hsa_circ_0000231 Promotes Colorectal Cancer Cell Growth Through Upregulation of CCND2 by IGF2BP3/miR-375 Dual Pathway

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Research

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Abstract

Background

circular RNAs (circRNAs) recently have been emerged as vital regulators for involvement of initiation and progression of diverse kinds of human cancers. This study aimed to investigate the role of circRNAs in colorectal cancer (CRC).

Methods

The expression profile of circRNAs in 5 pairs of CRC tissues and adjacent normal tissues were analyzed by Microarray. Quantitative real-time PCR and in situ hybridization and Base Scope Assay were used to determine the level and prognostic values of hsa_circ_0000231. Then, functional experiments *in vitro* and *in vivo* were performed to investigate the effects of hsa_circ_0000231 on cell proliferation.

Mechanistically, fluorescent in situ hybridization, dual luciferase reporter assay, RNA pull-down and RNA immunoprecipitation experiments were performed to confirm the interaction between hsa_circ_0000231 and IGF2BP3 or has_miR-375.

Results

hsa_circ_0000231 was evidently up-regulated in CRC primary tissues, which was indicated to poor prognosis of CRC patients. The results demonstrated that hsa_circ_0000231 could promote CRC cell proliferation as well as tumorigenesis *in vitro* and *in vivo*. Mechanistic analysis showed that hsa_circ_0000231 might on the one hand act as a ceRNA (competing endogenous RNA) of miR-375 to regulate cyclin D2 (CCND2), and on the other hand bind to IGF2BP3 protein to protect CCND2 from being degraded.

Conclusion

Our findings suggest that hsa_circ_0000231 facilitated CRC progression by sponging miR-375 or binding to IGF2BP3 to modulate CCND2. This discovery implied that has_circ_0000231 may be a potential new diagnostic and therapeutic biomarker for CRC.

Background

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the fourth most commonly diagnosed cancer in women worldwide¹. The annual new cases of colorectal cancer account for the third place in malignant tumors, and the deaths account for the fourth place². CRC is the fifth most commonly diagnosed cancer and the fifth most common cause of death by cancer in China in 2014, with

age-standardized incidence rate of 17.52 per 100000 and age-standardized mortality rate of 7.91 per 100 000, respectively³.

Colorectal cancer has become a major disease that seriously threatens human health, and it also creates a huge social and economic burden⁴. The treatment of colorectal cancer mainly adopts a comprehensive treatment mode centered on surgery, but the curative effect is still unsatisfactory. The molecular mechanism of the development of colorectal cancer is not completely clear, which is one of the important reasons for its high incidence and poor prognosis⁵. Recent studies have found that a new family of non-coding RNAs, circular RNA (circRNA), plays an important role in the molecular regulation of tumorigenesis and progression. It has a covalently closed circular structure at the 3' UTR and 5' UTR, and is widely and diversely present in eukaryotic cells, and has an endogenous RNA molecule that regulates gene expression⁶⁻⁹. circRNA functions mainly by competing for the endogenous RNA (ceRNA) mechanism, that is, circRNA acts as a "molecular sponge", and competitively binds miRNA through a miRNA response element (MRE) to inhibit miRNA function. miRNAs degrade or inhibit the expression of target genes through RNA-induced silencing complex (RISC), which relies on Ago2 protein¹⁰.

MicroRNAs (miRNAs) are small non-coding RNAs with a size of 18-25 nucleotides, which function as post-transcriptional regulators of target mRNAs¹¹. It is well known that the increase of oncogenic miRNAs expression in cancer leads to the downregulation of tumor-suppressive genes, whereas the decrease of tumor-suppressive miRNAs enhances the expression of oncogenes. The researches demonstrated that miRNAs can participate in tumorigenesis and progression of various cancers, including colorectal cancer¹²⁻¹⁴. However, the upstream regulators of miRNAs are poorly understood. Pandolf et al. present a theory called the competing endogenous RNA (ceRNA) hypothesis that lncRNAs, mRNAs and pseudogenes could communicate with and regulate each other by competitively binding to the microRNA response elements (MREs), which provides a new mechanism of gene regulation¹⁵. It has been reported that circRNA could also serve as ceRNA to sequester away the miRNA from its target genes^{16, 17}. It also has been reported that circRNAs could bind RNA protective protein¹⁸. However, biological functions of most circRNAs and the underlying mechanisms in pathogenesis and progression including CRC remain largely unclear.

In the present study, we found that 425 significantly differentially expressed circRNA in CRC tissues. has_circ_0000231 upregulation was further discovered to sponge miR-375 and IGF2BP3 to regulate CRC progression. hsa_circ_0000231 may play a key role in the development of colorectal cancer and participate in the molecular regulation of colorectal cancer by regulating the cell cycle.

Methods

Ethics statement

This study was conducted in accordance with the ethical standards, the Declaration of Helsinki, and national and international guidelines, and was approved by the authors' institutional review board, which

adheres to generally accepted international guidelines for animal experimentation.

circRNA microarray

Five pairs of CRC tumor tissues and the corresponding adjacent noncancerous tissues, were utilized for circRNA microarrays. The specimens were obtained from patients undergoing surgery in the Peking University People's Hospital in 2014; detailed information is shown in Additional file 1: Table S1.

Patients and samples

160 CRC patients who were diagnosed and underwent surgery in Peking University People's Hospital between 2014 and 2017 were included in this study. Fresh colorectal tumor tissues and matched normal colorectal mucosa tissues were obtained from all the 160 patients. The specimens were obtained and immediately frozen in liquid nitrogen and stored at -80°C until RNA or protein extraction.

Cellular fluorescence in situ hybridization

The cells were fixed with 4% paraformaldehyde for 15min, washed with PBS three times for 5min each time. 0.5% TritonX-100 was permeabilized at room temperature for 20min, and washed with PBS three times for 3min each time. Add 3% citric acid freshly diluted pepsin and digest at room temperature for 15min, then expose the nucleic acid fragment, rinse with PBS and add 20 μl of pre-hybrid solution, then pre-hybridize at 50°C for 2-4 hours. The hsa_circ_0000231 or miR375-specific probe hybridizes at a constant temperature of 50°C . Hybridization was carried out by SSC at 37°C water temperature, biotinylated mouse anti-digoxigenin was added dropwise, and PBS was washed 3 times for 3min each time, and the excess paper was absorbed by the absorbent paper. After adding DAPI staining for 10min, the specimen was subjected to nuclear staining, washed three times with PBS for 3min each time, and the excess paper was absorbed by the absorbent paper. The liquid was sealed with a liquid containing a fluorescent quencher, and the image was observed and collected under a fluorescence microscope.

Base Scope assay

Base Scope assays were performed in accordance with the manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA, USA). Tissues were sectioned at 5- μm thickness, placed onto Super frost Plus slides (Fisher Scientific, Loughborough, UK), and allowed to dry overnight at 25°C . Sections were then baked at 60°C for 1 h before deparaffinizing in xylene (twice for 5 min) and ethanol (twice for 2 min) and dried by baking at 60°C for 2 min. Then, hydrogen peroxide was applied for 10 min at 25°C , target retrieval was performed for 15 min at 100°C , and RNAscope[®] Protease III was applied at 40°C for 30 min, samples were rinsed twice in distilled water between treatments. Base Scope probes (Mm-1700024F13Rik, cat#709881) with positive control (Hs-PPIB, cat # 701031) and positive control (DapB, cat # 701011) were then applied and samples were incubated for 2 h at 40°C in a HybEZ oven and then with reagents AMP0 (30 min at 40°C), AMP1 (15 min at 40°C), AMP2 (30 min at 40°C), AMP3 (30 min at 40°C), AMP4 (15 min at 40°C), AMP5 (30 min at 25°C), and AMP6 (15 min at 25°C). Slides were rinsed with wash buffer (twice for 2 min) between AMP incubation steps. Finally, slides were treated with Fast

Red for 10 min at 25 °C in the dark and counterstained with Gill's hematoxylin, dried for 15 min at 60 °C, and mounted in Catamount permanent mounting medium (Vector Labs, Burlingame, CA, USA).

Cell lines and cell culture

The human CRC cell lines SW480, SW620, HT29, HCT8, LoVo, colo-205, LS174T, HCT116, RKO and NCM460 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and subcultured and preserved by our lab. The frozen cells were quickly removed from the liquid nitrogen tank, thawed in a 37 °C water bath, then inoculated into a disposable cell culture flask (25cm²), fresh 5ml of DMEM medium was added, and then placed in 5% CO₂ incubator at 37 °C, and the liquid was changed once every 2 days. When the adherent cell fusion degree reached about 90%, the passage was carried out, and then the cells were counted, and then inoculated at a concentration of 1 × 10⁵/mL to continue to be cultured for use.

RNA extraction and quantitative real-time PCR

Total RNA from cell lines and tissue samples was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. For the plasma, the total RNAs were extracted using mirVanaTM PARISTM microRNA extraction kit (ABI) according to the manufacturer's instructions. For lncRNA quantification, GAPDH was used as internal control, and PrimeScriptTM RT Master Mix (TAKARA) were used for reverse transcription and real-time PCR. The primer sequences were listed in Additional file 1: Table S2. All reactions were performed in triplicate. The fold change for each gene relative to the control group was calculated using the 2^{-ΔΔCt} method.

Lentiviral short hairpin (sh)RNA particles

Recombinant lentiviral particles expressing hsa_circ_0000231 or hsa_circ_0000231 small interfering (si)RNA were obtained from GenePharm Co., Ltd. (Shanghai, China). SW480 Cells were grown to approximately 40% confluence and infected with lentiviral particles in complete medium for 48 h. To increase the infection efficiency, cells were co-treated with the cationic polymer polybrene (8g/mL in water). Neither shRNA nor polybrene affected cell viability. siRNA and shRNA had no off-target effects, and did not affect cell adherence, shape, or viability at the indicated multiplicity of infection.

Cell transfection

For *in vitro* studies, to interfere the expression of hsa_circ_0000231, siRNA interference sequences targeting hsa_circ_0000231 were designed and synthesized (Ribobio, Guangzhou, China), and a final concentration of 50nM were used for transient transfection; to overexpress hsa_circ_0000231, full-length human hsa_circ_0000231 cDNA was cloned into the pcDNA3.1 expression vector (Genechem, Shanghai, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to manufacturer's instructions.

For *in vivo* assays, hsa_circ_0000231 overexpression cell line was used. The hsa_circ_0000231 gene was cloned in to a lentivirus vector LV-GFP-Puro, and SW480 cells were used for infection. Stable transfection cells were established by puromycin antibiotic selection applied for 7 days, with a concentration of 2.5 ug/ml. The hsa_circ_0000231 overexpression cells and control cells were named SW480-LV-hsa_circ_0000231 and SW480-LV-NC, respectively.

Transfection and grouping of cells: Cell transfection was performed in strict accordance with the instructions of Invitrogen's Lipofectamine® 3000 Transfection Reagent: Transfection was performed when the cytoplasm was inoculated with a confluence of about 80%—the Lipofectamine 3000 reagent was diluted with OPTI-MEM culture medium and mixed. Dilute the DNA expression plasmid to be transfected with OPTI-MEM culture medium, then add P3000 reagent and mix well—add diluted DNA expression plasmid in equal volume to each diluted dilution of Lipofectamine 3000 reagent and incubate at room temperature 5min. Add the DNA-liposome mixture to the cell suspension and carefully mix—continue to culture in 5% CO₂ incubator at 37°C.

Cell proliferation assay

SW480 and SW620 cells (3×10^3 cells) were seeded in 96-well plates in complete medium and infected with hsa_circ_0000231, hsa_circ_0000231 siRNA. Cell proliferation assay was performed with Cell Counting Kit-8 (CCK8) according to the manufacturer's protocol, and detected at 0, 24, 48, 72 and 96 h. Cells in each group were tested for 5 replicates. Cell proliferation was evaluated by the cell counting kit-8 method according to the manufacturer's instructions using a microplate reader (Molecular Devices, Sunnyvale, CA, United States) to measure the absorbance.

For colony formation assay, transfected cells were seeded into each well of a 6-well plate on day 0 then incubated for another 14d. Then, the wells were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet. The form colonies were counted and analyzed by Image J software.

CCK-8 method for detecting cell proliferation curve

The proliferation of colorectal cancer cells in each group was detected by CCK-8 method. After transfection for 24 hours, the cells were seeded in 96-well plates at 5×10^3 cells/well, and 10μl of CCK-8 solution was added to each well. Then, incubate in a 5% CO₂ incubator at 37°C. When the cells were grown to the 2d, 3d, 4d, and 5d, the absorbance of the cells at 450nm was measured using an enzyme reader to represent the proliferative activity of the cells during this period. The proliferation curve of each group of cells was plotted, and the difference in cell proliferation rate of each group was compared.

Western blot analysis

Cells were lysed in RIPA buffer, centrifuged at high speed, and then underwent protein quantification using a bicinchoninic acid assay. Cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes.

Membranes were incubated with primary antibodies followed by horseradish peroxidase-labeled secondary antibody. GAPDH was used as a loading control. The total protein of CRC cells was extracted with RIPA buffer and separated by 10% SDS-PAGE, then electrotransferred onto a PVDF membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% skimmed milk powder and incubated with primary antibodies against CCND2 (1:1000), IGF2BP3 (1:1000), (Abcam, Burlingame, CA, USA), RB (1:500), and GAPDH (1:5000) (Cell Signaling Technology, Beverly, MA, USA) at 4 °C overnight and then incubated with secondary antibodies (1:5000) (Cell Signaling Technology, Beverly, MA, USA) at room temperature for 2 h. Finally, the bands were examined by Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). The antibodies used in the experiments are shown in Additional file 1: Table S3.

Luciferase reporter assay

hsa_circ_0000231 and CCND2 fragments containing two putative wild-type or mutated miR-375-binding sites were amplified by PCR and cloned downstream of the luciferase gene in the pGL3 vector (Promega, Madison, WI, USA). The constructed reporter vectors were verified by sequencing. Luciferase reporter assays were performed by transiently co-transfecting HEK293T cells in 24-well plates with the reporter vectors, miR-216a, and the Renilla luciferase construct using Lipofectamine 2000 (Invitrogen, Waltham, USA). After 48 h transfection, cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and normalized to that of Renilla luciferase.

Biotin-labeled RNA pull-down and mass spectrometry analysis

Biotin-labeled RNA for linear sequence of hsa_circ_0000231 and CCND2 was generated by an *in vitro* transcription reaction with the Biotin RNA Labeling Mix (Roche, Mannheim, Germany) and T7 RNA polymerase (Roche, Mannheim, Germany), and then treated with RNase-free DNase I (Takara, Japan). After incubation with guide oligonucleotide targeting circular junction, the linear probe was then circularized using T4 RNA ligase I, treated with RNase R. After purified with RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA), the biotin-labeled RNA probe (3 µg) was then incubated with cell extracts from CRC cells at room temperature (RT) for 2 h, and treated with 35 µl of Streptavidin C1 magnetic beads (Invitrogen) for 1 h. After washed, the retrieved protein was detected by western blot or mass spectrometry analysis (CapitalBio Technology, Beijing, China).

RNA immunoprecipitation (RIP)

RIP was conducted with Magna RIP kit (Millipore, Billerica, MA, USA) following manufacturer's instructions. SW480 cells were harvested 48 h after transfection of miR-375 mimics or miR-NC, and lysed in complete RNA lysis buffer, then cell lysates were incubated with magnetic beads which were conjugated with anti-Argonaute2 (AGO2) (Millipore, Billerica, MA, USA) or negative control IgG antibody (Millipore, Billerica, MA, USA) at 4 °C for 4 h. The beads were washed using washing buffer. Then immunoprecipitated RNA and protein were purified and enriched to detect the target RNAs and AGO2 by qRT-PCR and western blot.

Nude mouse model of ectopic tumors

Balb/C nude (nu/nu) mice at 6 wk old were purchased from Beijing weitonglihua Experimental Animal Technology Co., Ltd. Tumors were generated by the subcutaneous injection of 2×10^6 SW480 cells infected with hsa_circ_0000231 overexpression cells, or control lentivirus particles and suspended in 50L of PBS into the dorsal region near the thigh. 5 mice were included in each group. Mice were then weighed and assessed for tumor size every 7wk by measuring the tumor length and width.

Statistical Analysis

The data generated in this study were all analyzed by SPSS22.0 statistical software. The measurement data were expressed by mean $\pm$ standard deviation ($\bar{x} \pm s$). The two groups were compared by T test for statistical analysis. Analysis of variance of factors, count data were analyzed by χ^2 test. The survival rates were evaluated by Kaplan-Meier method and tested by log-rank test. The effects of clinical variables on the overall survival of CRC patients were determined by univariate and multivariate Cox proportional hazards regression model. In the multivariate Cox proportional hazards regression model, age, T stage, N stage, clinical stage distant metastasis and the expression of hsa_circ_0000231 were adjusted for variable analysis. The correlation between groups was analyzed by Pearson correlation, and $p < 0.05$ was used as a criterion for statistically significant differences.

Results

hsa_circ_0000231 is up-regulated in CRC specimens and associated with the progression and poor prognosis of CRC patients

In order to understand the expression profiles of circRNA in CRC, we performed Arraystar circular RNA chip in paired CRC tissues and para-cancerous tissues from 5 patients with CRC. With a cut-off criteria of fold change > 1.5 and $p < 0.05$, we found that a total of 425 circRNAs were differentially expressed, among which 278 were upregulated while 147 were downregulated (Figure 1A-B). Besides, according to the classification analysis of circRNAs, the exon-related circRNAs were the most (Figure 1C—Additional file 1: Table S4). And remarkably, it was found that hsa_circ_0000231 was the most upregulated (4.557 folds) circRNA, which was spliced from ARHGAP12 located at [chr10:32197099-32199491](#) and finally formed a circular transcript of 794nt according to the annotation of circBase (<http://www.circbase.org/>).

A randomized selection of 10 circRNAs with significant differential expression in 10 pairs of colorectal cancer tissues showed quantitative PCR detection and the results were consistent with the chip results, indicating that the chip analysis results were credible (Figure 1D). Further quantitative PCR was performed to detect the expression of hsa_circ_0000231 in nine groups of colorectal cancer cell lines include SW480, SW620, SW620, LoVo, HT29, HCT116, RKO, LS174T, COLO-215, HCT8, along with human normal colorectal epithelial cell-derived NCM460 cells. The results showed that hsa_circ_0000231 could be detected in the above cell lines. And their expression level in cancer cell lines is higher than that of NCM460 cells (Figure 1E).

Then expression of hsa_circ_0000231 in 160 colon cancer patients and adjacent normal tissues was detected by quantitative PCR. The results showed that the expression of hsa_circ_0000231 in tumor tissues was higher than that in adjacent normal tissues, and the mean difference was 1.39 times (Figure 1F).

Statistical analysis of the expression of hsa_circ_0000231 in colorectal cancer tissues and clinical pathological parameters of patients revealed that the expression level of hsa_circ_0000231 was correlated with tumor size, depth of invasion and TNM stage of patients (Table1); Then, we conducted a survival analysis to determine the relationship between hsa_circ_0000231 and total survival (OS) and disease-free survival (DFS) in patients with CRC. It was found that patients with high expression of hsa_circ_0000231 in colorectal cancer had significantly shorter OS and lower DFS, and the differences were statistically significant (Figure 1G-H). Further univariate and multivariate Cox regression analysis showed that TNM stage and hsa_circ_0000231 expression levels were independent prognostic factors for CRC patients (Table S5).

has_circ_0000231 promotes CRC cell proliferation and modulates cell cycle, colony formation but no apoptosis

The effect of hsa_circ_0000231 on cell proliferation was detected by CCK8 assay. Compared with the control group, the proliferation ability of has_circ_0000231 siRNA group and both hsa_circ_0000231 and si-mRNA group in SW480 cells or SW620 cells were significantly weakened ($P < 0.05$) (Figure 2A-B).

Flow cytometry analysis with Annexin V/PI double staining showed that SW480 and SW620 cells transfected with each group of siRNAs respectively, there was no significant difference in the apoptotic rate in si-circ_0000231 group compared with the negative control (si-NC) group of SW480 and SW620 cells (Figure 2C-D).

After transfection with siRNAs in each group, there was no significant difference in the apoptotic rate between the si-mRNA group S, the si-circ_0000231 group and the si-both group SW480 cells and SW620 cells compared with the negative control group (si-NC) (Figure 2E-F).

Cell cycle detection by flow cytometry revealed that the percentage of G0/G1 cells obviously increased after inhibition of the expression of hsa_circ_0000231 in SW480 and SW620 cells. These results indicate that knockdown of hsa_circ_0000231 can induce G0/G1 phase arrest in CRC cell lines (Figure 2G-H).

hsa_circ_0000231 facilitates tumorigenesis and proliferation of CRC cells *in vivo*

To determine the effects of hsa_circ_0000231 on tumor growth *in vivo*, SW480 cells were stably transfected with over expression or mock vector and infected with lentivirus negative control (LV-NC) or LV-circ and then subcutaneously injected into female nude mice. As a result, the tumors derived from cells overexpressing hsa_circ_0000231 were bigger and heavier than those of the control group (Figure 2

I-K). These results confirmed the oncogenic role of hsa_circ_0000231 in development of CRC, suggesting that hsa_circ_0000231 might play a great part in the progression of CRC.

CCND2 might be a downstream target of hsa_circ_0000231 in CRC

To further clarify how hsa_circ_0000231 functions in CRC, given that hsa_circ_0000231 affects CRC cell cycle process, specific siRNA was used to interfere hsa_circ_0000231, then the Cell Cycle related genes PCR chip (Cell Cycle PCR array, Qiagen company) testing found that 18 gene expression of the 84-cell cycle-related genes more than doubled compared to the control cells compared with control group. The 10 mostly decreased genes were depicted at Figure S1 A. The downregulation of CCND2, CCND1, CDK6, CDKN3 and CCND2 was the most obvious (the downregulation factor was more than 3 times). qPCR experiment verified that the mRNA expression trends of CCND2, CCND1, CDK6, CDKN3 and CCND2 were consistent with the cell cycle chip results, and the expression levels were all down-regulated (Figure S1 B). CCND2 was found to be the most upregulated cell cycle-related gene in CRC tissues. As is known that cell cycle can act as a convergence point for oncogenic signaling pathways and aberrant cell cycle progression is a crucial characteristic of cancers. Hence, we focused our attention on hsa_circ_0000231 and CCND2 in tumorigenesis and progression of CRC in the present study.

qRT-PCR was used to detect CCND2, and the expression was found to be significantly reduced after interference with hsa_circ_0000231 (Figure S1 C). Then expression of CCND2 in 160 CRC cohort was detected by qPCR. The results showed that the expression of CCND2 in tumor tissues was notably higher than that in adjacent normal tissues (Figure S1 D). Then survival analysis was performed to reveal that patients with high expression of CCND2 in CRC patients had significantly shorter OS (Figure S1 E). Besides, the expression of CCND2 was positively correlated with hsa_circ_0000231 (Figure S1 F). And the expression level of CCND2 was correlated with tumor size and TNM stage (Table 1). In nude mice study, Western blot revealed that the expression of CCND2 in hsa_circ_0000231 overexpressed tumor tissues was much higher than that in mock group (Figure S1 G-H).

hsa_circ_0000231 functions as a sponge for miR-375

To elucidate the molecular mechanism underlying hsa_circ_0000231 regulating CCND2, given that circRNAs might act as a sponge for microRNAs further modulating downstream targets, we next predicted the potential targets of hsa_circ_0000231 by miRNA target prediction software made by Arraystar according to TargetScan and miRanda database. The results showed that hsa_circ_0000231 possesses conserved target site of miR-375 with a high score (Figure 3A). Considering that circRNAs could serve as miRNA sponges in the cytoplasm, we performed FISH assay and Base Scope Assay in CRC cells and tissues to observe the subcellular localization of hsa_circ_0000231. It was found that most of hsa_circ_0000231 were located in cytoplasm (Figure 3B-C). Then, the levels of miR-375 were examined in 160 pairs of CRC tissues and adjacent non-cancerous tissues, the results indicated that miR-375 was markedly downregulated in CRC tissues compared with adjacent non-tumor tissues (Figure 3D), and the expression of miR-375 was negatively correlated with hsa_circ_0000231 (Figure 3E). Therefore, we supposed that hsa_circ_0000231 might serve as a competing endogenous RNA (ceRNA) for miR-375.

In order to confirm the bioinformatics prediction analysis, dual-luciferase reporter assay was applied in SW480 cells. The full-length of hsa_circ_0000231-WT and mutant version without miR-375 binding sites were subcloned into luciferase reporter vector psiCHECK2 (Figure3F). The results indicated that miR-375 mimics could significantly decrease the luciferase activity of WT group but not mutant one (Figure3G), suggesting that there might be a direct interaction between hsa_circ_0000231 and miR-375.

It has been widely known that miRNAs regulate target gene expression by binding to Argonaute 2 (AGO2), the key component of RNA-induced silencing complex (RISC). Thus, an anti-AGO2 RNA immunoprecipitation (RIP) assay was conducted in SW480 cells to pull down the RNA transcripts which bind to AGO2 with anti-AGO2 antibody, and IgG as a negative control. Indeed, AGO2, hsa_circ_0000231 and miR-375 were all efficiently pulled down by anti-AGO2 antibodies compared with IgG, moreover, both hsa_circ_0000231 and miR-375 were significantly enriched in cells transfected with miR-375 mimics compared with miR-NC group (Figure3H-I).

To further verify the bond effect of hsa_circ_0000231 and miR-375, we performed a circRNA pull-down assay with specific biotin-labeled hsa_circ_0000231 probes. Interestingly, a specific enrichment of hsa_circ_0000231 and miR-375 was detected by qRT-PCR in the hsa_circ_0000231 probe group compared with control probe (Figure3J-K).

miR-375 suppresses tumor growth of CRC cells *in vitro* and *in vivo*

To explore the role of miR-375 in CRC cells, we transfected mimics-NC and miR-375 mimics into SW480 and SW620 cells, respectively. CCK8 assay was performed to detect the effect of miR-375 on cell proliferation. The results showed that compared with the control group, the proliferation ability of miR-375 mimics group and in SW480 cells or SW620 cells were markedly decreased ($P < 0.05$) (Figure S2A-B). Then cell cycle detection by flow cytometry revealed that the percentage of G0/G1 cells obviously increased after overexpression of miR-375 in SW480 and SW620 cells. (Figure S2 C-D).

CCND2 is directly targeted by miR-375 and indirectly regulated by hsa_circ_0000231

According to the TargetScan (<http://www.targetscan.org>), CCND2 and hsa_circ_0000231 share the same MRE of miR-375 (Figure 4A). Then we found miR-375 mimics could markedly reduce the expression of CCND2, while miR-375 inhibitors significantly enhanced the level of CCND2 in SW480 cells, and the increase or decrease of CCND2 expression induced by miR-375 mimics or inhibitors could be markedly reversed by hsa_circ_0000231 overexpression or knockdown respectively in SW480 cells detected by qPCR and Western Blot assay (Figure 4B-D). These data suggest that hsa_circ_0000231 could regulate the expression of CCND2 through serving as a ceRNA for miR-375 in CRC.

To confirm our suggestion, we then conducted dual luciferase reporter assay to validate this prediction, the results showed that the activity of luciferase reporter vector carrying the CCND2 3'UTR-WT sequence was significantly decreased by miR-375 mimics compared with miR-NC groups (Figure 4E-F). Moreover, the luciferase activity was recovered after transfection with hsa_circ_0000231 in the miR-375 +

CCND2 3'UTR-WTgroup (Figure 4G).Furthermore, an anti-AGO2 RIPassay was performed to detect the relationship betweenmiR-375 and CCND2, and the resultshowed miR-375 pull-down by CCND2 was specificallyenriched in SW480cells. (Figure 4H-I). Then we usedRNA pull-down in SW480 cells and the analysis demonstrated that endogenousCCND2 was significantly pulled down by biotinylatedprobes against miR-375(Figure 4J-K).

IGF2BP3 could bind to has_circ_0000231 and CCND2, as anRBP to prevent RNA degradation

circBaseand Circular RNA interactome databaseswere used to predict RBPs that could bind to has_circ_0000231. It was found that IGF2BP3 has a binding site with has_circ_0000231, and interestingly It can be perfectly matched to the 3'-UTR region of the CCND2 gene mRNA which is also the seed sequence region of miR375.To verify the combination of IGF2BP3 with has_circ_0000231 and CCND2, an anti-IGF2BP3 RIPassay was performed to detect the expression ofhas_circ_0000231and CCND2, and the resultshowed both has_circ_0000231 andCCND2 weresignificantlyenriched in SW480and SW620 cells (Figure 5A-B).Still, in SW480 and SW620 cell lines, we conducted RNA pull-down assay and the analysis demonstrated that IGF2BP3was remarkably pulled down by biotinylatedprobes against both has_circ_0000231 and CCND2(Figure 5C-D).

To validate whether IGF2BP3 playedas a protective role increase the expression of has_circ_0000231 and CCND2 in CRC cells, we overexpressed IGF2BP3andfoundtheoverexpressionofIGF2BP3 could markedly enhanced the expressions of has_circ_0000231and CCND2inSW480 and SW620 cells (Figure5E-F). These data suggest that IGF2BP3mayprotect the expression of CCND2 as RBP binding both has_circ_0000231 and CCND2 in CRC. MoreoverWestern BlotassaywasconductedtoconfirmthattheupregulationordownregulationofIGF2BP3 could markedly enhanced ordecreasedthe expressions of CCND2, andtheeffectcausedbyIGF2BP3couldbe partially reversedbymimicsorinhibitorofmiR-375in SW480and SW620 cells(Figure5G-J).

has_circ_0000231 promotes CRC proliferation through has_circ_0000231/IGF2BP3/miR-375/CCND2 axis

To ensure whether has_circ_0000231 serves its biological function through has_circ_0000231/IGF2BP3/miR-375/CCND2 axis, rescue experiments were applied using miR-375 mimics and inhibitors. The results indicated that themiR-375 mimics reversed the proliferationpromoting effects ofhas_circ_0000231overexpression in SW480 cells, whereas miR-375 inhibitors could rescue the proliferationssuppressingeffects of induced byknockdownofhas_circ_0000231 in SW620 cells by CCK8 assays. And interestingly, the effects caused byincrease or decreaseof has_circ_0000231 could be partially rescued by si-IGF2BP3 or overexpressionofIGF2BP3in SW480 and SW620 cells, respectively(Figure 6A-B). In summary, these data demonstrated that has_circ_0000231 might serve as a ceRNA for miR-375and can also be protected by IGF2BP3 to regulate CCND2 expression,which led to cell cycle out of control and developmentof CRC (Figure 6C).

Discussion

It has been confirmed that circRNA is abnormally expressed in various tumor tissues such as esophageal cancer¹⁹, gastric cancer²⁰, pancreatic cancer²¹, breast cancer²² and liver cancer²³, and it is associated with cell proliferation invasion and metastasis, and even patient prognosis. At present, the reports of circRNA in colorectal cancer are mostly related to the relationship between circRNA and clinicopathological indicators, and there are few in-depth studies on molecular regulation mechanisms. It has been found that circ0000069 promotes proliferation, invasion and migration of colorectal cancer cells and is associated with age, lymph node metastasis, and TNM staging in patients with colorectal cancer²⁴. However, the molecular mechanism by which circular RNA regulates target gene expression by molecular sponge action in colorectal cancer is not fully understood. In the present study, we discovered overexpression of circ_0000231 in CRC tissues and cell lines which indicated poor prognosis of patients with CRC. Furthermore, multivariate analysis showed that the high has_circ_0000231 and TNM stage were two independent prognostic factors for a poor survival in CRC patients.

The ceRNA hypothesis suggests that RNA transcripts, including mRNAs, lncRNAs, pseudogenes and circRNAs could crosstalk with and regulate expression each other via competing for shared miRNA response elements (MREs), building a new complicated post transcriptional regulatory network and mechanism¹⁵. Growing evidences indicated that some circRNAs could serve as sponges for miRNAs to regulate the expression of miRNA target genes in multiple human diseases including colorectal cancer²⁵. For example, it was reported that hsa_circ_001680 promoted colorectal cancer development by functioning as a sponge for miR-340 to influence the expression of BMI1²⁶. Thus, fully understanding how circ_0000231 functioned in CRC would provide a novel insight into oncogenesis mechanism for us. In our study, we found that hsa_circ_0000231 contained the MRE of miR-375 through bioinformatics analyses. hsa_circ_0000231 and miR-375 were co-located in cytoplasm of CRC cells and tissues. Further, dual-luciferase reporter, anti-AGO2 RNA immunoprecipitation and RNA pull-down assays confirmed that has_circ_0000231 could interact with miR-375 directly which indicated that circ_0000231 played an oncogenic role via sponging miR-375 in CRC. Consistent with our results, it is reported that miR-375 is significantly downregulated in colorectal cancer patient tissues and cell lines and negatively correlated with the degree of malignancy of colorectal cancer²⁷. Our findings demonstrated that has_circ_0000231 serves as an oncogene by sponging miR-375 in CRC and revealed the significance of interaction between has_circ_0000231, and miR-375 in tumorigenesis and development of CRC.

According to ceRNA hypothesis, circRNA could act as a ceRNA to modulate the expression of miRNA target gene. We found that CCND2, a vital cell cycle regulator, and has_circ_0000231 are co-overexpressed in CRC, which further support that cell cycle is closely related to tumorigenesis and development of CRC²⁸. Moreover, bioinformatics analysis indicated that CCND2 is one of the potential targets of miR-375 using miRcode and TargetScan. Next, a dual-luciferase reporter assay confirmed that miR-375 could directly target the 3'-untranslated regions of CCND2. Additionally, upregulation of miR-375 led to knockdown of CCND2 at mRNA and protein levels, whereas downregulation of miR-375 displayed an opposite effect. It is known that CCND2 mainly regulates the cell cycle progression²⁹. We found that down-regulation of has_circ_0000231 resulted in G1/S phase cell cycle arrest. CCND2 plays roles in determining

progression through checkpoints at G1/S and G2/M that dictate whether a cell can proceed with DNA replication and cell division. Our data suggest that has_circ_0000231 knockdown might inhibit the CCND2, which leads to decreased activity and G1/S phase cell cycle arrest. It is well-demonstrated that the dysregulation of CCND2 activity is implicated in multiple cancers, including colorectal cancer³⁰. It has also been demonstrated that overexpression of CCND2 was related to poor prognosis in CRC³¹.

Consistent with former studies, we found that CCND2 was significantly regulated in CRC tissues and overexpression of CCND2 was correlated with shorter overall survival time. To validate the crosstalk between hsa_circ_0000231 and CCND2, we uncovered that overexpression of hsa_circ_0000231 could increase CCND2 in both mRNA and protein levels, while knockdown of hsa_circ_0000231 exhibited a reverse effect. Furthermore, these effects could be partially abolished by miR-375 mimics or inhibitors, respectively, which might support our hypothesis that hsa_circ_0000231 functions as a ceRNA to promote CCND2-mediated proliferation and via decoying miR-375 in CRC.

Besides, RNA Binding Protein (RBP) plays an important role in the regulation of post-transcriptional gene expression, and circRNA may act as an RBP "super sponge" by binding to RBP, thus changing splicing pattern or mRNA stability. In our research, Circular RNA interactome database was used to forecast that IGF2BP3 may combine with hsa_circ_0000231 by RBA-protein binding sites, further study found that IGF2BP3 can also combine with CCND2 through <http://starbase.sysu.edu.cn> prediction. IGF2BP3 is a member of the RNA binding protein family, and functions in many biological processes such as mRNA localization, translation and stability maintenance³². Former studies have found that the expression of IGF2BP3 is associated with the occurrence, metastasis and poor prognosis of malignant tumors³³. As for the regulatory mechanism of IGF2BP3 on target genes, some studies have suggested that IGF2BP3 protein can bind to the 3'UTR of target gene mRNA and compete with miRNA for binding sites, thus protecting the mRNA of target genes from miRNA degradation, including maintaining the stability of CCND2 mRNA and promoting CCND2 translation^{34, 35}.

Therefore, it was speculated that IGF2BP3 could enhance the stability of has_circ_0000231 and protect CCND2 from degradation by miR-375. To confirm our previous suggestion, we speculated that IGF2BP3 may bind to has_circ_0000231 and CCND2 at the same time, and the binding site is consistent with the binding site of miR-375 and CCND2. RIP, RNA pull-down, qRT-PCR and Western Blot assays were further performed to confirm that the expression of hsa_circ_0091073 and CCND2 could be upregulated by increasing IGF2BP3, while the expressions of hsa_circ_0091073 and CCND2 were down-regulated by interfering with the expression of IGF2BP3 gene. Meanwhile, it was also found that IGF2BP3 protects CCND2 from the regulation of miR-375, and the regulation effect of miR-375 on CCND2 is opposite to that of IGF2BP3. Therefore, IGF2BP3 might not only bind to has_circ_0000231, but also inhibit the degradation of CCND2 mRNA by miR-375 at the 3'UTR binding site of CCND2 mRNA, thus protecting the stability of CCND2 mRNA.

In conclusion, our results suggest that increased hsa_circ_0000231 expression is a frequent event and a potential independent prognosis marker for CRC. We firstly demonstrated that hsa_circ_0000231 might

sponge miR-375 to modulate CCND2 expression, and IGF2BP3 can protect hsa_circ_0000231 and CCND2, leading to tumorigenesis and development of CRC. Our findings suggested that hsa_circ_0000231 could be a valuable prognosis marker and promising diagnosis and therapy targets for CRC in the future. The regulatory network involving hsa_circ_0000231/IGF2BP3/miR-375/CCND2 axis might highlight a better understanding for potential mechanism of pathogenesis and progression of CRC.

Conclusion

In conclusion, we find that hsa_circ_0000231 facilitated CRC progression by sponging miR-375 or binding to IGF2BP3 to modulate CCND2. This discovery implied that hsa_circ_0000231 may be a potential new diagnostic and therapeutic biomarker for CRC.

List Of Abbreviations

circRNA: circular RNA, CRC: colorectal cancer, ceRNA: competing endogenous RNA, CCND2: cyclin D2, MRE: miRNA response element, miRNA: microRNA, siRNA: small interfering RNA, RIP: RNA immunoprecipitation, AGO2: anti-Argonaute2, RBP: RNA binding protein

Declarations

Ethics approval and consent to participate

This study was performed according to the recommendations in the Guide for the Chinese Ethics Review Committees. The protocol was approved by the Ethics Committee of Peking University People's Hospital. Written informed consent was obtained from each subject. The animal experiment was carried out under ethics approval of Peking University People's Hospital.

Consent for publication

All contributing authors agree to the publication of this article.

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WZ and ZLS conceived the study and participated in its design and coordination; ZW, BW and ZZ performed the experiments; HRZ and YY collected and analyzed the clinical data. QW, KWJ and SW analyzed and interpreted the data; WZ drafted the manuscript; BW and ZLS revised the manuscript; all authors read and approved the final manuscript.

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References

1. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wallace MB. Colorectal cancer., 2019:1467-80.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020., 2020.
3. Chen H, Li N, Ren J, Feng X, Lyu Z, Wei L, Li X, Guo L, Zheng Z, Zou S, Zhang Y, Li J, Zhang K, Chen W, Dai M, He J, Group OCSP. Participation and yield of a population-based colorectal cancer screening programme in China. GUT 2019; 68:1450-7.
4. Navarro M, Nicolas A, Ferrandez A, Lanás A. Colorectal cancer population screening programs worldwide in 2016: An update. World J Gastroenterol 2017; 23:3632-42.
5. Lai Y, Wang C, Civan JM, Palazzo JP, Ye Z, Hyslop T, Lin J, Myers RE, Li B, Jiang B, Sama A, Xing J, Yang H. Effects of Cancer Stage and Treatment Differences on Racial Disparities in Survival From Colon Cancer: A United States Population-Based Study. GASTROENTEROLOGY 2016; 150:1135-46.
6. Su M, Xiao Y, Ma J, Tang Y, Tian B, Zhang Y, Li X, Wu Z, Yang D, Zhou Y, Wang H, Liao Q, Wang W. Circular RNAs in Cancer: emerging functions in hallmarks, stemness, resistance and roles as potential biomarkers. MOL CANCER 2019; 18.
7. Shang Q, Yang Z, Jia R, Ge S. The novel roles of circRNAs in human cancer. MOL CANCER 2019; 18.
8. Naeli P, Pourhanifeh MH, Karimzadeh MR, Shabaninejad Z, Movahedpour A, Tarrahimofrad H, Mirzaei HR, Bafrani HH, Savardashtaki A, Mirzaei H, Hamblin MR. Circular RNAs and gastrointestinal cancers: Epigenetic regulators with a prognostic and therapeutic role. Critical Reviews in Oncology/Hematology 2020; 145:102854.

9. Naeli P, Pourhanifeh MH, Karimzadeh MR, Shabaninejad Z, Movahedpour A, Tarrahimofrad H, Mirzaei HR, Bafrani HH, Savardashtaki A, Mirzaei H, Hamblin MR. Circular RNAs and gastrointestinal cancers: Epigenetic regulators with a prognostic and therapeutic role. *Critical Reviews in Oncology/Hematology* 2020; 145:102854.
10. Kristensen LS, Andersen MS, Stagsted L, Ebbesen KK, Hansen TB, Kjems J. The biogenesis, biology and characterization of circular RNAs. *NAT REV GENET* 2019; 20:675-91.
11. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. *NAT REV DRUG DISCOV* 2010; 9:775-89.
12. Sakaguchi M, Hisamori S, Oshima N, Sato F, Shimono Y, Sakai Y. miR-137 Regulates the Tumorigenicity of Colon Cancer Stem Cells through the Inhibition of DCLK1. *MOL CANCER RES* 2016; 14:354-62.
13. Lv C, Li F, Li X, Tian Y, Zhang Y, Sheng X, Song Y, Meng Q, Yuan S, Luan L, Andl T, Feng X, Jiao B, Xu M, Plikus MV, Dai X, Lengner C, Cui W, Ren F, Shuai J, Millar SE, Yu Z. MiR-31 promotes mammary stem cell expansion and breast tumorigenesis by suppressing Wnt signaling antagonists. *NAT COMMUN* 2017; 8:1036.
14. Panza E, Ercolano G, De Cicco P, Armogida C, Scognamiglio G, Botti G, Cirino G, Iannaro A. MicroRNA-143-3p inhibits growth and invasiveness of melanoma cells by targeting cyclooxygenase-2 and inversely correlates with malignant melanoma progression. *BIOCHEM PHARMACOL* 2018; 156:52-9.
15. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA Hypothesis: The Rosetta Stone of a Hidden RNA Language? *CELL* 2011; 146:353-8.
16. Wu P, Mo Y, Peng M, Tang T, Zhong Y, Deng X, Xiong F, Guo C, Wu X, Li Y, Li X, Li G, Zeng Z, Xiong W. Emerging role of tumor-related functional peptides encoded by lncRNA and circRNA. *MOL CANCER* 2020; 19.
17. Zhong Y, Du Y, Yang X, Mo Y, Fan C, Xiong F, Ren D, Ye X, Li C, Wang Y, Wei F, Guo C, Wu X, Li X, Li Y, Li G, Zeng Z, Xiong W. Circular RNAs function as ceRNAs to regulate and control human cancer progression. *MOL CANCER* 2018; 17.
18. Zang J, Lu D, Xu A. The interaction of circRNAs and RNA binding proteins: An important part of circRNA maintenance and function. *J NEUROSCI RES* 2020; 98:87-97.
19. Jiang C, Jiang C, Xu D, Xu D, You Z, You Z, Xu K, Xu K, Tian W, Tian W. Dysregulated circRNAs and ceRNA network in esophageal squamous cell carcinoma. *Frontiers in Bioscience - Landmark* 2019; 24:277-90.
20. Xin L, Liu L, Liu C, Zhou LQ, Zhou Q, Yuan YW, Li SH, Zhang HT. DNA-methylation-mediated silencing of miR-7-5p promotes gastric cancer stem cell invasion via increasing Smo and Hes1. *J CELL PHYSIOL* 2020; 235:2643-54.
21. Xu Y, Yao Y, Gao P, Cui Y. Upregulated circular RNA circ_0030235 predicts unfavorable prognosis in pancreatic ductal adenocarcinoma and facilitates cell progression by sponging miR-1253 and miR-1294. *Biochem Biophys Res Commun* 2019; 509:138-42.

22. Pan M, Li M, You C, Zhao F, Guo M, Xu H, Li L, Wang L, Dou J. Inhibition of breast cancer growth via miR-7 suppressing ALDH1A3 activity concomitant with decreasing breast cancer stem cell subpopulation. *J CELL PHYSIOL* 2020; 235:1405-16.
23. Qiu L, Huang Y, Li Z, Dong X, Chen G, Xu H, Zeng Y, Cai Z, Liu X, Liu J. Circular RNA profiling identifies circADAMTS13 as a miR-484 sponge which suppresses cell proliferation in hepatocellular carcinoma. *MOL ONCOL* 2019; 13:441-55.
24. Guo JN, Li J, Zhu CL, Feng WT, Shao JX, Wan L, Huang MD, He JD. Comprehensive profile of differentially expressed circular RNAs reveals that hsa_circ_0000069 is upregulated and promotes cell proliferation, migration, and invasion in colorectal cancer. *Onco Targets Ther* 2016; 9:7451-8.
25. Tu FL, Guo XQ, Wu HX, He ZY, Wang F, Sun AJ, Dai XD. Circ-0001313/miRNA-510-5p/AKT2 axis promotes the development and progression of colon cancer. *AM J TRANSL RES* 2020; 12:281-91.
26. Jian X, He H, Zhu J, Zhang Q, Zheng Z, Liang X, Chen L, Yang M, Peng K, Zhang Z, Liu T, Ye Y, Jiao H, Wang S, Zhou W, Ding Y, Li T. Hsa_circ_001680 affects the proliferation and migration of CRC and mediates its chemoresistance by regulating BMI1 through miR-340. *MOL CANCER* 2020; 19:20.
27. Alam KJ, Mo JS, Han SH, Park WC, Kim HS, Yun KJ, Chae SC. MicroRNA 375 regulates proliferation and migration of colon cancer cells by suppressing the CTGF-EGFR signaling pathway. *INT J CANCER* 2017; 141:1614-29.
28. Park SY, Lee CJ, Choi JH, Kim JH, Kim JW, Kim JY, Nam JS. The JAK2/STAT3/CCND2 Axis promotes colorectal Cancer stem cell persistence and radioresistance. *J Exp Clin Cancer Res* 2019; 38:399.
29. Decker T, Schneller F, Hipp S, Miething C, Jahn T, Duyster J, Peschel C. Cell cycle progression of chronic lymphocytic leukemia cells is controlled by cyclin D2, cyclin D3, cyclin-dependent kinase (cdk) 4 and the cdk inhibitor p27. *LEUKEMIA* 2002; 16:327-34.
30. Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2002; 1602:73-87.
31. Li WC, Wu YQ, Gao B, Wang CY, Zhang JJ. MiRNA-574-3p inhibits cell progression by directly targeting CCND2 in colorectal cancer. *Biosci Rep* 2019; 39.
32. Nielsen J, Christiansen J, Lykke-Andersen J, Johnsen AH, Wewer UM, Nielsen FC. A Family of Insulin-Like Growth Factor II mRNA-Binding Proteins Represses Translation in Late Development. *MOL CELL BIOL* 1999; 19:1262-70.
33. Lederer M, Bley N, Schleifer C, Huttelmaier S. The role of the oncofetal IGF2 mRNA-binding protein 3 (IGF2BP3) in cancer. *SEMIN CANCER BIOL* 2014; 29:3-12.
34. Jonson L, Christiansen J, Hansen T, Vikesa J, Yamamoto Y, Nielsen FC. IMP3 RNP safe houses prevent miRNA-directed HMGA2 mRNA decay in cancer and development. *CELL REP* 2014; 7:539-51.
35. Ennajdaoui H, Howard JM, Sterne-Weiler T, Jahanbani F, Coyne DJ, Uren PJ, Dargyte M, Katzman S, Draper JM, Wallace A, Cazarez O, Burns SC, Qiao M, Hinck L, Smith AD, Toloue MM, Blencowe BJ, Penalva LO, Sanford JR. IGF2BP3 Modulates the Interaction of Invasion-Associated Transcripts with RISC. *CELL REP* 2016; 15:1876-83.

Tables

Table 1 Relationship between has_circ_0000231 and CCND2 expression and clinicopathological data

clinicopathological features		expression of hsa_circ_0000231		P value	expression of CCND2		P value
		Low	High		Low	High	
Gender				0.791			0.262
	Male	44	39		48	43	
	Female	36	41		32	37	
Age at diagnosis				0.162			0.715
	≤ 60	19	27		21	19	
	>60	61	53		59	61	
Tumor size (cm)				0.032*			0.260
	≤ 5	18	8		14	9	
	>5	62	72		66	71	
Location				0.733			0.391
	proximal	26	24		22	27	
	distal	54	56		58	53	
Differentiation				0.750			0.078
	Well-moderate	44	46		41	52	
	Poor	36	34		39	28	
Depth of invasion				0.122			0.032*
	T1-T2	13	21		8	18	
	T3-T4	67	59		72	62	
Lymph node metastasis				0.002*			0.514
	No	7	22		11	14	
	Yes	73	58		69	66	
TNM stage				0.015*			0.043*
	I-II	9	21		16	7	
	III-IV	71	59		64	73	
distant metastasis				0.276			0.514
	No	65	70		69	66	

Yes	15	10	11	14
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Figures

Figure1

Figure 1

Expression profiles of circRNA and the correlation between has_circ_0000231 and poor prognosis of CRC

A. The cluster heat maps displayed the increased and decreased circRNAs. Each column indicates a sample while each row indicates an individual circRNA. The red and green strips represent high and low expression, respectively.

B. The volcano plot visualizes the expression of circRNA between CRC tissues and adjacent.

C. Classification of differentially expressed circRNA between CRC tissues and adjacent.

D. qRT-PCR assay was used to verify the results of circRNA microarray assay.

E. Relative expression of has_circ_0000231 in CRC cell lines was determined by qRT-PCR.

F. Relative expression of has_circ_0000231 in CRC tissues (CRT) and adjacent normal tissues (CRN) was detected by qRT-PCR (n = 160).

G. Kaplan-Meier survival curve of overall survival in 160 patients with CRC according to the has_circ_0000231 expression. Patients were divided into high expression and low expression group by

median expression. H. Kaplan-Meier survival curve of progression-free survival in 160 patients with CRC according to the hsa_circ_0000231 expression. Patients were divided into high expression and low expression group by median expression.

Figure2

Figure 2

hsa_circ_0000231 promotes cell proliferation and tumor growth in vitro and in vivo in CRC A and B. CCK8 assay was performed to detect the effect of hsa_circ_0000231 on cell proliferation. C and D. Colony formation assays were executed to detect the proliferation of cells transfected with indicated vectors. E and F. Apoptosis rate was analyzed by flow cytometry after downregulation of hsa_circ_0000231. G and H. The cell cycle progression was analyzed by flow cytometry after downregulation of hsa_circ_0000231. I. Images of xenograft tumors of each group (n = 5). J. Tumor volume was shown. K. Tumor weight was shown. Data were showed as mean ± SD, *p< 0.05, **p< 0.001.

Figure 3

Figure 3

hsa_circ_0000231 functions as a sponge for miR-375 in CRC A. The miR-375 binding site on hsa_circ_0000231 predicted by targetScan and miRanda. B. FISH assay was performed to observe the cellular location of hsa_circ_0000231 (red) in cells (magnification, $\times 200$, scale bar, $50 \mu\text{m}$) C. BaseScope assay was performed to observe the cellular location of hsa_circ_0000231 (red) in CRC tissues (magnification, $\times 100$, scale bar, $100 \mu\text{m}$) D. Relative expression of miR-375 in CRC tissues (Tumor) and adjacent non-tumor tissues (Normal) was tested by qRT-PCR ($n = 160$). E. Pearson correlation analysis of hsa_circ_0000231 and miR-375 expression in 160 CRC tissues. F. Schematic illustration of hsa_circ_0000231-WT and hsa_circ_0000231-Mut luciferase reporter vectors. G. The relative luciferase activities were detected in SW620 cells after transfection with hsa_circ_0000231-WT or hsa_circ_0000231-Mut and miR-375 mimics or miR-NC, respectively. H and I. Anti-AGO2 RIP was executed in SW620 cells after transfection with miR-375 mimic or miR-NC, followed by western blot and qRT-PCR to detect AGO2 protein, hsa_circ_0000231 and miR-375, respectively. J and K. RNA pull-down was executed in

SW620 cells, followed by qRT-PCR to detect the enrichment of hsa_circ_0000231 and miR-375. Data were showed as mean ± SD, *p< 0.05,**p< 0.001.

Figure 4

Figure 4

CCND2 is directly targeted by miR-375 and indirectly regulated by hsa_circ_0000231 A. Schematic illustration of CCND2 3'UTR-WT and CCND2 3'UTR-Mut luciferase reporter vectors. B. Relative expression of CCND2 was detected by qRT-PCR in cells transfected with indicated vectors, miRNAs or inhibitors. Cand D. Western blot assay was performed to determine the relative expression of CCND2 transfected with indicated vectors, miRNAs or inhibitors. E. Schematic illustration of CCND2 3'UTR-WT and CCND2 3'UTR-Mut luciferase reporter vectors. F. The relative luciferase activities were detected in 293 T cells after transfected with CCND2 3'UTR-WT or CCND2 3'UTR-Mut and miR-375 mimics or miR-NC, respectively. G. The luciferase activity was recovered after transfection with hsa_circ_0000231 in the miR-375 + CCND2 3'UTR-WT group H and I. Anti-AGO2 RIP was executed in SW620 cells after transfection with miR-375 mimic or miR-NC, followed by western blot and qRT-PCR to detect AGO2 protein, hsa_circ_0000231 and miR-375, respectively. J and K. RNA pull-down was executed in SW620 cells, followed by qRT-PCR to

detect the enrichment of hsa_circ_0000231 and miR-375. Data were showed as mean $\pm$ SD, * p < 0.05, ** p < 0.001.

Figure5

Figure 5

IGF2BP3 could bind to has_circ_0000231 and CCND2, as an RBP to protect the stability of RNA A and B. Anti-IGF2BP3 RIP was executed in SW620 and SW480 cells followed by western blot and qRT-PCR to detect IGF2BP3 protein, hsa_circ_0000231 and CCND2, respectively. C and D. RNA pull-down was executed in SW620 and SW480 cells, followed by qRT-PCR to detect the enrichment of hsa_circ_0000231 and CCND2, respectively. E and F. qRT-PCR was used to detect expression of hsa_circ_0000231 and CCND2 after overexpression of IGF2BP3. G-J. Western Blot assay was conducted to confirm the relative expression of CCND2 transfected with indicated vectors, miRNAs or inhibitors in SW480 cells. Data were showed as mean $\pm$ SD, * p < 0.05, ** p < 0.001.

Figure 6

hsa_circ_0000231 promotes CRC proliferation through hsa_circ_0000231/IGF2BP3/miR-375/CCND2 axis A and B. The cell proliferations were determined after transfection with indicated vectors, miRNAs or inhibitors by CCK-8 assays, respectively. C. Schematic diagram of how hsa_circ_0000231 promotes CRC tumorigenesis and progression. D. Data were showed as mean $\pm$ SD, * p < 0.05, ** p < 0.001.

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