

## ORIGINAL ARTICLE

# MicroRNA-29c induces G1 arrest of melanoma by targeting CDK6

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## Summary

**Purpose:** Melanoma is a malignant skin tumor that can easily metastasize, while no effective treatment exists for this disease. This study explored the mechanism of microRNA-29c in inhibiting melanoma cell growth.

**Methods:** Bioinformatics analysis and polymerase chain reaction (PCR) experiments were performed to analyze the expression of microRNA-29c in various samples. The Cell Counting Kit-8 (CCK-8) experiment was used to detect cell viability. The mimic and inhibitor of microRNA-29c were transfected into melanoma cells to achieve microRNA-29c overexpression or knockdown so as to observe the biological effect on the melanoma cells. Flow cytometry was used to detect cell cycle, while the luciferase reporter gene assay was used for predicting microRNA-29c target genes. West-

ern blot was performed to determine the cellular protein expression.

**Results:** microRNA-29c was highly expressed in melanoma cells. Overexpression of microRNA-29c inhibited cell viability and induced G1 cell cycle arrest. Conversely, cell proliferation and cycle progression were promoted by transfection of microRNA-29c inhibitor in melanoma cells. In addition, CDK6 served as a microRNA-29c target gene. G1 phase of melanoma cells was blocked by knockdown of CDK6.

**Conclusions:** microRNA-29c can inhibit the growth of melanoma cells by targeting CDK6, which could trigger G1 arrest of melanoma cells.

**Key words:** CDK6, G1 arrest, melanoma, microRNA-29c

## Introduction

Melanoma is a malignant skin tumor that originates from melanocytes and is prone to metastasis [1,2]. At present, no effective therapies exist for melanoma, while the effects of radiotherapy and chemotherapy are not as satisfactory as expected. Although biological therapies have achieved good therapeutic effects in recent years, the complex pathogenesis of melanoma and its high rate of metastasis and recurrence make it difficult to further improve the therapeutic results.

Therefore, it is of great importance to investigate the possible pathogenesis of melanoma and find a novel biomarker that can represent the development on the disease.

microRNAs are non-encoding small RNAs consisting of 18 to 23 nucleotides. They can bind to the mRNAs of target genes to form dimers. By silencing complexes, microRNAs are able to degrade mRNAs or inhibit their transcription and translation so as to regulate target gene expression [2-4]. The regulation of microRNAs in gene expression is a complex process in that one microRNA can regulate numerous genes whereas one gene can be regulated by multiple microRNAs. microRNAs can regulate cell proliferation, differentiation, apoptosis and other physiological activities in melanoma cells and, therefore, microRNAs are likely to be a promising target of diagnosis and treatment

of malignant melanoma [5]. Many studies have revealed that microRNA-372, microRNA-202, microRNA-340, microRNA-509 and microRNA-195 can regulate tumor cell growth [6-9]. These demonstrate that microRNAs may play a vital role in the development of melanoma. Using bioinformatics analysis, we found that microRNA-29c was highly expressed in melanoma. However, whether microRNA-29c has a regulatory role in the growth of melanoma is still unknown.

This study mainly focused on the regulatory role of microRNA-29c in melanoma cell growth, and firstly discovered that CDK6 was a downstream gene of microRNA-29c. These results may provide new evidence to elucidate the role of microRNA-29c and CDK6 in melanoma progression.

## Methods

### *Human samples*

Thirty malignant melanoma tissues and 10 paracancer tissues were collected from the Suzhou Wujiang District First People's Hospital. No patients had received chemotherapy or radiotherapy before surgery. All pathological tissues were studied by at least two pathologists. All surgery samples were removed and immediately frozen at -80°C. This study was approved by the ethics committee of Suzhou Wujiang District First People's Hospital. Signed written informed consents were obtained from all participants before the study entry.

### *Cell culture*

The human melanoma cell lines A375, SK-MEL-1 and SK-MEL-5 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Human epithelial melanocyte HEMa-LP cell line was obtained from Invitrogen (Carlsbad, CA, USA) and cultured in 254 medium (Cascade Biologies). All cells were incubated in a 5% CO<sub>2</sub> incubator at 37°C.

### *Cell transfection*

The microRNA-29c mimic, inhibitor as well as their negative controls (NC) were synthesized by Genepharma (Shanghai, China). Small interference RNA of CDK6 (si-CDK6) and its negative control (si-NC) were also synthesized by Genepharma (Shanghai, China). According to the manufacturers' instructions, the mimic, inhibitor, negative control (NC), si-CDK6 and si-NC were transfected into cells in 6-well plates using Lipofectamine<sup>TM</sup> 2000 (Invitrogen, Carlsbad, CA, USA). After 6-h culture, fresh medium was added and 24 hrs after transfection, cells were collected for further analysis.

### *Luciferase reporter assay*

The mutant and wild-type CDK6 3-UTR regions were bound downstream of the microRNA-29c luciferase

reporter. After transfection for 24 hrs, fluorescence activities were detected according to the instructions of Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA).

### *Cell counting kit-8 (CCK-8) assay*

After digested, A375 cells were centrifuged and resuspended in culture medium. Counted cells were put in 96-plates well with 2000 cells per well. After 24 hrs, 100 µL of medium were retained in each well and 10 µL of CCK8 reagent (Dojindo, Kumamoto, Japan) were added. Then, cells were placed in a 5% CO<sub>2</sub> incubator at 37°C for another 2 hrs. A microplate reader was used to measure the absorbance values at 450 nm.

### *Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)*

Cells or tissue samples were lysed using the TRIzol (Invitrogen Carlsbad, CA, USA) and total RNA was isolated. Two µL of each RNA sample were taken for quantification on Nanodrop (IMPLEN GmbH, Munich, Germany). A 20 µL reaction system containing 1 µg of RNA was prepared, and the RNAs were reverse-transcribed into cDNAs by PrimeScript RT reagent Kit (TaKaRa, Otsu, Japan). The complementary deoxyribose nucleic acid (cDNA) and primers were then mixed with SYBR Primix Ex Taq II Kit (TaKaRa, Otsu, Japan). qRT-PCR was performed on the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The microRNA-29c, U6 RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were all synthesized by Shanghai Shenggong Bioengineering Co., Ltd. (Shanghai, China).

### *Flow cytometry*

The cells were put in 6 cm dishes, and collected when the confluence was close to 70%. After fixed with 70% ethanol for 48 hrs, cells were washed once with 1 mL of PBS. Propidium iodide (PI) (50 µg/mL) containing RNase (100 µg/mL) was added into cells. The mixture was gently vortexed and incubated in the dark for 30 min at room temperature. The mixed solution was filtered into a flow tube. The results were examined using flow cytometer (BDFACS Verse <sup>TM</sup> System, San Jose, CA, USA).

### *Western blot*

The sample was lysed with radioimmunoprecipitation assay (RIPA) lysate and total protein was extracted. The concentration of cellular proteins was detected with the Bio-Rad Protein assay (Beyotime, Shanghai, China). Equal amount of protein was electrophoresed on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the proteins in the gel were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and further incubated with primary antibody at 4°C overnight. The primary antibodies were: CDK6 (Cell Signaling Technology, #13331, Danvers, MA, USA) and Actin (Cell Signaling Technology, #3700, Danvers, MA, USA). In the next day, the immunoblots were incubated with the secondary antibodies for another 1 hr at room temperature.

Membranes were washed 6 times with TBST (Phosphate Buffer Solution with 0.05% Tween 20) and protein bands were analyzed by Find-do x 6 Tanon (Tanon, Shanghai, China).

#### Statistics

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze all experimental data. The results were statistically analyzed by *t*-test and one-way analysis of variance (ANOVA). Pearson correlation analysis and the Kaplan-Meier method were used for calculating the survival.  $P < 0.05$  was considered as statistically significant. All experiments were repeated three times.

## Results

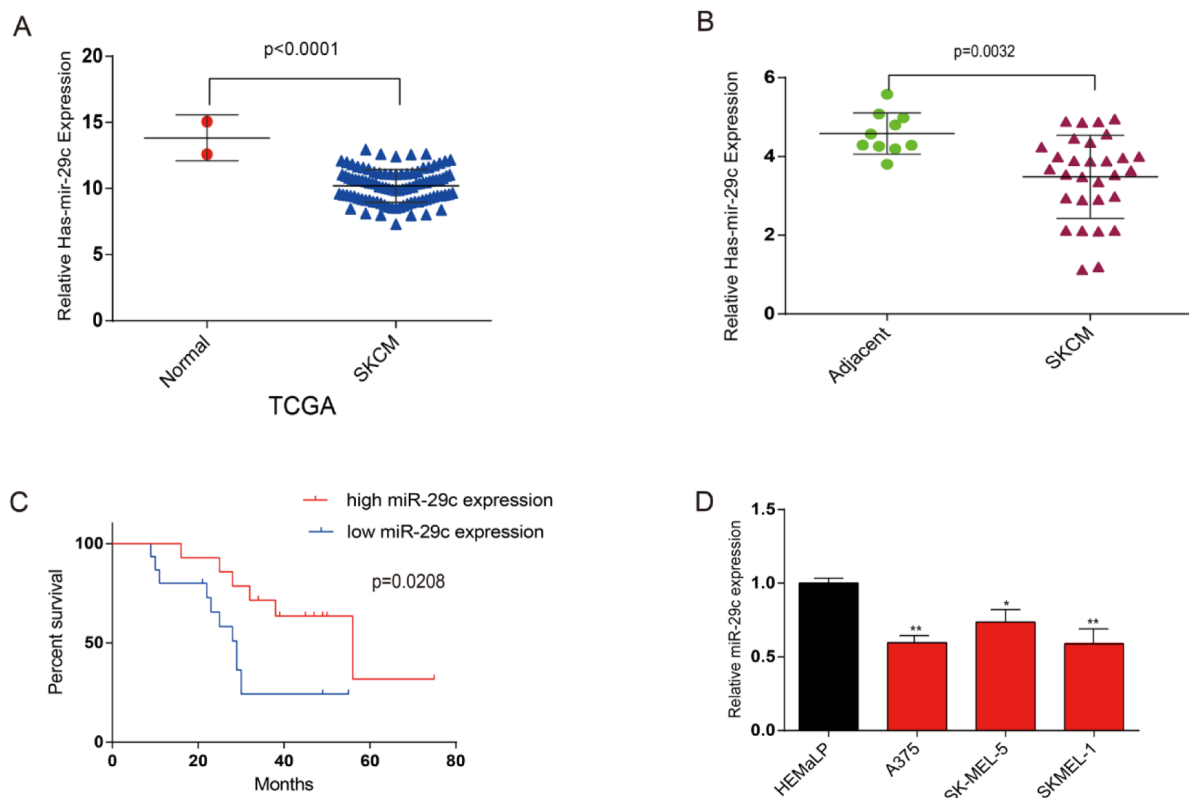
*microRNA-29c was lowly expressed in melanoma samples and was associated with poor patient prognosis*

We first performed a statistical analysis in the TCGA (The Cancer Genome Atlas) database to detect microRNA-29c levels in skin cutaneous melanoma (SKCM) tissues. The results demonstrated

that the levels of microRNA-29c in SKCM tissues were markedly downregulated compared to normal tissues (Figure 1A). Analysis of the expression of microRNA-29c in 30 melanoma tissues and 10 paracancer tissues also showed that microRNA-29c expression was decreased in melanoma tissues (Figure 1B). In addition, low expression of microRNA-29c was associated with the grade of melanoma differentiation, but not with age, gender, family history or ulceration (Table 1). Kaplan-Meier analysis suggested that patients with lower expression of microRNA-29c had a worse prognosis (Figure 1C). We also found low expression of microRNA-29c in A375, SK-MEL-1 and SK-MEL-5 cells compared to normal cells (Figure 1D). These results indicate that microRNA-29c may play a pivotal role in the development of cutaneous melanoma.

*microRNA-29c induced G1 arrest in melanoma cells*

To further explore the effect of microRNA-29c on the biological function of melanoma cells, mimics



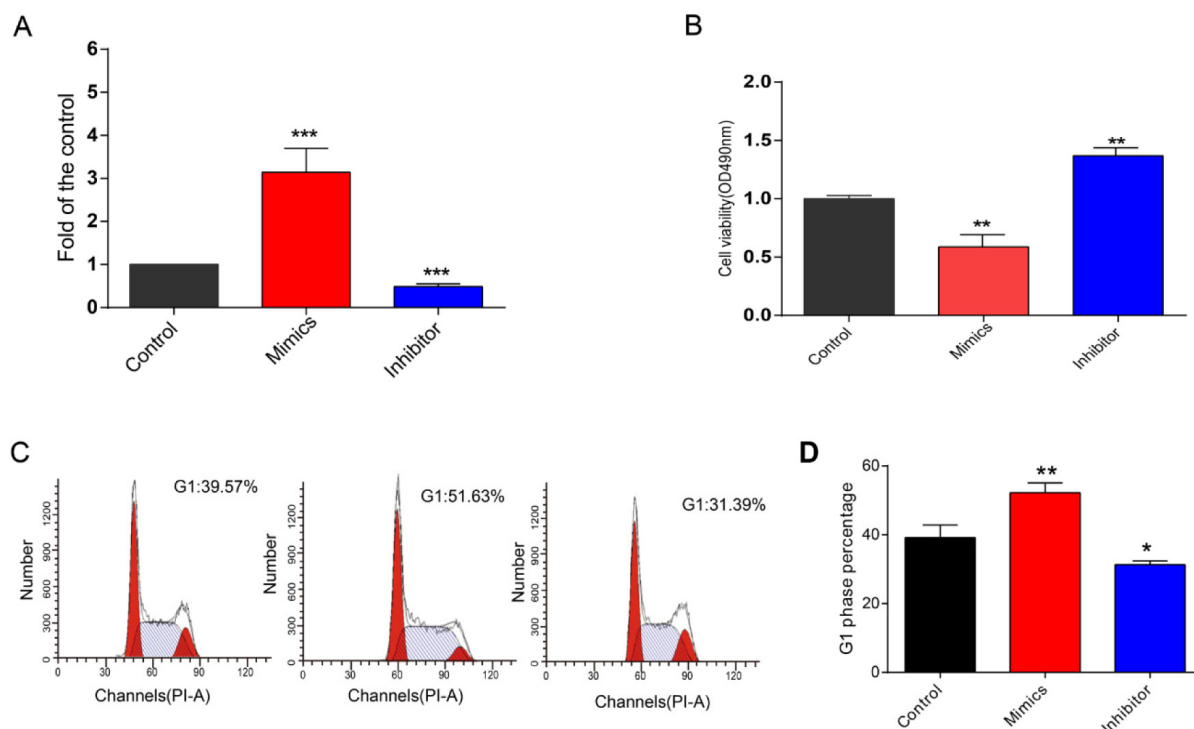
**Figure 1.** MicroRNA-29c was lowly expressed in melanoma samples and was associated with poor patient prognosis. **(A):** The microRNA-29c levels of normal tissues (n=2) and skin cutaneous melanoma (SKCM) tumor tissues (n=97) in the TCGA database. These results show that the levels of microRNA-29c in SKCM tissues were significantly downregulated compared to normal tissues. **(B):** PCR analysis of microRNA-29c levels in 30 melanoma tissues and 10 paracancer tissues showing that microRNA-29c expression was decreased in melanoma tissues. **(C):** The overall survival curves of patients with high or low levels of microRNA-29c. The results show that patients with lower expression of microRNA-29c had a worse prognosis. **(D):** The Figure shows microRNA-29c levels in melanoma cell lines (A375, SK-MEL-1, SK-MEL-5) and human epithelial melanocytes (HEMa-LP). (\* $p < 0.05$ , \*\* $p < 0.01$ ).

and inhibitors of microRNA-29c were transfected into A375 tumor cells (Figure 2A). Mimics and inhibitors of microRNA-29c were also used to explore the role of microRNA-29c in cell proliferation. CCK8 experiments showed that overexpression of microRNA-29c inhibited cell viability, whereas knockout of microRNA-29c enhanced cell

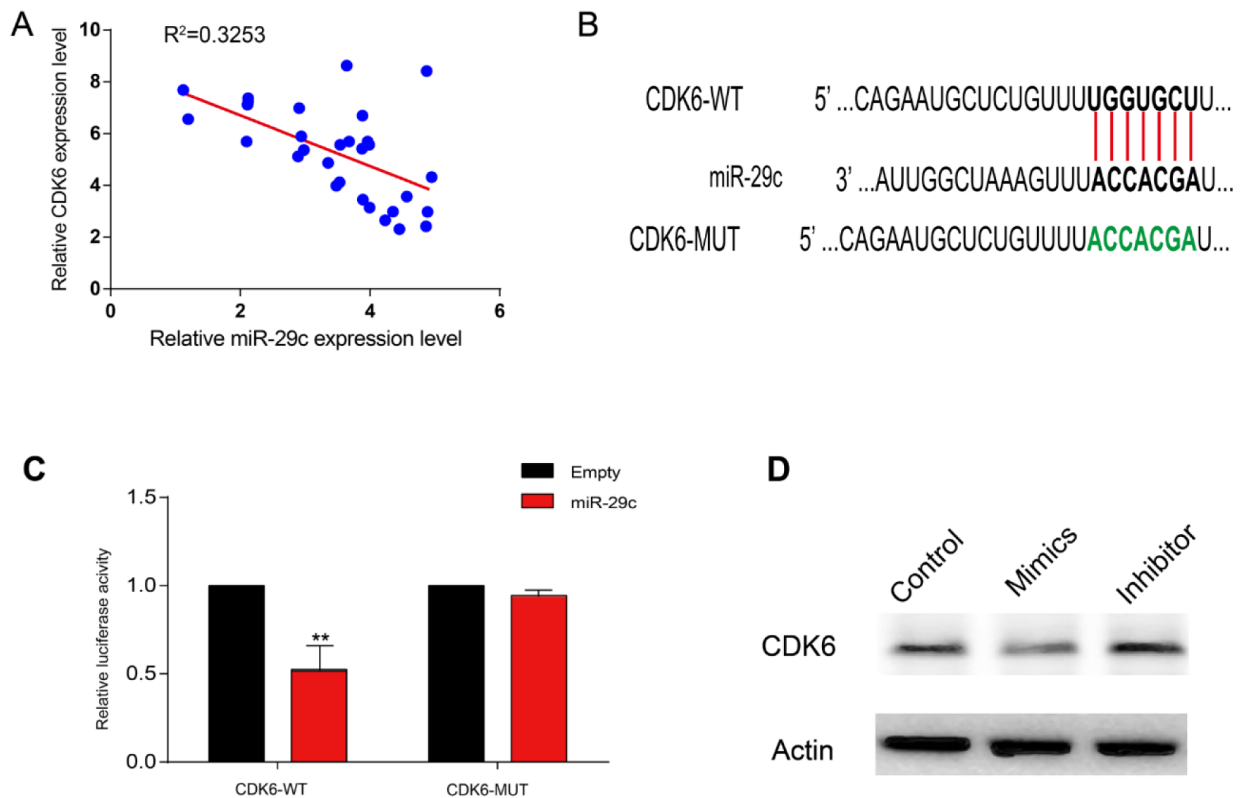
viability (Figure 2B). The flow cytometry results showed that overexpression of microRNA-29c increased the proportion of G0/G1 cells in melanoma cells, while the proportion were strikingly decreased when inhibitors were added (Figure 2C). These suggest that microRNA-29c can induce G1 arrest in melanoma cells.

**Table 1.** Correlation between miR-29c expression and clinicopathological characteristic (n =30)

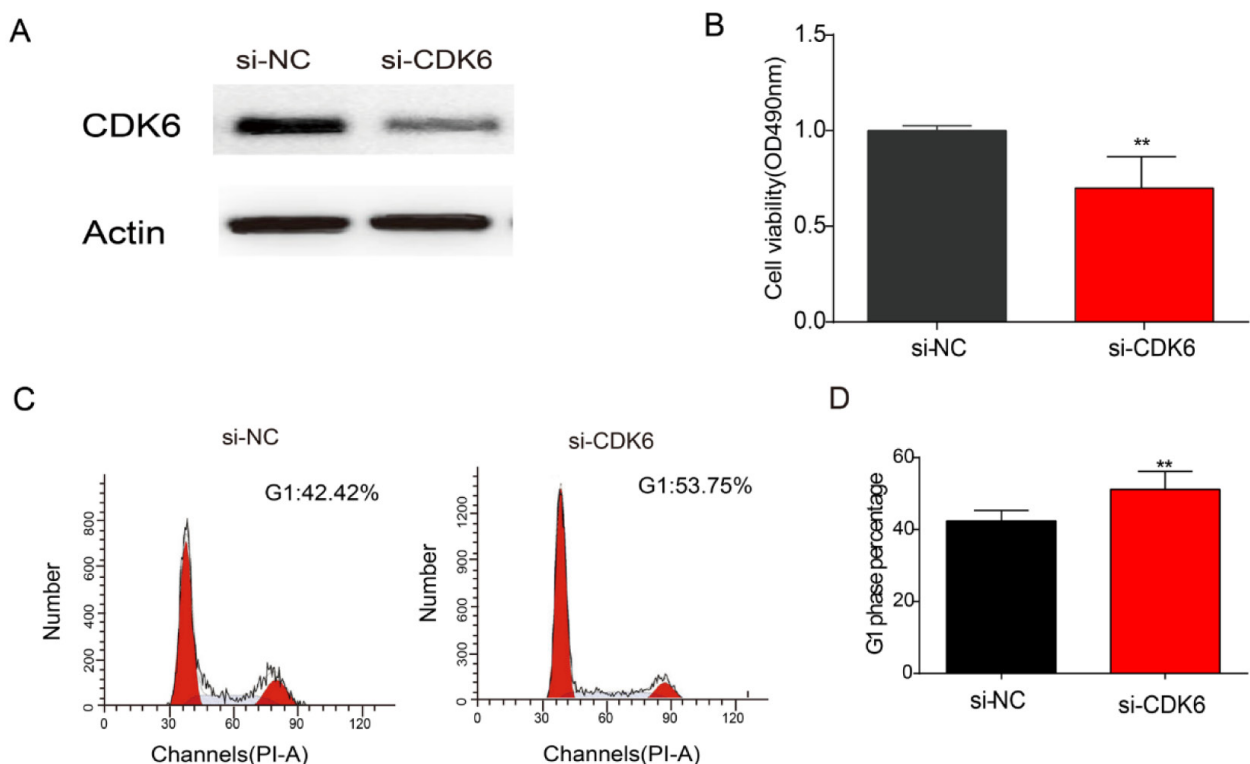
Characteristics	Low miR-29c expression, n	High miR-29c expression, n	p value
Age, years			0.249
<50	6	8	
≤50	9	7	
Gender			0.660
Male	8	10	
Female	7	5	
TMN stage			0.020
I-II	7	5	
III	8	10	
Ulcer			0.471
Yes	8	6	
No	7	9	
Family history			0.837
Yes	1	2	
No	14	13	



**Figure 2.** MicroRNA-29c induced G1 arrest in melanoma cells. **(A):** The relative microRNA-29 levels in melanoma cells treated with microRNA-29c mimics or inhibitors. **(B):** The result of CCK8 in melanoma cells treated with microRNA-29c mimics or inhibitors for 24 hrs. **(C,D):** Cell cycle progression of melanoma cells treated with microRNA-29c mimics or inhibitors (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 3.** CDK6 was a target gene of microRNA-29c. **(A):** Pearson correlation analysis of microRNA-29c and CDK6 levels in 30 melanoma patients. **(B):** Fluorescein activity of microRNA-29c receptors in melanoma cells. **(C):** The protein expressions of CDK6 in melanoma cells treated with microRNA-29c mimics or inhibitors. **(D):** The protein expressions of CDK6 in melanoma cells treated with microRNA-29c mimics or inhibitors (\*\* $p<0.01$ ).



**Figure 4.** Knockdown of CDK6 induced G1 arrest in melanoma cells. **(A):** The protein expressions of CDK6 and Actin in melanoma cells treated with si-NC and si-CDK6. **(B):** The result of cell counting Kit-6 (CDK6) in melanoma cells treated with si-NC and si-CDK6. **(C and D):** The result of flow cytometry in melanoma cells treated with si-NC and si-CDK6 (\*\* $p<0.01$ ).



### *CDK6 was a downstream gene of microRNA-29c*

Pearson's correlation coefficient in 30 melanoma samples showed a negative correlation between microRNA-29c and CDK6 (Figure 3A). We used TargetScan, microRNAanda and the PicTar programs to search for microRNA-29c target genes and found that microRNA-29c can directly bind to the 3'UTR region of CDK6 (Figure 3B). To further verify the correlation between microRNA-29c and CDK6, dual-Luciferase Reporter Assay was performed to investigate the role of microRNA-29c on regulating CDK6. Consistent with the prediction, the wild-type CDK6 3'UTR luciferase activity was inhibited by microRNA-29c, however, the mutated CDK6 3'UTR luciferase activity did not alter strikingly (Figure 3C). The CDK6 protein expression was downregulated in melanoma cells treated with microRNA-29c mimic, whereas the addition of microRNA-29c inhibitor increased CDK6 expression (Figure 3D). These results confirmed that CDK6 was a downstream gene of microRNA-29c.

### *Knockdown of CDK6 induced G1 arrest in melanoma cells*

We transfected small interfering RNA of CDK6 in cells to verify whether the viability of melanoma cells was inhibited by CDK6 downregulation (Figure 4A). What was found was that downregulation of CDK6 expression remarkably inhibited the viability of melanoma cells (Figure 4B). To further explore its underlying mechanism, flow cytometry in melanoma cells was performed and the results showed that the percentage of G0/G1 phase in melanoma cells was strikingly increased after knockdown of CDK6, comparing with the control (Figure 4C). These results indicate that microRNA-29c can induce G1 arrest of melanoma cells by downregulating the expression of CDK6, thus inhibiting the melanoma cells' growth.

## **Discussion**

Melanoma is a very aggressive cutaneous tumor with low patient survival rate. It usually has a rapid onset and high metastatic potential spreading to important organs such as the brain, liver and lungs. In this study, we found that microRNA-29c was lowly expressed in melanoma tissues and had an obvious correlation with the prognosis of patients. In addition, microRNA-29c can block melanoma cell cycle in the G1 phase. We used two methods to find potential target genes for microRNA-29c. First, by using a variety of bioinformatics methods (TargetScan, microRNAanda and the PicTar), we predicted the target genes of microRNA-

29c and found that CDK6 was one of microRNA-29c target genes. After comparing the sequence homology between different species, we knocked out CDK6 in melanoma cells and found that G1 phase of melanoma cells was blocked. These results indicate that microRNA-29c can induce G1 arrest of melanoma cells by downregulating CDK6 and inhibit the growth of melanoma.

MicroRNAs are a group of non-coding small RNAs that regulate more than one-third of human genes [10,11]. In 1993, Lee et al. [12] found a 22-nucleotide, noncoding small RNA-lin-4 in *Caenorhabditis elegans*. This microRNA did not encode any protein, but it was an essential component of nematode development. Accumulated evidence demonstrated that microRNAs can act as proto-oncogenes or anti-oncogenes *in vivo* by regulating different signaling pathways, including cell proliferation, apoptosis, migration, invasion, drug resistance and angiogenesis. Studies have revealed that microRNA-21 can act as a crucial part in the development of melanoma by targeting genes such as TIMP3, PDCD4, BCL-2, and PTEN [13,14]. Higher expression of microRNA-21 may predict worse non-progressive survival and overall survival of the patient within 5 years, and can be used to determine patient prognosis [13]. Contrary to the role of microRNA-21, microRNA-125b can inhibit the proliferation, survival, apoptosis, and migration of tumor cells through regulating c-Jun, MLK3 and MKK7 genes, thus playing a suppressor role *in vivo*. MicroRNA-125b may also cause resistance to Wirtufenib [15]. In addition, the increased expression of microRNA-155 in melanoma tissue can promote the apoptosis of tumor cells and inhibit their proliferation by targeting SK1 expression [16]. The expression of microRNA-205 and microRNA-211 were decreased in the melanoma tissues, a fact that can target the expression of E-cadherin and NFAT3 to promote tumor invasion [17,18]. In this study, we discovered that microRNA-29c was lowly expressed in melanoma and that patients with lower microRNA-29c had poor prognosis. Through bioinformatics analysis, we believe that CDK6 is one of the microRNA-29c target genes.

Abnormal regulation of cyclins is one of the major causes of human tumorigenesis, while the CDKs family has a vital role in regulating the cell cycle [19,20]. A large number of studies have demonstrated that CDK6 gene amplification or mutation enhanced its activity in various tumor tissues [21]. Tang et al. [22] analyzed 92 tissue samples of patients with pancreatic endocrine tumors. The results demonstrated that most of the tumor tissues had high levels of CDK4/6 and cyclin, which were often accompanied with elevated pRB. The

CDK4/6 inhibitor (PD0332991) can inhibit tumor growth in mice transplanted with human pancreatic endocrine QGP1 tumor cells. This suggests that the inhibitor has antitumor effect [22]. In addition, studies have shown that CDK6 is closely related to the development of non-small cell lung cancer, glioma, ovarian cancer and lymphomas [23-26]. We knocked down CDK6 intracellularly and found that melanoma cells' proliferation was inhibited and the cells were arrested in the G1 phase. Our study shows that CDK6 is expected to be one of the targets in cancer therapy.

## Conclusions

Overexpression of microRNA-29c can inhibit the growth of melanoma cells by inducing G1 cell cycle arrest by downregulating CDK6. CDK6 is a target gene of microRNA-29c. This study provides new theoretical evidence for the role of microRNA-29c and CDK6 in the treatment of melanoma.

## Conflict of interests

The authors declare no conflict of interests.

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