ORIGINAL RESEARCH RETRACTED ARTICLE: Hsa_circRNA_000166 Promotes Cell Proliferation, Migration and Invasion by Regulating miR-330-5p/ELK1 in Colon Cancer

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class of non-RNAs, which are Introduction: Circular RNAs (circRNAs), a not lir widely expressed in human cells, have essential les in the development and progression of cancers. The aim of this study is to figure of circ 02,766 in colon cancer (CC) the I development and the signaling pathway volved.

Materials and Methods: HT29 and H 116 cells are transfected with siRNA of circRNA, miRNA mimics and inhibitors. Cele roliferation, migration and invasion were examined using CCK-8 assay and transwell assay, pectively. Luciferase reporter assay was circRNA and miRNA. CC cells were implanted into nude mice used to validate the targets subcutaneously to detect the or growth.

Results: hsa circRNA 0001 vas signizantly upregulated in the human CC tissue and in the on of hsa energy 000166 reduced cell viability, colony formation, CC cell lines. K decreased tumor size and weight in vivo. Luciferase reporter migration and inva on in y 30-5p was the target of circRNA 000166. miR-330-5p could bind to 3' assay rev and that n gion (3 TR) of ELK1 to downregulate both mRNA and protein expression of untr slated K1. Dual objition CircRNA 000166 and miR-330-5p inhibited the suppression of cell on, migration and invasion induced by si-circRNA 000166. pro

Conclusion: The data of this study demonstrated that the hsa circRNA 000166 could upregulate the expression of gene ELK1 by sponging miR-330-5p, which may contribute a better understanding of the regulatory circRNA/miRNA/mRNA network and CC pan genesis.

Keywords: colon cancer, circRNA 000166, miRNA-330-5p, ELK1



Introduction

Colon cancer (CC) is the third most common cancer (1.8 million new cases reported in 2018) in the world and the fourth one to cause death.^{1,2} Global burden of CC is estimated to be augmented by 60%, which means more than 2.2 million new cases and 1.1 million deaths in 2030.³ CC is considered as a "lifestyle" disease because the mortality and morbidity are associated with diet, obesity and carcinogenesis.⁴⁻⁶ Many signaling pathways are involved in the development of CC such as PTEN-Akt, NF-kB, AMPK-COX-2, as well as ELK1.^{7–9} ELK1 is one of the transcription factors belonging to ETS family,¹⁰ which regulates cell proliferation, angiogenesis, differentiation and apoptosis.¹¹ Upregulation of ELK1 has been found to promote cervical cancer,¹² thyroid cancer progression¹³ and urothelial tumorigenesis.¹⁴ Therefore, ELK1 expression and activation plays a crucial role in tumorigenesis.



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microRNAs (miRNAs), a class of noncoding RNAs with ~22 nucleotides, can induce translational suppression through binding to the 3'-untranslated region (3'UTR) of their target mRNAs.¹⁵ Dysregulation of miRNAs is linked to carcinogenesis.¹⁶ More and more studies are focusing on interactions between circular RNAs (circRNAs) and miRNAs since biological effects of circRNAs are mainly mediated by miRNAs.17,18 circRNAs are a novel class of noncoding RNAs with covalently closed continuous loops, which make circRNAs more stable than linear microRNAs.¹⁹⁻²² Utilizing the high-throughput sequencing technology, more and more circRNAs have been found to involve in pathological process such as myocardial infarction, apoptosis, depression, as well as carcinomas.²³⁻²⁶ circRNAs can act as a real sponge of miRNAs to regulate gene expression²⁶⁻²⁸ and the circRNA-miRNA-mRNA network might play a key role in cancer related and non-cancer pathways.^{29,30}

Recent studies have proved a global increase of circRNA expression in both CC cell lines and tumor tissues.^{1,31} However, little is known about the role of circRNAs in the development of CC. This study aims to explore the role of circ_000166 on CC progression and the signaling pathway involved.

Materials and Methods Cell Culture

Normal human colon mucosal epithelial cellane NCU 460 and six colon cancer cell lines (HT29, HUT116 HCT) LoVo, SW420 and SW620 cells) dere purchased from American Type Culture Collection ATCC, USA. The cells were cultured in Eagles MEM (Signe-Aldrich, USA) containing 10% fetal boying serum (FBS, Incurgen, USA) and 1% penicillin/strengmycin/davitrogen, USA) at 37 °C with 95% air and 5% CC

RNA Extract and Quantitative Real-Time PCR (qRIPCR) Assay

Total RNA was thracted using Trizol reagent (TaKaRa, China) according to the manufacturer's instructions. 500 ng of total RNA was reverse transcribed to cDNA with the PrimeScript RT Master Mix (TaKaRa, China). The relative RNA expression was examined using the SYBR Premix Ex Taq II Kit (TaKaRa, China) on the StepOnePlus system (Applied Biosystems, USA). The primer sequences (Sigma-Aldrich, USA) used in this study are shown in Table 1. The data were calculated by means of the $2^{-\Delta\Delta Ct}$ method.

Table I Primer Sequences for qRT-PCR

Gene	Primer Sequences	
GAPDH	Forward: CCACATCGCTCAGACACCAT Reverse: CCAGGCGCCCAATACG	
circRNA_000166	Forward: CCATATTGAATCACAGTGCGT Reverse: ACAGCGCAGTAAGGTGCTCG	
U6	Forward: CGCTTCGGCAGCACATATAC Reverse: TTCACGAATTTGCGTGTCAT	
miR-330-5p	Forward: TCTCTGGGCCTGTGTCTTAGGC	
ELKI	Forward: CCTTGCC, AND STATGAC Reverse: CCTTC, SGTACTAC, TGAC	

RNase R Treatment

Total RNA (10 μ g) was included with or without 3 U· μ g⁻¹ of RNast R (Epicetre Bielechnologies, USA). After incubation 37°C for Lemm, the RNA was subsequently purified by RNeasy MinElute Cleaning Kit (Qiagan, commany) and can subjected to qRT-PCR.

Ce Viability and Colony Assay

Cell w. reeds into sterile 96-well plates. After transfecbase cell proliferation was measured at 0, 24, 48, 72 and 9 hours (h) using the Cell Counting Kit-8 (CCK-8) assay (Dojido, Japan) according to the manufacturer's instrucons. Briefly, 10 μ L of CCK-8 solution was added to each well. The solution was then measured spectrophotometrically at 450 nm after 2-hour incubation at 37°C.

For colony formation assay, a total of 2000 stably transfected cells were seeded into 6-well plates and cultured for 2 weeks under standard conditions. Then, the colonies were washed with PBS, fixed with methanol and then stained with crystal violet. The number of clone spots was counted in 10 random view fields using a microscope (Olympus, Japan).

Cell Invasion and Migration Assays

Transwell assay was used to examine cell migration and invasion. For cell invasion assay, 1×10^6 of HT29 and HCT116 cells were seeded into the upper chamber of a 24-well insert (8-µm pore size; Corning Inc., USA) precoated with Matrigel. The upper chamber was filled with serum-free medium while the lower chamber was filled with FBS-contained medium. The cells in the upper chamber were removed and the invading cells were fixed with methanol and stained with crystal violet after incubation for 48 h. Cells

from five random fields were counted under a 200× microscope. For cell migration assay, the upper chambers were not coated with Matrigel, and the following protocols were the same as what was conducted for cell invasion assay.

Plasmid and Luciferase Reporter Assay

This protocol followed the published paper.³² Briefly, the full-length of *ELK1* 3'-UTR containing (wt) and scrambled (mut) miR-330-5p binding sequence was inserted downstream of the firefly luciferase gene in psiCHECK2 to generate the psiCHECK2-*ELK* 3'UTR-wt or cirRNA_000166 wt plasmid and psiCHECK2-*ELK* 3'UTR-mut plasmid or cirRNA_000166 mut, respectively. The wt and mut plasmids subsequently were co-transfected into CC cells with negative control, miR-330-5p mimics, si-circRNA_000166 along with control Renilla luciferase expression plasmid (phRL-TK) using Lipofectamine 2000 (Invitrogen, USA). After 24 h, luciferase and renilla signals were assayed using the Dual-luciferase reporter Assay System (Promega, USA) according to the manufacturer's instructions.

Western Blotting

Protein was extracted using RIPA cell lysis buffer (Beyotime, China). 10 μ g of protein was electrophered on a 10% polyacrylamide gel (SDS-PAGE) and transforred to PVDF membranes (Hybond; USA). Membranes was blocked for 1 h with 5% milk and there probed with the indicated primary antibodies and the appropriate second ary antibodies (Cell Signaling Technolog, el SA). Finally, blots were detected using a characteristic reagent kit (Merck KGaA, Germany).

Tumor Xenograts in Nude Nice

Male BALB/c nucleonics (6–8 weeks) were purchased from Guangelong Marcal Laboratory Animal Center (Foshan, unina) and kep under the environment of 23 $\pm 2^{\circ}$ C, $\pm 5 \pm 15^{\circ}$ chumidity, 12 h light/12 h dark cycle. Negative unital cells of treated cells with the indicated lentivirus vector with a concentration of 1×10^{7} /mL diluted in PBS, 0.1 mL of this solution was injected subcutaneously on the back flank of each mouse at day 0. Tumor size was measured with a caliper every 7 days until 35 days. The tumor weight was weighed every 7 days until 35 days.

All experiment procedures were approved and carried out following the ethical standards under a protocol approved by the Committee on Animal Welfare of Suqian First Hospital, and were executed conforming to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (No. 85–23, 1996).³³

Bioinformatics Analysis

CircInteractome (<u>https://circinteractome.nia.nih.gov/</u>) was used to predict miRNA-330-5p binding sites to the hsa_circRNA_000166 and TargetScan (<u>http://www.targets</u> <u>can.org/</u>) was used to predict the potential miR-330-5p binding sites to 3'UTR of *ELK1* to study the possible crossing network among circPLA, https// https//

Statistical Anal sis

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Results have been presented as mean \pm SEM. All statistical analysis was performed via the Pearson chisquared (1st, two-tailed sectent's *t*-test, or analysis of variance (ANO A) GraphPad Prism 7.0. (GraphPad Sectionary, USA). *p* 0.05 was considered statistically significant.

Upregulated in Colon Cancer Cell Lines and Tissues

To investigate the dysregulated circRNAs in CC tissue, we analyzed 10 pairs of human CC tissue and their adjacent normal tissue from GSE126094 database and figured out the top 15 upregulated and downregulated circRNAs (Figure 1A). hsa_circRNA_000166 was chosen for further study. qRT-PCR results of 30 pairs of human CC tissue and their adjacent normal tissue showed that circRNA_000166 expression was elevated in CC tissue (Figure 1B). The qRT-PCR data from six CC cell lines (HT29, HCT116, HCT8, LoVo, SW420 and SW620 cells) also demonstrated that circRNA_000166 expression was higher than that in NCM 460 cells (Figure 1C).

RNase R treatment was used to confirm the circular characteristics of circRNA_000166. The results manifested that the circRNA_000166 expression did not change while the linear control gene GADPH expression was significantly reduced with the treatment of RNase R in both HT29 and HCT116 cells (Figure 1D). Further experiments demonstrated that circRNA_000166 was mainly localized in cytoplasm (Figure 1E)





circRNA_000166 Knockdover Inchied Colon Cancer Proliferation Migration and Invasion

To figure out the effects of *c* RNA 000166 CC, Scramble RNA (si-NC) and circ. NA_0/166 siRNA (si-cicRNA _000166) was transfected to TT29 and HCT116 cells. The vircR 000 56 expression was sigdata demonstrat nificantly red ced (Figure 2A), is cating that the function of circRNA 00. 66 as mine a. CCK-8 assay revealed that downregulation criticRNA 000166 decreased the proliferative ability of HT29. HCT116 cells (Figure 2B). Inhibition of circRNA 000166 reduced the number of clone spots in HT29 and HCT116 cells compared with the control group (Figure 2C). 1×10^{6} of HT29 and HCT116 cells were transfected with si-NC and si-circRNA 000166, and then implanted into nude mice subcutaneously. Subcutaneous tumor size and tumor weight were smaller in the si-circRNA 000166 group compared with si-NC group (Figure 2D). Transwell assay without and with Matrigel was used to

as the pering analysis of the top 15 upregulated and downregulated circRNAs in CC; (**B**) using qb the period of the top 15 upregulated and downregulated circRNA_000166 expression in different CC cell lines 016 expression and GAPDH in HT29 and HCT116 cells treated with RNase R; (**E**) to r, ns: no significance.

examine HT29 and HCT116 cells migration and invasion, respectively. Both migrated cells and invaded cells were decreased in si-circRNA_000166 group (Figure 2E and F).

circRNA_000166 Sponged miR-330-5p

Many studies have revealed that circRNAs can act as a sponge of miRNAs to regulate gene expression.^{26–28} A complementary sequence was observed between circRNA_000166 and miR-330-5p predicted using CircInteractome database (Figure 3A). Luciferase reporter assay indicated that miR-330-5p expression was reduced in WT circRNA_000166 transfected cells (Figure 3A). Furthermore, RNA pull-down assay demonstrated that circRNA_000166 were enriched in miR-330-5p group (Figure 3B). Inhibition of circRNA_000166 induced upregulation of miR-550-3p (Figure 3C).

qRT-PCR assay was conducted to examine miR-330-5p expression in 10 pairs of human CC tissue and their adjacent normal tissue. The data showed that miR-330-5p expression was decreased in CC tissue compared with



Figure 2 circRNA_0 66 knockdown inhibited colon cancer proliferation, migration and invasion. (A) qRT-PCR analysis of knockdown efficiency of hsa_circ_000166 in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166. (B) CCK-8 assay results of cell viability in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166; (C) Colony formation in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166; (C) Colony formation in HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166; (C) Tumor size and weight in nude mice implanted subcutaneously with 1 × 10⁶ of HT29 and HCT116 cells transfected with si-NC and si-circRNA_000166 (E) HT29 and HCT116 cell migration using transwell assay; (F) HT29 and HCT116 cell invasion using transwell assay. *p < 0.05; **p < 0.01.

Abbreviations: si-NC, Scramble siRNA; si-circRNA_000166, siRNA of circRNA_000166.

normal tissue (Figure 3D). Consistently, TCGA database demonstrated miR-330-5p expression was lower in CC tissue than that in normal tissue (Figure 3E). A negative

correlation was observed between the expression of circRNA_000166 and miR-330-5p human CC tissue (Figure 3F).



Figure 3 circRNA_000166 sponged miR-330-5p. (A) the pute of using sites between circRNA_000166 and miR-330-5p. Relative miR-330-5p expression in cells cotransfected with wt or mut circRNA_000166 and put-330-5p using ciferase reporter assay; (B) Enrichment of circRNA_000166 using RNA pull-down experiments; (C) Relative miR-330-5p expression in circRNA_00016 mockdown cells, (C) Relative miR-330-5p expression in CC tissue and their adjacent normal tissue; (E) Relative miR-330-5p expression from TCGA database; (F) pearma rank-order contraction between miR-330-5p and circRNA_000166. **p < 0.01. Abbreviations: ns, no significance. miR-NC, negative carbol of miR-330-5p; wt, wild type; mut, mutant.

circRNA_00016c Recalated Colon Cancer Programsion by Stronging miR-330 op

The results of RT cR snowed that miR-330-5p expression was significantly pregulated in si-circRNA_000166 group compared with si-NC group and was reduced in si-circRNA _000166 + miR-330-5p inhibitor group compared with si-circRNA_000166 group (Figure 4A). CCK-8 assay indicated that knockdown of circRNA_000166 decreased the proliferation of HT29 and HCT116 cells at 24h, 48h, 72h and 96h, and that cell viability was increased in si-circRNA _000166 + miR-330-5p inhibitor group compared with sicircRNA_000166 group (Figure 4B). Similar results were also observed in colony growth of HT29 and HCT116 cells (Figure 4C). Cell migration and invasion data revealed that both cell migration and invasion were inhibited by si-circRNA _000166 and restored dual inhibition of circRNA_000166 and miR-330-5p (Figure 4D and E).

circRNA_000166 Promotes ELK1 Expression via Sponging miR-330-5p

The miRNA target prediction website <u>http://www.targets</u> <u>can.org</u> (TargetScan) was used to predict the direct target mRNA of miR-330-5p. The results demonstrated that there were miR-330-5p binding sites in the 3'-untranslated region (3'UTR) of ELK1 (Figure 5A). The results of luciferase assay demonstrated that overexpression of miR-330-5p reduced the luciferase activity of wt ELK1



Figure 4 circRNA_166 accelerated colon cancer progression by targeting miR-330-5p. (A) qRT-PCR analysis of relative miR-330-5p expression in HT29 and HCT116 cells transfected with si-VC, si-circRNA_000166 and si-circRNA_000166 + miR-330-5p inhibitor; (B) CCK-8 assay of cell viability in HT29 and HCT116 cells transfected with si-circRNA_000166 + miR-330-5p inhibitor; (C) Colony formation in HT29 and HCT116 cells transfected with si-circRNA_000166 + miR-330-5p inhibitor; (D) HT29 and HCT116 cell migration using transwell assay; (E) HT29 and HCT116 cell invasion using transwell assay. **p < 0.01 versus (vs) si-NC; ## p < 0.01 vs si-circ_000166.

transfected cells while reduction of luciferase activity was not observed in the 3'-UTR of ELK1 mutant group (Figure 5A).

mRNA and protein expression of ELK1 was examined by qRT-PCR and Western blotting, respectively. In the cells

transfected with miR-330-3p inhibitor and si-circRNA _000166, both mRNA level and protein expression of ELK1 was upregulated in HT29 and HCT116 cells (Figure 5B and C). On the contrary, in the cells transfected with miR-330-3p mimic and circRNA_000166 + miR-330-5p mimics, both



Figure 5 circRNA 000166 p oted ELK xpression through sponging miR-330-5p. (A) The putative binding sites between ELK1 and miR-330-5p. Relative ELK1 ut circRNA 000166 using luciferase reporter assay; (B) Relative ELK mRNA and protein expression in HT29 and HCT116 cells expression in cells transfected with t an depublicors; (C) Relative ELK mRNA and protein expression in HT29 and HCT116 cells transfected with vector, p mimics; (D) Relative ELK1 expression in 30 pairs of CC tissue and their adjacent normal tissue. Spearman's ranktransfected with miR-NC miR-330 nimics and 000166 circRNA 000166 and miR-32 , and between circRNA_000166 and ELK1. **p < 0.01. order correlation 30-5p and veen mi s, no signif nce. miR-NC, miRNA negative control. Abbreviation

mRNA level and petein expression of ELK1 was downregulated compared with the cells transfected with miR-330-3p inhibitor and circRNA_000166 (Figure 5B and C). ELK1 mRNA expression was elevated in CC tissue (Figure 5D). Spearman's rank-order correlation results manifested a negative correlation between the expression of ELK1 expression and miR-330-5p, and a positive correlation between ELK1 expression and circRNA_000166 in 30 pairs of human CC tissue (Figure 5D).

Discussion

circRNAs are stable in cells because of their covalent closedloop structure without a 5'end and a poly-A tail, which protected circRNAs from ribonuclease degradation.³⁴ Many studies have shown that circRNAs may be involved in miRNA inhibition and tumorigenesis, including CC.^{34–36} For example, circular RNA PIP5K1A promotes CC cell invasion and migration via sponging miR-1273a.³⁷ Overexpression of circCCDC66 accelerated CC cell proliferation, migration and invasion.³⁸ As a sponge of miR-6778-5p, CircRNA CBL.11 could regulate YWHAE expression, resulting in suppressing cell growth of CRC.³⁹ All of these evidences suggest that circRNAs play an important role in CC development. However, the role of circRNA_000166 in CC has not been reported. The present study is the first to indicated that hsa_circRNA_000166 was upregulated both in CC tissue and cell lines, suggesting that hsa_circRNA_000166 contributed to CC progress. Inhibition of hsa_circRNA_000166 reduced CC cell proliferation, migration and invasion, resulting in colon tumor growth arrest, demonstrating hsa_circRNA_000166 might be a therapeutic target of CC.

ELK1, characterized by a conserved DNA-binding domain, or Ets domain, was regarded as a transcription factor engaged mainly in the regulation of cell growth, differentiation, and migration.^{40,41} It is reported that tumor-derived CXCL5 promotes CC cell migration via activation of ERK/ELK1/Snail pathway, suggesting that activation of ELK1 might contribute to human CC metastasis.⁴² In cervical cancer cells, upregulation of ELK1 enhanced cell proliferation, migration and invasion.43 All these publications proved that increased expression of ELK1 promoted cancer progress and metastasis. In this study, ELK1 expression was higher tissue, positively correlated with circRNA 000166, and high level of circRNA 000166 in CC accelerated proliferation, migration and invasion h in itro al in vivo, indicating that ELK1 might play ar mortan role in CC development and metrasis, h was indirectly regulated by circRNA 0 66.

miR-330-5p has been reported a play a role in many cancers, such as cervice cancer, and pencreatic cancer.^{44–}⁴⁶ In this study, TargetScan and luciferase assay proved that ELK1 was the direct arget of miR-330-5p, which is coincident with previous study comiR-330-5p in cervical cancer.⁴⁴ everex ression of anR-330-5p suppressed cell growth of non-act cell lung cancer (NSCLC).⁴⁷ The present stale showed that miR-330-5p expression was lower in CC usue than that in normal tissue and inhibition of miR-330-5p hereased CC cell proliferation, migration and invasion. These findings revealed that high level of miR-330-5p expression could inhibit CC cancer progress.

Accumulated studies have revealed that circRNAs can act as a sponge of miRNAs to regulate gene expression.^{26–28} miR-330-5p was also regulated by circRNAs. For example, increasing expression of circPTN rescued the inhibition of proliferation and downregulation of SOX9/ITGA5 in glioma cells through binding to miR-330-5p.⁴⁸ In NSCLC, circFARSA targeted miR-330-5p and miR-326 to relieve their inhibitory effects on oncogene fatty acid synthase.⁴⁹ This is the first study to report the interaction between circRNA_000166 and miR-330-5p, and the interaction between 3'UTR of ELK1 and miR-330-5p. Upregulation of ELK1 by circRNA_000166 was mediated by miR-330-5p to promote CC cell proliferation, migration and invasion, thereby accelerating CC progress.

In conclusion, our data demonstrated that the hsa_circRNA_000166 upregulated the expression of ELK1 as sponge of miR-330-5p, responding CC cell proliferation, migration and investor. Then findings may contribute to a better understanding of between the regulatory miRNA network and CC path genesis. The hsa_circRNA_00016 may be a point of biomarker and future therapeutic larget of CC.

Disclesi

The authors report no conflicts of interest in this work.

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