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Original Article

Circ-0001313/miRNA-510-5p/AKT2 axis promotes the development and progression of colon cancer

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Abstract: Circular RNAs (circRNAs) have recently emerged as novel and potentially promising therapeutic targets in a serious of cancers. However, the expression pattern and biological function of circRNAs in colon cancer remain largely elusive. This study firstly analyzed circRNA microarray of colon cancer and selected circ-0001313 as the study object. We aim to comprehensively investigate the expression pattern and biological function of circ-0001313 in the progression of colon cancer. Relative levels of circ-0001313 and miRNA-510-5p in colon cancer tissues and cell lines were determined with qRT-PCR. The binding relationship between miRNA-510-5p to circ-0001313 and AKT2 was predicted by bioinformatics analyses and further confirmed by dual-luciferase reporter gene assay. Regulatory effects of circ-0001313/miRNA-510-5p/AKT2 axis on colon cancer cells were evaluated by EdU assay and flow cytometry. Consistent with the microarray analysis, circ-0001313 was highly expressed in colon cancer tissues and cell lines. Knockdown of circ-0001313 attenuated proliferative ability, but induced apoptosis of colon cancer cells. Furthermore, we confirmed that circ-0001313 competitively bound to miRNA-510-5p, thus upregulating its target gene AKT2. Moreover, western blot analyses revealed that circ-0001313 also affects the expression of Bcl-2 family proteins and the activation of PI3K/Akt signaling pathway. In conclusion, our study revealed that circ-0001313 regulates the pathogenesis of colon cancer by sponging miRNA-510-5p to upregulate AKT2 expression.

Keywords: Colon cancer, circ-0001313, miRNA-510-5p, AKT2

Introduction

Colon cancer is a common gastrointestinal tumor posing a serious hazard to human health [1]. Globally, the morbidity and mortality of colon cancer rank third and fourth, respectively. Relative pathogenic factors of colon cancer have been widely explored in recent years. It is suggested that interventions on these pathological factors could prevent the occurrence of colon cancer to a great extent [2]. However, the comprehensive pathogenesis of colon cancer remains unclear so far, while its morbidity and mortality have been annually elevated [3]. Hence, seeking effective targets is of substantially clinical value for diagnosing and treating colon cancer in the early stage.

Recently, clinical research emphasis has been put on circular RNAs (circRNAs) associated with the pathogenesis of tumors. Unlike linear RNA with 3' and 5' ends, circRNA, the primary gene transcription subtype, forms a loop covalently closed through specific splicing. Almost all circRNAs with stable expression are observed in species at all diversities [4] that are not affected by RNA exonuclease [5]. The most pronounced function of circRNA is miRNA sponge as a competing endogenous RNAs (ceRNA) [6]. CircRNA has correlation with numerous diseases via the ceRNA network, such as bladder carcinoma [7], osteosarcoma [8], human cartilage degradation [9], and coronary artery disease [10].

Table 1. Patient cohort description

Feather	Number	Low	High	*P* value
All cases	30	15	15	
Age (years)				0.7152
< 65	14	6	8	
≥ 65	16	9	7	
Gender				1
Male	17	8	9	
Female	13	7	6	
Tumor size (cm)				0.0283
< 3	8	2	6	
3-5	7	2	4	
> 5	15	11	3	
Differentiation grade				0.0352
Well	4	1	3	
Moderate	13	4	9	
Poorly	13	10	3	
Tumor number				0.7125
Solitary	13	6	7	
Multiple	17	9	8	
Tumor capsular				0.3091
Incomplete	1	1	0	
Complete	29	14	15	
TNM stage				0.1432
I~II	16	10	6	
III~IV	14	5	9	

Total data from 30 colon cancer patients were analyzed. For the expression of hsa_circ_0001313 was assayed by qRT-PCR, the median expression level was used as the cutoff. Data were analyzed by chi-squared test. *P*-value in bold indicates statistically significant.

The high expression of circ-0001313 was seen in colon cancer tissues and cells in this research. Circ-0001313, as a ceRNA, adjusts the pathogenesis of colon cancer by sponging miR-510-5p, thereby upregulating AKT2 and further regulating colon cancer cell behaviors, so it can be concluded that circ-0001313 may serve as a new marker for diagnosing and treating colon cancer in the early stage.

Materials and methods

Ethical statement

The study was approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology. Patient consent was written informed consent, in compliance with the Declaration of Helsinki.

Clinical specimens

Thirty pairs of colon cancer tissues and non-tumor normal tissues were gained from cases pathologically diagnosed with colon cancer undergoing surgeries at Tongji Medical College, Huazhong University of Science and Technology from 2011 to 2018. Informed consents in written forms were collected from these cases prior to this research. Samples (30 colon cancer tissues and 30 non-tumor normal tissues) were placed at -80°C for further exploration. Lymph node metastasis (TNM) staging system was adopted in this research. This experiment gained the approval from the Ethics Committees of Tongji Medical College, Huazhong University of Science and Technology. The clinical case data are listed in **Table 1**.

Cell culture and transfection

Colon cancer cell lines (SW620, HCT116, SW480, HT-29, LoVo) and normal colon epithelial cell line (NCM460) were provided by ATCC, USA. Cells were cultured in DMEM (Gibco BRL, Grand Island, NY, USA) with 10% FBS (Gibco, Carlsbad, CA), and were maintained in an incubator with 5% CO_2 at 37°C. Lipofactamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied for cell transfection. Transfection plasmids were provided by GenePharma (Shanghai, China). Detailed sequences were depicted in **Table 2**.

RNA isolation and qPCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and SYBR PremixEx Taq (Vazyme, Shanghai, China) was utilized to examine transcript abundance. Primers were constructed by GenePharma (Shanghai, China). All the PCR primers were listed in **Table 2**.

RNase R digestion

Total RNA (5 μg) was fostered using 3 U/μg of RNase R (Epicentre Biotechnologies, Shanghai, China) at 37°C for 15 min, followed by RNase R digestion twice as mentioned above.

Ethynyldeoxyuridine (EdU) analysis

Cells were seeded in the 96-well plate (1×10^5 per well). EdU-labeled solution (KeyGen Bio-

Table 2. Sequences of primers for qRT-PCR and siRNA related sequence

Name		Sequence
circ-0001313	Forward	5'-CGAGACAGACGACGACAAAA-3'
	Reverse	5'-TTGACGGTCATCTTCTATTTGC-3'
AKT2	Forward	5'-ACCACAGTCATCGAGAGGACC-3'
	Reverse	5'-GGAGCCACACTTGTAGTCCA-3'
GAPDH	Forward	5'-AGAAGGCTGGGGCTCATTTG-3'
	Reverse	5'-AGGGGCCATCCACAGTCTTC-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
miR-510-5p	Forward	5'-ACACTCCAGCTGGGCACTAACGGTGAGAGG-3'
	Reverse	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGAGTCC-3'
circ-0001313 siRNA	Sense	5'-ATTAGAGCATCAGGAAACAGTTT-3'
	Antisense	5'-ACTGTTTCCTGATGCTCTAATTT-3'
miR-510-5p mimics	Sense	5'-UACUCAGGAGAGUGGCAAUCAC-3'
	Antisense	5'-GAUUGCCACUCUCCUGAGUAUU-3'
miR-510-5p inhibitor	Sense	5'-GUGAUUGCCACUCUCCUGAGUA-3'

tech, Nanjing, China) was supplied per well and incubated at 37°C for 2 h. DAPI was added for nuclei staining.

Western blotting

After cell lysis, proteins were collected, followed by quantification and SDS-PAGE (12% gel) with 50 μg protein sample/lane. Thereafter, membrane (PVDF) transfer was conducted for proteins, followed by incubation with primary antibodies at 4°C overnight and with secondary antibodies for 2 h. Ultimately, ECL system (Thermo Fisher Scientific, USA) was employed for color development of membrane bands, and these bands were then assessed using Quantity One software (Bio-Rad Laboratories, San Diego, CA, USA). Primary antibodies of Caspase-9, cleaved Caspase-9, Bcl-2, Bcl-W, A1, Bad, AKT, phospho-AKT, PI3K, phospho-PI3K, mTOR, phospho-mTOR, AKT2 and GAPDH, as well as secondary antibodies were provided by Abcam (Shanghai, China).

Detection of caspase-9 activity

Colorimetric substrates (Ac-DEVD-AMC) were added for incubation of the lysed cells at 37°C in dark for 1 h, after which the optical density (405 nm) was tested with the use of a microplate reader. In this assay, the Caspase-9 activation kit (R&D Systems, Minneapolis, MN, USA) was employed.

Apoptosis examination

Annexin V/PI kits (KeyGEN, Nanjing, China) were applied to detect apoptotic cells. Fluorescence-activated cell sorter was used for determining the apoptotic rate.

Dual-luciferase reporter gene assay

Predicted binding sequences of circ-0001313/AKT2 to miR-510-5p and mutative sequences were inserted into pGL3 vector. Cells were co-transfected with wild-type/mutant sequences miR-510-5p mimics/NC and for 48 h. Luciferase intensity was finally determined through a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Bioinformatics analysis

For NCBI Gene Expression Omnibus (GEO) data, the GSE121895 was used for identifying gene expression difference. $P < 0.05$ and the log2 fold change (log2FC) > 1 or < -1 was considered statistically significant.

Statistical processing

Quantitative data are manifested as the mean ± standard deviation. SPSS 24.0 software (SPSS, Chicago, USA) was adopted to perform comparisons of data *via* the χ^2 test and Fisher's exact test under a small sample size. The Pearson test was performed to determine the

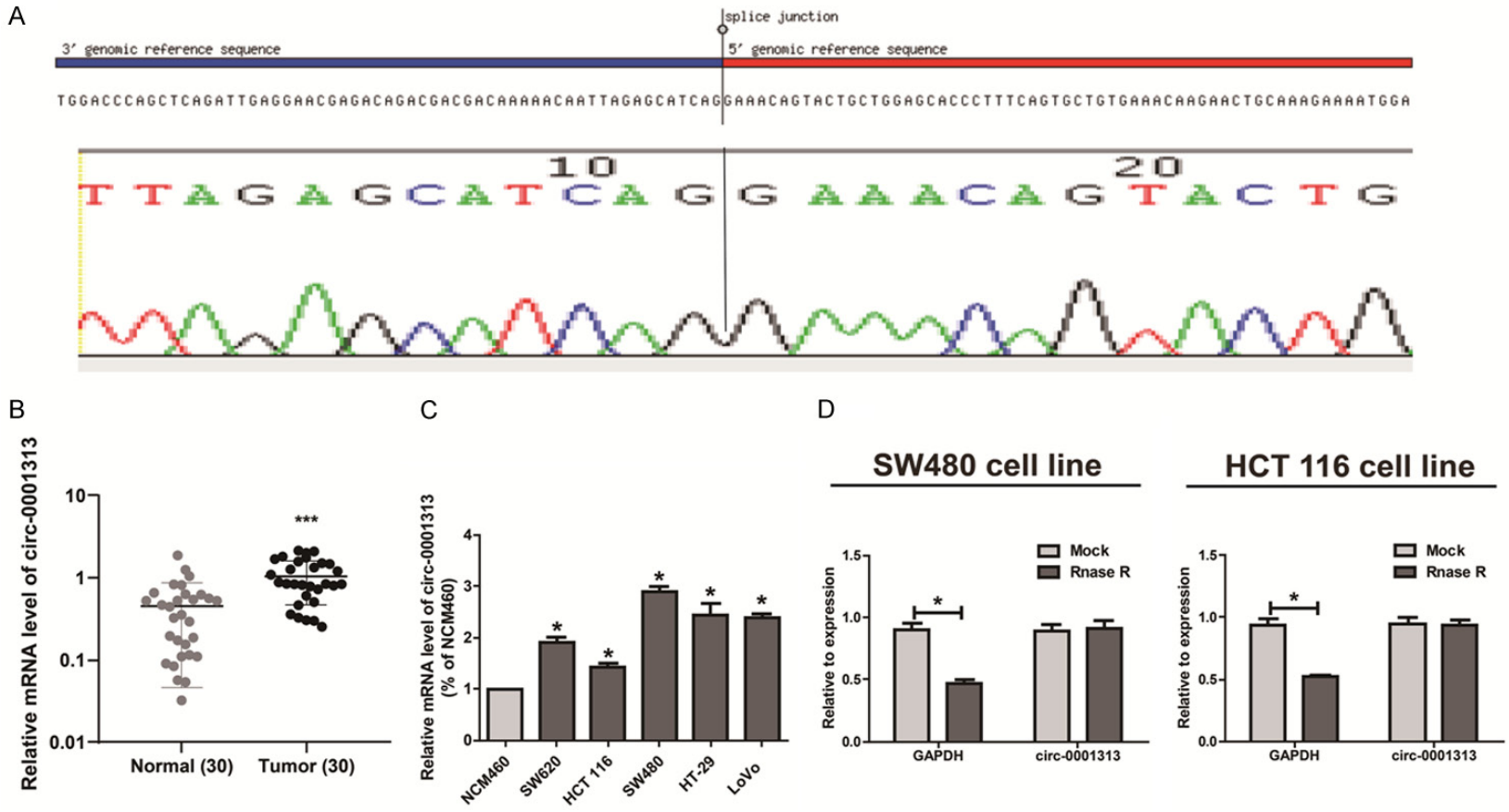

Figure 1. Characteristics and expression of circ-0001313 in colon cancer. A. The sequence of circ-0001313 in circBase (upper part) was consistent with that in Sanger sequencing (lower part). B. Expression level of circ-0001313 in colon cancer tissues and paired paracancerous tissues (n = 30). C. Expression level of circ-0001313 in colon cancer cell lines (SW620, HCT116, SW480, HT-29, LoVo) and normal colon epithelial cell line (NCM460) detected by qRT-PCR. D. Circ-0001313 in colon cancer cells was resistant to RNase R digestion. *P < 0.05, ***P < 0.001. Data were shown as mean ± SD from three independent experiments.

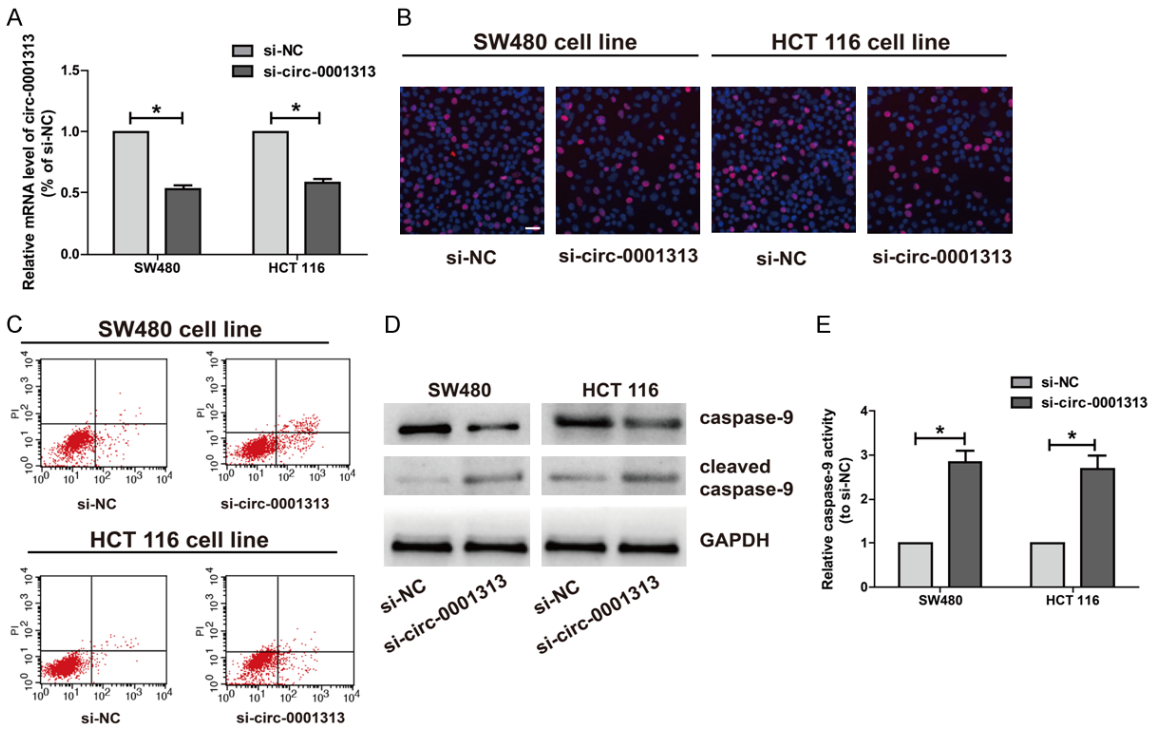

Figure 2. Circ-0001313 promoted proliferation and inhibited apoptosis of colon cancer cells. A. Expression level of circ-0001313 in colon cancer cells transfected with si-circ-0001313 or si-NC detected by qRT-PCR. B. EdU assays were performed to determine the proliferation of colon cancer cells transfected with siRNA circ-0001313 or si-NC (scale bars 50 μm). C. Flow cytometry performed to determine apoptosis of colon cancer cells transfected with si-circ-0001313 or si-NC. D, E. Caspase-9 activity in colon cancer cells transfected with si-circ-0001313 or si-NC. si-circ-0001313, circ-0001313 siRNA; si-NC, siRNA negative control. *P < 0.05. Data were shown as mean ± SD from three independent experiments.

relationship between circ-0001313 expression and AKT2. For all tests, intergroup differences were analyzed by the unpaired two-sided *t*-test. $P < 0.05$ represented a statistically significant difference.

Results

Circ-0001313 exhibited a high expression in colon cancer tissues

GSE121895 microarray analysis demonstrated that circ-0001313 displayed a high expression in colon cancer tissues. Moreover, it was also discovered the upregulated circ-0001313 in colon cancer tissues relative to non-tumor matched tissues, and circ-0001313 expression in colon cancer cell lines was higher than normal colon epithelial cell line (**Figure 1A-C**). In particular, SW480 with the highest circ-0001313 expression and HCT116 with lowest circ-0001313 expression were selected for the subsequent experiments. Features of circ-0001313 were verified since it was resistant to RNase R digestion (**Figure 1D**).

Circ-0001313 reduction repressed proliferation and boosted apoptosis of colon cancer cells

To explore the biological function of circ-0001313 in colon cancer cells, SW480 and HCT116 cells were transfected with siRNA circ-0001313. Firstly, its transfection rate was determined by qRT-PCR (**Figure 2A**). Next, it was discovered in EdU assay that circ-0001313 reduction evidently repressed the proliferation rate of colon cancer cells relative to controls (**Figure 2B**). According to the results of flow cytometry, circ-0001313 silence greatly induced colon cancer cell apoptosis (**Figure 2C**). Thereafter, protein levels of genes associated with apoptosis were measured, and it was discovered that transfection of siRNA circ-0001313 upregulated cleaved Caspase-9 activity, implying that the apoptosis is stimulated (**Figure 2D** and **2E**).

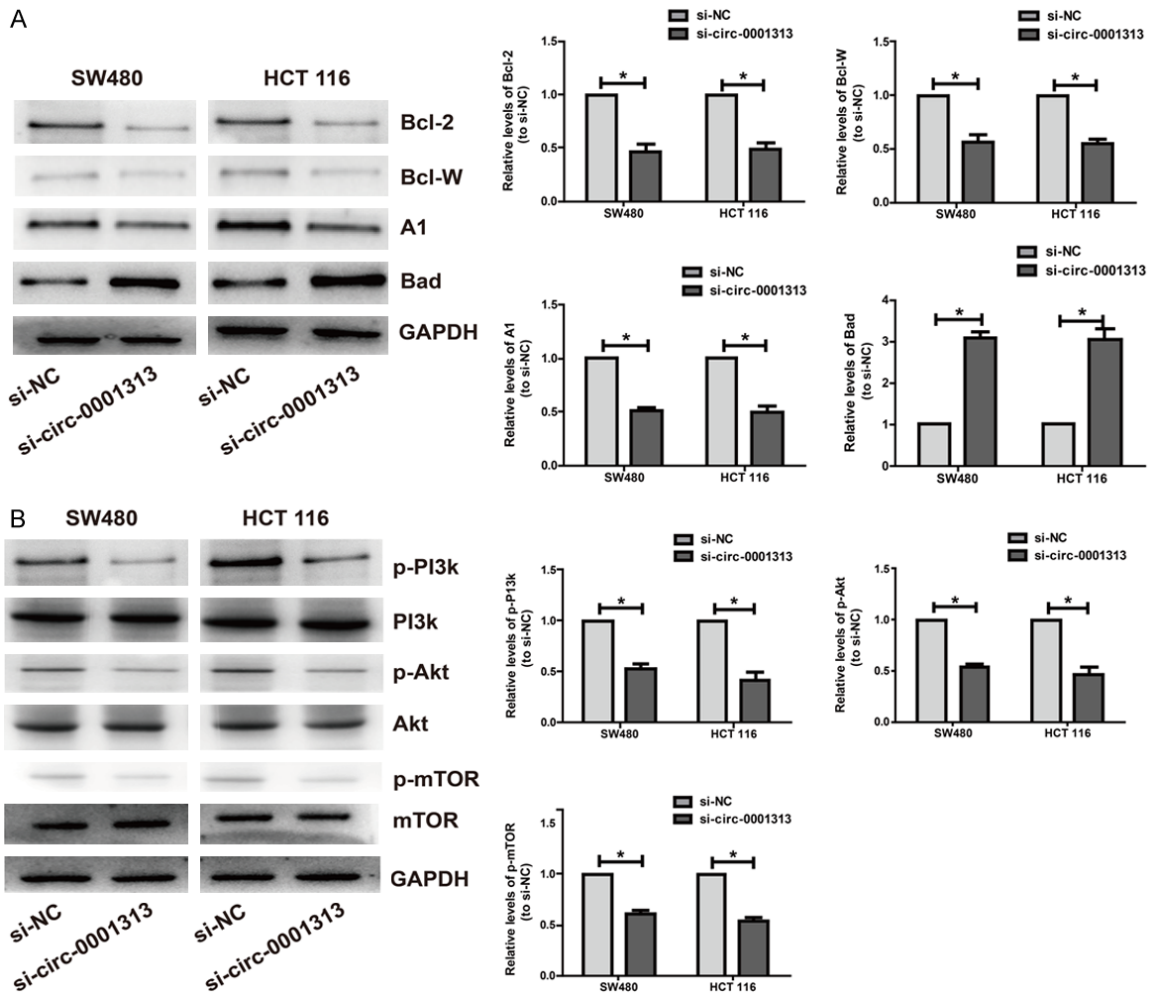

Figure 3. Circ-0001313 regulated Bcl-2 family and PI3K/AKT/mTOR pathway. A. Western blot analyses of Bcl-2, Bcl-W, A1 and Bad in colon cancer cells transfected with si-circ-0001313 or si-NC. B. Western blot analyses of PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR in colon cancer cells transfected with si-circ-0001313 or si-NC. si-circ-0001313, circ-0001313 siRNA; si-NC, siRNA negative control. *P < 0.05. Data were shown as mean ± SD from three independent experiments.

Circ-0001313 adjusted Bcl-2 family and PI3K/AKT/mTOR pathway

Bcl-2 family and the PI3K/AKT/mTOR pathway substantially regulate apoptosis [11, 12]. Therefore, the regulation of circ-0001313 on them was assessed. Circ-0001313 silence markedly downregulated genes that suppressed apoptosis in Bcl-2 family (Bcl-2, Bcl-W and A1), but conversely, upregulated gene Bad that promotes apoptosis (**Figure 3A**). In the meantime, transfection of siRNA circ-0001313 downregulated p-AKT, p-PI3K and p-mTOR in colon cancer cells, while their total levels did not change (**Figure 3B**). It can be seen that circ-0001313 may influence colon cancer cell apoptosis *via* regulating Bcl-2 family and inhibiting PI3K/AKT/mTOR pathway.

Circ-0001313 directly bound to miR-510-5p

CircRNAs could function as ceRNAs to sponge corresponding miRNAs, so as to adjust their biological roles [7, 13]. Bioinformatics prediction in accordance with circinteractome (https://circinteractome.nia.nih.gov) revealed that several miRNAs have the matching sequence with circ-0001313 3'-UTR and miR-510-5p has very high scores among them. Here, potential binding sites between circ-0001313 and miR-510-5p were forecasted through bioinformatics analysis, which were continuously to be confirmed by dual-luciferase reporter gene assay. The data unfolded a decreased luciferase intensity in SW480 and HCT116 cells co-transfected with wild-type circ-0001313 and miR-510-5p mimics. How-

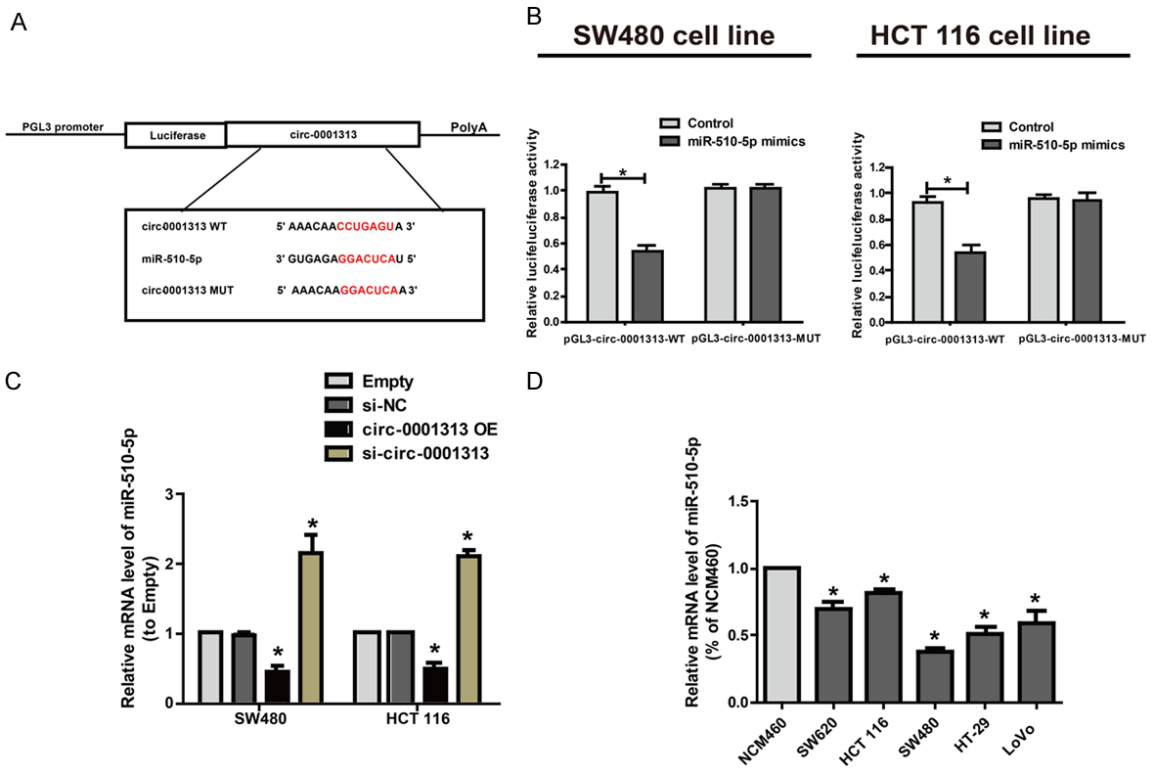

Figure 4. Circ-0001313 directly bound to miR-510-5p in colon cancer cells. A. Potential binding sites between circ-0001313 and miR-510-5p. B. Dual-luciferase reporter gene assay in colon cancer cells co-transfected with wild-type/mutant circ-0001313 and miR-510-5p mimics/negative control. C. MiR-510-5p level in colon cancer cells transfected with siRNA circ-0001313, pcDNA circ-0001313 or si-NC detected by qRT-PCR. D. Expression level of miR-510-5p in colon cancer cell lines (SW620, HCT116, SW480, HT-29, LoVo) and normal colon epithelial cell line (NCM460) detected by qRT-PCR. *P < 0.05. Data were shown as mean ± SD from three independent experiments.

ever, after co-transfection with mutant circ-0001313 and miR-510-5p mimics, no pronounced change was observed in luciferase intensity (**Figure 4A** and **4B**). Circ-0001313 was verified to be negatively associated with miR-510-5p as circ-0001313 overexpression pulled down miR-510-5p, whereas circ-0001313 reduction decreased miR-510-5p (**Figure 4C**). Furthermore, miR-510-5p exhibited a low expression in colon cancer cells (**Figure 4D**). All in all, it can be concluded that miR-510-5p directly binds to circ-0001313 in colon cancer cells.

Circ-0001313 regulated AKT2 expression via inhibiting miR-510-5p

MiR-510-5p's biological role is rarely reported so far, so the underlying downstream of miR-510-5p on Targetscan and miRDB was forecasted in the first place. Through screening, it was found that AKT2 was probably a target (**Figure 5A**). Besides, the luciferase intensity was confirmed by dual-luciferase reporter gene assay to become weakened after co-transfection with wild-type AKT2 and miR-510-5p mimics, whereas it was unchangeable after co-transfection with mutant AKT2 and miR-510-5p mimics (**Figure 5B**). Afterwards, levels of AKT2 mRNA and protein were determined in colon cancer cells treated with miR-510-5p NC, miR-510-5p mimics, siRNA circ-0001313 or pcDNA circ-0001313, respectively. AKT2 was markedly downregulated by miR-510-5p overexpression or circ-0001313 knockdown. Conversely, AKT2 expression was upregulated after miR-510-5p knockdown or circ-0001313 overexpression (**Figure 5C** and **5D**). QRT-PCR data denoted that AKT2 expression was notably upregulated in colon cancer cells relative to NCM460 cells (**Figure 5E**). Then AKT2 was evidently raised in colon cancer tissues relative to the non-tumor matched tissues (**Figure 5F**). In addition, a prominent correlation between the expression levels of circ-0001313 and AKT2

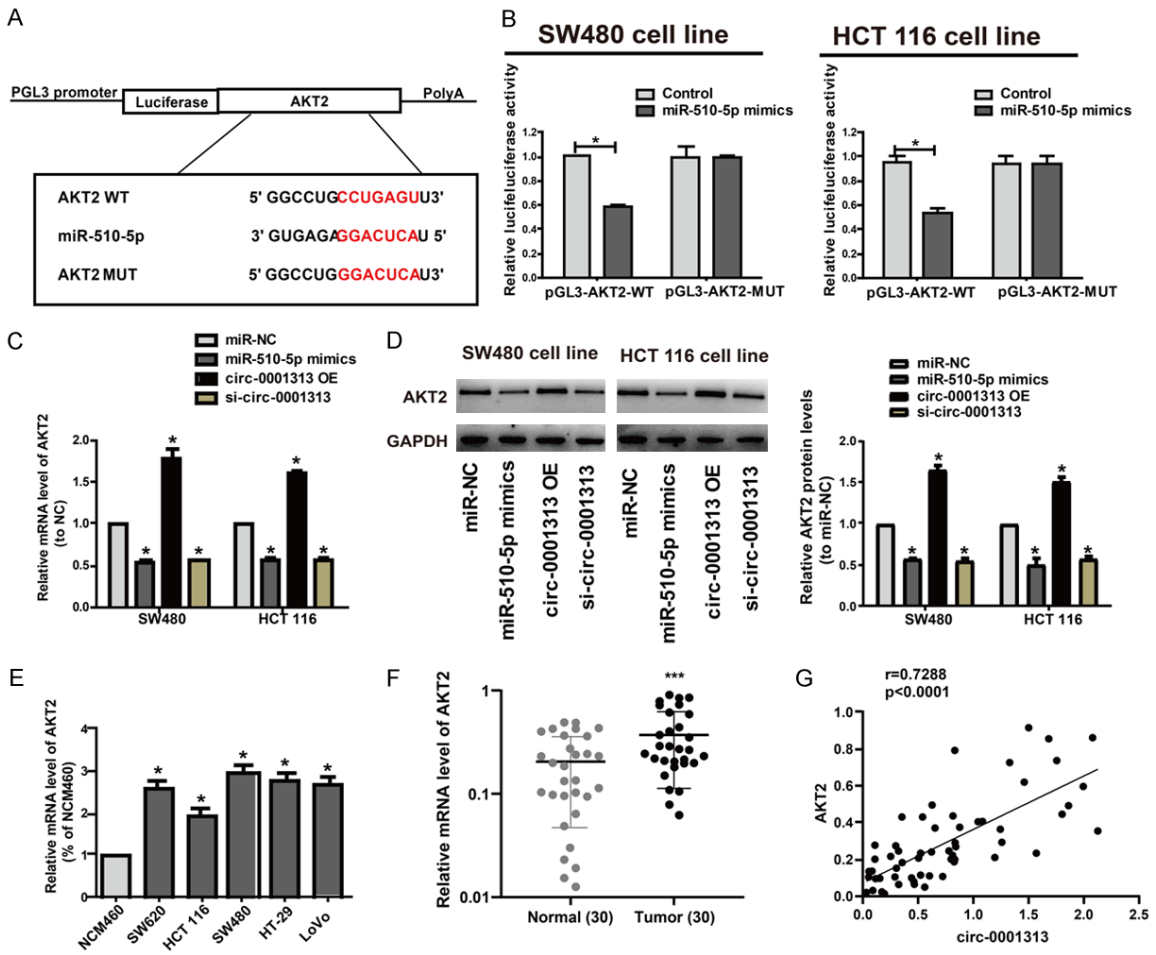

Figure 5. Circ-0001313 upregulated AKT2 *via* sponging miR-510-5p in colon cancer cells. A. Potential binding sites between AKT2 and miR-510-5p. B. Dual-luciferase reporter gene assay in colon cancer cells co-transfected with wild-type/mutant AKT2 and miR-510-5p mimics/negative control. C, D. The mRNA and protein level of AKT2 in colon cancer cells transfected with miR-510-5p mimics, miR-510-5p NC, siRNA circ-0001313 or pcDNA circ-0001313. E. Expression level of AKT2 in colon cancer cell lines (SW620, HCT116, SW480, HT-29, LoVo) and normal colon epithelial cell line (NCM460) detected by qRT-PCR. F. Expression level of AKT2 in colon cancer tissues and paired paracancerous tissues. G. Bivariate correlation analysis of the relationship between circ-0001313 and AKT2 expression level. *P < 0.05, ***P < 0.001. Data were shown as mean ± SD from three independent experiments.

was observed (**Figure 5G**). The above findings imply that circ-0001313 adjusts AKT2 expression through repressing miR-510-5p in colon cancer cells.

AKT2 reduction triggered colon cancer cell apoptosis

Biological function of AKT2 in colon cancer cells was specifically analyzed. First of all, transfection of siRNA AKT2 pronouncedly decreased AKT2 expression in SW480 and HCT116 cells (**Figure 6A** and **6B**). What's interesting was that the impact of AKT2 on colon cancer cell apoptosis as circ-0001313 was similar, and there was an opposite influence with miR-540-5p. Silence of AKT2 remarkably induced apoptosis, while miR-510-5p knockdown markedly inhibited apoptosis (**Figure 6C** and **6D**). More importantly, miR-510-5p could reverse the regulation of circ-0001313 on the apoptosis of colon cancer cells, and the trend of Caspase-9 activity was identical to the result obtained from flow cytometry (**Figure 6E** and **6F**). The above data imply that circ-0001313 induces colon cancer *via* the circ-0001313/miR-510-5p/AKT2 axis.

Discussion

The epigenetic regulation of DNA on disease progression has become the research empha-

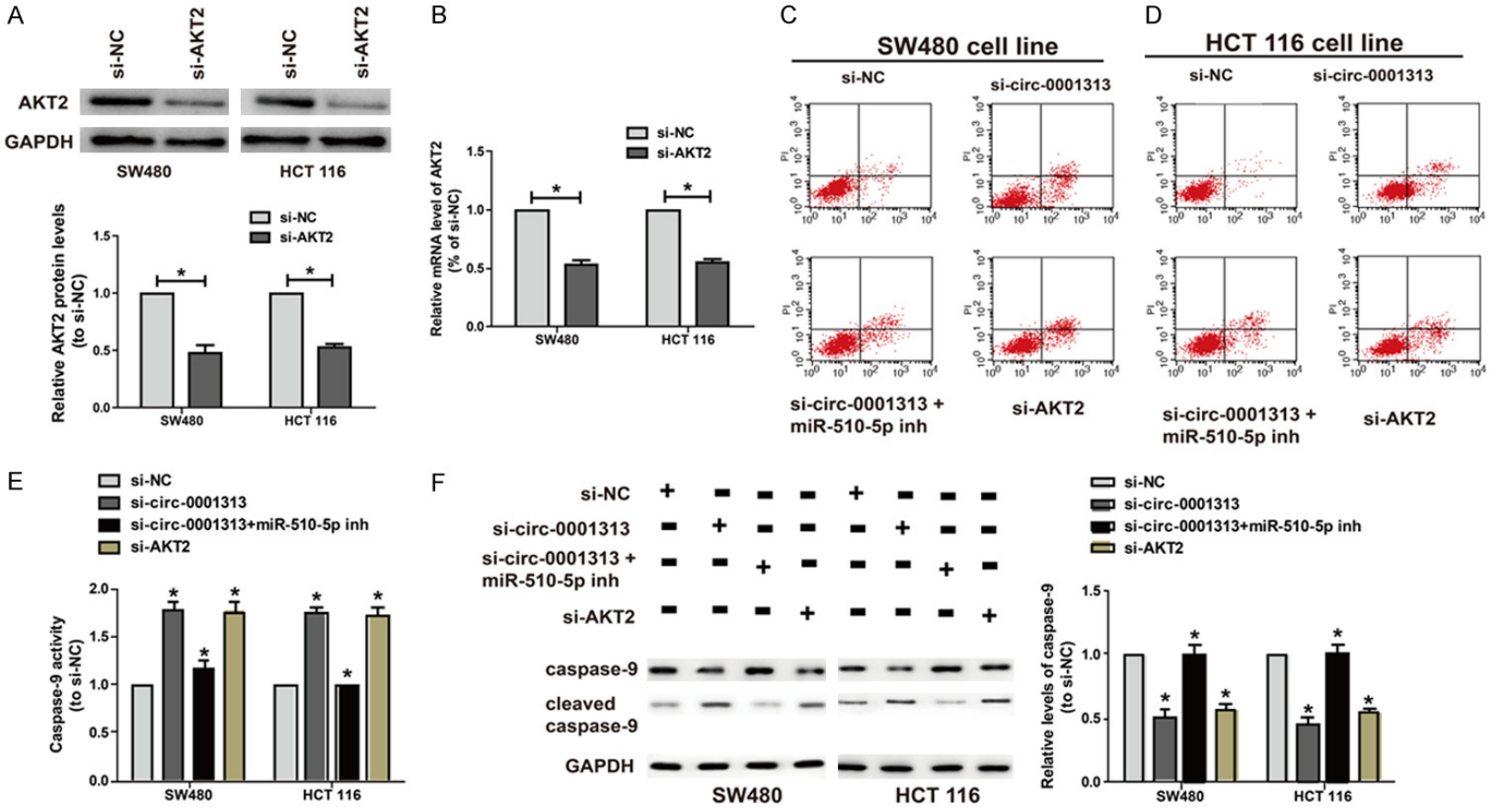

Figure 6. Knockdown of AKT2 induced apoptosis in colon cancer cells. A. Western blot analysis of AKT2 in colon cancer cells transfected with si-AKT2 or si-NC. B. The mRNA level of AKT2 in colon cancer cells transfected with si-AKT2 or si-NC detected by qRT-PCR. C, D. Flow cytometry determined apoptosis in transfected colon cancer cells. Silence of AKT2 or circ-0001313 remarkably induced apoptosis, while miR-510-5p knockdown markedly inhibited apoptosis. E, F. Caspase-9 activity in transfected colon cancer cells. Silence of AKT2 or circ-0001313 remarkably stimulated the activity of caspase-9, while miR-510-5p knockdown markedly inhibited it. si-circ-0001313, circ-0001313 siRNA; si-AKT2, AKT2 siRNA; si-NC, siRNA negative control. *$P < 0.05$. Data were shown as mean ± SD from three independent experiments.

sis. Through observation, multiple circRNAs, including has_circ_0055625 [14], has_circ_001988 [15], circAGO2 [16], etc., involved in the pathogenesis of colon cancer may be considered as targeting for diagnosing and treating colon cancer. In this research, circ-0001313 displayed a high expression in colon cancer tissues and cells. Circ-0001313 reduction remarkably blocked the proliferation rate but stimulated the apoptosis of colon cancer cells. The pivotal role of circ-0001313 in the progression of colon cancer can be concluded.

MiRNAs are small ncRNAs adjusting gene expressions [17]. MiRNAs are involved in cell cycle progression as well as cell differentiation and adjustment, and have an inextricable association with numerous diseases, especially tumors [18-20]. In this study, complementary sequences of target miRNA to circ-0001313 were first predicted by bioinformatics analysis and further verified by dual-luciferase reporter gene assay. Low expression of miR-510-5p in colon cancer cells was verified by qRT-PCR. Detected using the same method, AKT2 was forecasted to be the direct target of miR-510-5p. A train of functional assays illustrated that circ-0001313 triggered colon cancer by sponging miR-510-5p to elevate AKT2.

As a putative oncogene, AKT2 encodes the gene that belongs to the subfamily of serine/threonine kinases containing SH2-like (Src homology 2-like) domains [21, 22]. Multiple AKT2-related diseases have been found, such as bladder cancer [23], oral cancer [24], pancreatic cancer [25], breast cancer [26], colon cancer [27] and etc. This research proved that downregulation of AKT2 activated caspase-9 and finally increased the apoptosis rate of colon cancer cells.

In conclusion, circ-0001313 is raised in colon cancer and has an oncogenic impact by sponging miR-510-5p to elevate AKT2 as a ceRNA. It is considered that circ-0001313 may serve as a target for treating colon cancer.

Disclosure of conflict of interest

None.

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