided into circ-0079593 high-expression group and low-expression group to analyze their clinical information. It was found that patients in circ-0079593

![Figure 1](image_url). Circ-0079593 is highly expressed in melanoma tissues and cell lines. A, The expression level of circ-0079593 in melanoma tissue and normal control tissues. B, The expression levels of circ-0079593 in melanoma cell lines and normal control cell lines. C, The correlation between the expression level of circ-0079593 and the prognosis of melanoma patients. *p<0.05; **p<0.01.
high-expression group showed a marked low survival rate in comparison with those in the low-expression group (Figure 1C), suggesting that highly expressed circ-0079593 may predict a poor prognosis of melanoma patients.

**Overexpression of Circ-0079593 Accelerates Melanoma Cell Proliferation and Migration**

The circ-0079593 lentiviral vector in A375 and A2058 were transfected, and the transfection efficiency was detected by qRT-PCR. The results showed that LV-circ-0079593 remarkably increased circ-0079593 expression in melanoma cells (Figure 2A). Subsequently, CCK-8 results revealed that this upregulation of circ-0079593 remarkably elevated the proliferation rate of melanoma cells (Figure 2B). Meanwhile, cell migration, as well as invasiveness, also showed an enhanced tendency after overexpression of circ-0079593, measured by transwell assay (Figure 2C, 2D). The above results indicate that the overexpression of circ-0079593 in melanoma cells can prompt cell growth and metastasis.

**Circ-0079593 Can Be Combined With MicroRNA-433**

MiRNAs binding to circ-0079593 were predicted through the bioinformatics website (circular RNA interactome: https://circinteractome.nia.nih.gov), with the highest microRNA-433 binding score (Figure 3A). Hence, *in vitro*, a wild-type plasmid and a mutant plasmid of circ-0079593 were constructed, and the binding relationship of the two was tested by the Luciferase assay. As a result, the Luciferase activity of the circ-0079593-WT 3’UTR group was remarkably decreased after transfection with microRNA-433 mimics in A375 and A2058 cells, while no significant change was observed in Luciferase activity of circ-0079593-MUT 3’UTR group (Figure 3B), confirming that microRNA-433 does bind to circ-0079593. Subsequently, microRNA-433 expression in melanoma tissues and normal control ones was examined by qRT-PCR, and the former, in comparison to the latter, showed a marked reduction (Figure 3C). Meanwhile, in melanoma tissues, a negative correlation was found between the expression levels of circ-0079593 and microRNA-433 (R=-0.447, *p*<0.001) (Figure 3D). Additionally, *in vitro* experiments verified that the overexpression of circ-0079593 markedly inhibited microRNA-433 expression in melanoma cell lines (Figure 3E).

**Circ-0079593 Prompts Melanoma Cell Invasion and Proliferation by Binding to MicroRNA-433**

Subsequently, the impacts of microRNA-433 on metastasis and proliferation ability of melanoma cells were examined by *in vitro* experiments.
It was found that, in A375 cell line, overexpression of microRNA-433 gave rise to a reduction in cell proliferation rate, which could be partially restored by simultaneous overexpression of circ-0079593 (Figure 4A). Similarly, transfection with microRNA-433 mimics also caused the inhibition of cell migration capacity and invasiveness, which could also be reversed by simultaneous upregulation of circ-0079593 (Figure 4B-4C). The above observations demonstrate that circ-0079593 may accelerate melanoma progression by inhibiting microRNA-433 expression.

**MicroRNA-433 is Able to Bind EGFR**

To further explore the mechanism of how circ-0079593 works, the target gene that binds to microRNA-433 was predicted by bioinformatics, and functional analysis was performed. EGFR was then selected, and EGFR wild-type plasmid (EGFR WT) and the mutant plasmid (EGFR mut) were constructed (Figure 4D). The Luciferase assay indicated that transfection with microRNA-433 mimics in A2058 cells lowered the Luciferase activity of EGFR-WT 3’UTR group, whereas no significant difference was found in that of the EGFR-MUT 3’UTR group (Figure 4E), indicating that microRNA-433 can bind to the 3’UTR of EGFR. Subsequently, qRT-PCR confirmed that EGFR was highly expressed in melanoma tissues (Figure 4F). In addition, *in vitro* experiments suggested that in A2058 cells, EGFR mRNA and protein expressions were remarkably inhibited after the overexpression of microRNA-433, while the opposite result was observed after knockdown of microRNA-433 (Figure 4G-4H), which suggests that microRNA-433 may exert its biological function via degrading EGFR.

**Discussion**

Currently, melanoma is still the most common skin malignancy, with its incidence increasing in the past decade. Although the diagnosis and treatment of melanoma has improved with the continuous advancement of medical technology, the therapeutic effect is still not optimistic after radical resection. For its high malignancy, melanoma is often found to have distant metastases in the early stage, leading to a low 5-year overall survival rate. It was found that, in A375 cell line, overexpression of microRNA-433 gave rise to a reduction in cell proliferation rate, which could be partially restored by simultaneous overexpression of circ-0079593 (Figure 4A). Similarly, transfection with microRNA-433 mimics also caused the inhibition of cell migration capacity and invasiveness, which could also be reversed by simultaneous upregulation of circ-0079593 (Figure 4B-4C). The above observations demonstrate that circ-0079593 may accelerate melanoma progression by inhibiting microRNA-433 expression.

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survival rate. Therefore, it is particularly important to explore its potential molecular mechanisms to provide a theoretical basis for searching for new treatments of melanoma.

CircRNAs are considered to be a new hotspot in the field of lncRNAs research, which have been extensively studied. So far, many circular RNAs have been reported as biomarkers for tumor diagnosis or treatment. For example, Bachmayr-Heyda et al. have found by RNA-seq technology that some circRNAs are under expressed in colorectal cancer tissues, and circMTO1 can adsorb microRNA-9 to inhibit the progression of liver cancer. However, circRNAs have not been fully investigated in melanoma and still need to be further explored.

In this study, high-throughput sequencing results demonstrated that circ-0079593 was abnormally highly expressed in melanoma tissues, which was further verified by qRT-PCR analysis.

**Figure 4.** Circ-0079593 competes binding to miR-433 through EGFR. A, CCK8 assay shows that overexpression of miR-433 in A375 cells inhibits cell proliferation, and the inhibitory effect is reversed after simultaneous overexpression of circ-0079593. B-C, Overexpression of miR-433 in A375 cell line reduces cell migration and invasion ability, while simultaneous overexpression of circ-0079593 reverses that change. D, Bioinformatics website predicts the binding site of miR-433 to EGFR and an EGFR wild type plasmid and a mutant plasmid are constructed. E, Luciferase reporter gene results show that miR-433 can bind to the 3’-UTR of EGFR. F, QRT-PCR results show that EGFR is abnormally highly expressed in melanoma tissues. G, Overexpression of miR-433 in A2058 significantly down-regulates EGFR mRNA levels, while knockdown of miR-433 promotes them. H, Overexpression of miR-433 in A2058 significantly down-regulates EGFR protein levels, while knockdown of miR-433 promoted them. *p<0.05; **p<0.01.
in tissue specimens collected from melanoma patients. Meanwhile, by analyzing clinical information, it was found that high expression of circ-0079593 could predict a poor prognosis of melanoma patients. As we know, circ-RNAs can act as ceRNAs to bind to miRNAs to exert their biological functions, so it was hypothesized that circ-0079593 may participate in melanoma progression by binding to certain miRNAs to regulate the expression of the downstream genes. Studies have reported that microRNA-433 can play a tumor suppressing role in various diseases. So, it inhibits proliferation and invasion of oral squamous cell carcinoma by targeting HDAC628 and can suppress the development of gastric cancer29. Therefore, microRNA-433 was chosen as a possible binding target, and it was discovered that circ-0079593 could bind to microRNA-433 and that overexpression of circ-0079593 could remarkably inhibit microRNA-433 expression. In addition, cell function investigations demonstrated that microRNA-433 could inhibit the growth and metastasis of melanoma cells.

Furthermore, it was predicted from the bioinformatics website that circ-0079593 was able to competitively bind to microRNA-433 with EGFR, which has been confirmed to be highly expressed in many types of cancer. However, the expression of EGFR in melanoma tissues varies30. It has also been reported that the EGFR overexpression often occurs in advanced melanoma tissue samples31. Due to this, EGFR was found to be also abnormally highly expressed in melanoma tissues, which, however, could be inhibited by overexpression of microRNA-433, and vice versa. Therefore, it can be concluded that circ-0079593 may protect EGFR from degradation by binding to microRNA-433 and inhibiting its expression, but the biological function of EGFR in melanoma remains to be further studied in the future.

Conclusions

Circ-0079593 acts as an oncogene in the progression of melanoma, and the circ-0079593/microRNA-433/EGFR regulatory axis may be a potential target for the clinical diagnosis and treatment of melanoma.

Conflict of Interest
The Authors declare that they have no conflict of interests.

References


