Identification of circular RNA hsa_circ_0044556 and its effect on the development and progression of colorectal cancer

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Abstract

Background

Circular RNAs (circRNAs) are a novel class of noncoding RNAs. Increasing evidence indicates that circRNAs play an important role in the occurrence and development of tumors. However, the role of circRNAs in the development and progression of colorectal cancer (CRC) remains unclear.

Methods

First, we searched for differentially expressed circRNAs using a circRNA microarray in paired CRC and adjacent normal tissues. The circRNA hsa_circ_0044556 was screened out from the existing CRC circRNA microarray in the Gene Expression Omnibus database and our microarray. The clinical significance of hsa_circ_0044556 expression level in CRC patients was then investigated. Finally, the functions of the targets of this circRNA were determined in CRC cell lines.

Results

hsa_circ_0044556 was highly expressed in CRC patients and was positively correlated with tumor stage and lymph node metastasis. In CRC cell lines, the proliferation, migration, and invasion of cancer cells were inhibited by knocking down hsa_circ_0044556 expression.

Conclusion

hsa_circ_0044556 promoted the development and progression of CRC. It is possible that hsa_circ_0044556 will become a novel biomarker or therapeutic target for CRC.

Background

Colorectal cancer (CRC) is a malignant cancer that seriously endangers the health of humans. Currently, the global incidence rate of CRC ranks third among cancer-related diseases with up to 1.2 million new cases each year, and more than 0.6 million deaths are expected each year (1). From the perspective of the global incidence trend, the European and American regions are higher than the Asian and African regions, with the second place mortality rate (9.2%), and the developing countries are higher than the developed countries (2). In 2015, China's cancer statistics showed that the incidence and the mortality of CRC ranked fifth among cancer-related diseases in China. The incidence of CRC and the death toll are rising yearly while the population with this disease trends younger (3). Early diagnosis, accurate prognosis, and recurrence monitoring play important roles in cancer diagnosis and treatment. The 5-year survival rate of patients with advanced CRC is only 12%, while the 5-year survival rate of patients at the early stage can reach more than 90% (4). Therefore, early diagnosis can significantly improve the prognosis as well as the survival and quality of life of patients with CRC. On this basis, the in-depth study of the pathophysiology mechanisms underlying the occurrence and development of CRC will help us more comprehensively understand this cancer and open up new ideas and methods for the diagnosis and treatment of CRC. However, accurate and reliable prognosis and recurrence monitoring methods for CRC patients are still lacking in clinical practice, which makes it impossible to perform detailed posttreatment management and recurrence monitoring for patients.

At present, the fecal occult blood (OB) test, detection of carcinoembryonic antigen (CEA) and CA19-9 protein, and electronic colonoscopy are the main techniques for screening and diagnosis of CRC. However, the positive rate of diagnosis of fecal OB test is less than 30% (5); the abnormal increase in CEA is present in a variety of tumors, so CEA is not a specific molecular marker of CRC, and it is limited by low specificity and weak sensitivity (6). Although electronic colonoscopy is the gold standard for the diagnosis of CRC, it is an invasive examination with low patient compliance and high cost, limiting its use as a technical tool for broad-spectrum screening. Therefore, new CRC biomarkers and diagnostic methods that have good diagnostic efficacy and are suitable for...
broad-spectrum screening need to be further investigated.

Circular RNAs (circRNAs) are a new class of noncoding RNAs (ncRNAs), which are single-stranded circular RNAs with no free 5'-end cap or 3'-end poly (A) tail. They are produced by alternative splicing of a specific pre-mRNA (pre-mRNA). Most circRNAs consist of exons and may also contain intergenic or noncoding regions (7–10). circRNAs did not attract much attention from researchers at first. For a long time, circRNAs were considered by-products of incorrect alternative splicing. Then in 2012, Salzman et al. discovered a large number of circRNAs using high-throughput sequencing technology (7). Now it is recognized that circRNAs are not as rare as previously thought. In contrast, circRNAs are highly stable in cells with high expression, and sometimes their expression levels are even 10 times higher than those of their homologous messenger RNAs. It has been found that circRNAs can act as a miRNA sponge bound to RNA-binding protein to exert biological functions.

In recent years, circRNAs have been considered to play an important role in tumor progression (11). circRNAs are expected to become biomarkers for tumor diagnosis and prognosis due to their stable circular structure (12). Xu et al. found that hsa_circ_0001649 was expressed at abnormally low levels in intrahepatic cholangiocarcinoma tissues and could promote cell proliferation and tumor metastasis (13). Circ-ITCH can act as a sponge of miR-7 and miR-20a to inhibit the negative regulatory effect of the latter on the target gene ITCH, while ITCH has a tumor suppressor function by inhibition of the Wnt/β-catenin signaling pathway (14). The role of circRNAs in CRC remains unclear and deserves further investigation.

Materials And Methods

Tissue collection

In this study, tissue samples of 52 patients with CRC from the Third Xiangya Hospital of Central South University, China between May 2018 and December 2018 were collected. No patient received radiotherapy or chemotherapy before surgery, and the postoperative pathological diagnosis was adenocarcinoma (high, medium, and low differentiation). For each specimen, two copies of cancer tissues and adjacent normal tissues were collected. The surgical specimens were cryopreserved in liquid nitrogen immediately after excision. This study was approved by the Ethics Committee of the hospital, and all patients gave informed consent.

Cell culture: Human CRC cell lines (SW480, SW620, HCT116, HT29) and normal colonic epithelial cell lines (NCM460) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). SW480 and SW620 cells were cultured in L15 medium (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) containing 10% fetal bovine serum (FBS: Biological Industries Israel Beit-Haemek, Beit-Haemek, Israel). HCT116 and HT29 were cultured with McCoy's 5A medium (Nanjing KeyGen Biotech Co., Ltd.) containing 10% FBS. NCM460 was cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific ™, Beijing China) containing 10% FBS. All cells were incubated at 37 °C in a 5% CO₂ incubator.

Cell transfection

siRNA-1, siRNA-2, siRNA-3, shRNA-3, and negative-control siRNA were all designed by Suzhou GenePharma Co., Ltd., China. Cell transfection was done according to the manual of Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Tablet cloning assay

The tumor cell lines that were successfully transfected with shRNA-3 were harvested, counted, and adjusted to a cell concentration of 500 cells/ml, then were seeded in 6-well plates (1 x 10³/well), and 3 duplicate wells were set for each cell line. The 6-well plates were incubated at 37 °C in a 5% CO₂ incubator for 2 weeks. The cell culture medium was changed every 2 to 3 days. The 6-well plates were taken out after 2 weeks, and the medium was removed. Cells were then washed with phosphate-buffered saline (PBS) 2 times, dried naturally, and then fixed by adding 1 ml of 4% paraformaldehyde in each well. After cells were fixed at room temperature for 30 min, the paraformaldehyde solution was aspirated. Cells were dried and stained with 0.1% crystal violet for
30 min. After washing with PBS twice, the cells were photographed and counted.

**Cell Counting Kit-8 (CCK-8) assay**

SW480 and HCT116 (5 × 10^3/well) were mixed well in 100 µl normal normal medium containing 10% FBS, seeded into 96-well plates, and incubated at 37 °C in a 5% CO₂ incubator. Each well received 10 µL of CCK-8 reagent at the specified time point (0, 24, 48, and 72 hours). After incubation for another 3 hours, the absorbance at 450 nm of each well was measured by an EnVision microplate reader (PerkinElmer, Inc., Waltham, MA, USA).

**Scratch test**

A marker was used to draw horizontal lines with the help of a ruler on the back of a 6-well plate. The lines were evenly drawn to 1 cm long, and there were at least 5 lines for each well. A total of 5 × 10^5 cells were inoculated into each well of a 6-well plate. Cells were transfected when they reached a density of approximately 70% confluence. When the cells grew to just cover the entire well, a 10 µl pipette tip was used to make a scratch, with the help of a ruler, perpendicular to the horizontal lines on the back of the plate. The detached cells were washed with PBS, and then serum-free medium was added to continue the culture for 72 hours. Samples were taken at 0, 24, 48, and 72 hours, the cells were photographed, and the scratch width was measured.

**Migration and invasion assays**

Migration and invasion assays were performed using a Transwell chamber (8 µm, 24-well insert; Corning Incorporated, Corning, NY, USA). After 3 × 10^5 cells were mixed in 200 µl of serum-free medium, they were seeded in the upper chamber, 500 µl normal medium containing 20% FBS was added into the lower chamber, and then the plates were incubated for 48 hours at 37 °C in a 5% CO₂ incubator. The upper chamber and the lower chamber were washed twice with PBS. The cells in the upper chamber were cleaned with a cotton swab to remove cell debris. The chamber membrane was fixed with 4% paraformaldehyde for 30 min. The paraformaldehyde solution was aspirated and cells were dried, followed by 0.1% crystal violet stain for 30 min and two washes with PBS. Cells on the lower side of the chamber membrane were observed under an inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan). The cells were photographed and cell numbers were calculated.

**Reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR)**

Total RNAs in tissues and cells were extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA). RT-qPCR was performed according to the manual of the Toyobo RT kit (Toyobo Life Science, Osaka, Japan) and Hieff® qPCR SYBR® Green Master Mix (Yason Biotech Co., Ltd.). CircRNA primers were designed by Beijing Tsingke Biological Technology Co., Ltd. (Hunan, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, and the relative expression levels were calculated by the 2^−ΔΔCt method.

**CircRNA-miRNA-mRNA coexpression network construction**

circRNA-miRNA interactions were predicted using Arraystar’s homemade miRNA target prediction software (Rockville, MD, USA) based on TargetScan (15) and miRanda (16). The miRNAs were scored and sequenced using the miRNA support vector regression (mirSVR) algorithm to focus on target miRNAs (17). Therefore, for each circRNA, we identified the top 5 miRNAs in the miRVR score system to establish a top-5 circRNA-miRNA network (1 circRNA connecting to 5 miRNAs). To further predict the interactions between miRNA and mRNAs, the miRNA-mRNA overlapping set predicted by databases of miRDB (18), miRstarBase (19), and TargetScan were used to plot circRNA-miRNA-mRNA interaction networks in Cytoscape (version 3.4.0) (20).

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of circRNAs:** The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.ncifcrf.gov (Version6.7)) (21) is an online bioinformatics database that integrates biological data and analysis tools. It provides a complete set of gene and protein functional annotation information for users to extract biological
information. KEGG is a database resource used to categorize high-level functions and biological systems from large-scale molecular datasets generated by high-throughput experimental techniques (22). GO is an important bioinformatics tool for annotating genes and analyzing their biological processes (23). To analyze the functions of circRNAs, the online database of DAVID was used for biological analysis. \( P < 0.05 \) indicated statistical significance.

**Data analysis:** SPSS 19.0 software was used for statistical analysis (IBM Corp., Armonk, NY, USA): The data were imaged using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA), and the data are expressed as the mean \( \pm \) standard deviation. The differences between two groups were analyzed using the independent-sample t test, and intragroup differences were analyzed using one-way analysis of variance (ANOVA). \( P < 0.05 \) indicated that a difference was statistically significant.

**Results**

**Expression profiles of circRNAs in human CRC tissues**

To study the expression profile of circRNAs in human CRC tissues, we used circRNA microarray technology to detect and analyze the expression of circRNAs in 3 pairs of CRC tissues and adjacent normal tissues. We drew a box plot showing the density distributions of all datasets after normalization and found that the distributions of \( \log_2 \) ratios were similar between all test samples (Figure 1A). The unsupervised hierarchical clustering shows that circRNAs had different expression in CRC vs. adjacent normal tissues (Figure 1B). The differential expression of these circRNAs was further confirmed in the volcano plot. With cutoffs of \( \log_2 \) (fold change) \( \geq 1 \) and \( P < 0.05 \) for differentially expressed circRNAs compared with adjacent normal tissues, 66 circRNAs in CRC tissues were upregulated, while 77 circRNAs were downregulated (Figure 1C). Among the upregulated circRNAs, 52 were composed of exons, 9 were composed of introns, 1 was composed of intergenic regions, 2 were mixed circRNAs, and 2 were antisense circRNAs. Among the downregulated circRNAs, 67 were composed of exons, 4 were composed of introns, 1 was composed of intergenic regions, and 5 were mixed circRNAs [Supplementary Table 1].

**Identification of the target circRNAs in CRC**

The circRNAs with upregulated expression in our microarrays [Supplementary Table 1] were compared with the circRNAs with upregulated expression in the CRC circRNA microarray GSE126094 (24) from the GEO database (25). A total of 20 circRNAs were found to be upregulated in both microarrays, and the top 10 co-upregulated circRNAs are listed in Table 1. The top two circRNAs (hsa_circ_0004104 and hsa_circ_0044556) with upregulated expression in both microarrays were selected for further experiments. To further verify whether hsa_circ_0004104 and hsa_circ_0044556 had consistent expression between tissues and the microarray, we extracted total RNA from 10 pairs of CRC tissues and adjacent normal tissues for RT-qPCR validation. The results showed that compared with the adjacent normal tissue, hsa_circ_0044556 expression was upregulated in CRC tissues, which was consistent with the microarray results [Supplementary Figure S1]. The expression of hsa_circ_0004104 in CRC tissues and paracancerous tissues was not significantly different, which was inconsistent with the microarray results [Supplementary Figure S2].

**Expression characteristics of hsa_circ_0044556 in CRC**

To further validate the clinical significance of hsa_circ_0044556, 42 pairs of CRC tissues and adjacent normal tissues were collected. We confirmed that hsa_circ_0044556 was highly expressed in CRC tissues compared to adjacent normal tissues (Figure 2A) by RT-qPCR. hsa_circ_0044556 was significantly upregulated in 69.23% (36/52) of CRC tissues compared to adjacent normal tissues (Figure 2B). Based on the expression level of hsa_circ_0044556, the diagnostic value of hsa_circ_0044556 in distinguishing CRC from adjacent normal tissues was calculated using the receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) was 0.7274 (\( P < 0.0001 \)) (Figure 2C).

To further investigate whether the high expression level of hsa_circ_0044556 in patients was related to
clinicopathological parameters, Table 2 was developed. We can see from Table 2 that the expression level of hsa_circ_0044556 did not have a significant difference in terms of patient sex \( (P = 0.3548) \), age \( (P = 0.562) \), tumor size \( (P = 0.9865) \), or position \( (P = 0.8677) \), but a significant difference was observed for tumor stage \( (P = 0.0121) \) and lymph node metastasis \( (P = 0.0045) \) (Table 2).

Next, we used CRC cell lines for experiments. Compared to in the colonic epithelial cell line NCM460, hsa_circ_0044556 was highly expressed in the CRC cell lines of HCT116, SW480 and SW620 but was lower expressed in the CRC cell line HT29 (Figure 2D). Because hsa_circ_0044556 was expressed at a relatively higher level in the two cell lines HCT116 and SW480, these two cell lines were chosen for further experiments.

**Loop-forming validation and siRNA design of hsa_circ_0044556**

By reviewing the human reference genome, we found that hsa_circ_0044556 (chr17: 48271490-48272189) was composed of exons 21-24 of the collagen type I alpha 1 (COL1A1) gene and was located on human chromosome 17q21.33. By RT-qPCR, Sanger sequencing, and anti-ribonuclease R digestion in HCT116 and SW480 cells, we verified that hsa_circ_0044556 had a circular structure (Figure 3A, B and C). To evaluate the biological effects of hsa_circ_0044556 in CRC cells, we constructed three siRNAs covering the back-splicing region of hsa_circ_0044556. The results showed that the expression level of hsa_circ_0044556 in CRC cells transfected with siRNA-3 significantly decreased, while COL1A1 mRNA expression level did not change significantly (Figure 3D, E, F, and G). Therefore, hsa_circ_0044556 was chosen for further experiments, and shRNA-3 was constructed.

**Silencing hsa_circ_0044556 inhibits the proliferation, migration, and invasion of CRC cells**

The results of the tablet cloning assay and CCK-8 assay showed that silencing of hsa_circ_0044556 lowered the proliferation ability of HCT116 and SW480 cells (Figure 4A and B). The results of the scratch test and Transwell experiment showed that knockdown of hsa_circ_0044556 inhibited the migration and invasion capability of HCT116 and SW480 cells (Figure 4C, D, and E).

**CircRNA-miRNA-mRNA coexpression network of hsa_circ_0044556**

We hypothesized that hsa_circ_0044556 acts as a miRNA “sponge” to regulate circRNA-miRNA-mRNA networks. Using miRNA target prediction software, five miRNAs with the highest miRVR scores that might be bound by differentially expressed circRNAs were identified by TargetScan and miRanda [Supplementary Table 1]. The molecular interaction between hsa_circ_0044556 and the 5 miRNA targets is depicted in Figure 5A. Then, miRDB, miRTarBase, and TargetScan were used to predict the target genes that those five miRNAs might bind to. A total of 107 predicted genes [Supplementary Table 2] from the 3 databases were selected as the potential target genes of hsa_circ_0044556. Cytoscape analysis of the circRNA-miRNA-mRNA interaction network of hsa_circ_0044556 revealed that hsa-mir-214-3p and hsa-mir-761 exhibited the most complex interaction network, followed by hsa-mir-194-3p, hsa-mir-412-3p, and hsa-mir-362-5p (Figure 5B). Since predicted target miRNAs including hsa-mir-214-3p and hsa-mir-194-3p proved to be downregulated in cancer progression in ENCORI (26).

**Bioinformatic analysis of the predicted genes in Hsa_circ_0044556.**

Gene ontology (GO) analysis was performed on hsa_circ_0044556, and the functional roles of the top 10 most enriched target genes were investigated from the perspective of biological processes (Figure 6A). The results showed that hsa_circ_0044556 had a strong correlation with the cell cycle, responses to steroid hormone stimulation, and angiogenesis. KEGG analysis of hsa_circ_0014130 indicated that its top nine enriched pathways included prostate cancer, the cancer pathway, and the ErbB signaling pathway (Figure 6B). Seven target genes were enriched in the cancer pathway. These data suggested that hsa_circ_0044556 may play important roles in the malignant behavior of cancer by regulating the expression of target genes involved in these pathways.
In recent years, with the rapid development and extensive application of RNA sequencing technology, circRNAs have become a hotspot in the field of RNA research. Researchers have found that many exon transcripts can form circRNAs through nonlinear reverse splicing or gene rearrangement. Moreover, they account for a large percentage of all spliced transcripts (7). circRNAs may come from introns or exons (27). In mammals, exon-constituted circRNAs have two mechanisms of loop formation: lariat-driven circularization and intron-pairing-driven circularization (7, 28, 29). A covalently closed circular structure without a 5′-to-3′ polarity or poly(A) tail is then formed by reverse splicing of a typical splice.

More and more studies have investigated the potential functions of circRNAs in various diseases, such as nervous system diseases, cardiovascular diseases, and cancers (30–33). Some abnormally expressed circRNAs have been associated with the tumor development, invasion, metastasis, or prognosis of patients (34–40). In mammalian cells, compared with other ncRNAs, such as miRNAs and long noncoding RNAs, circRNAs have highly conserved sequences and high stability (41). These features might let circRNAs become ideal biomarkers and potential therapeutic targets for disease diagnosis.

In this study, high-throughput circRNA microarrays were used to study the expression of circRNAs in human CRC. The results showed that the expression of circRNAs in CRC tissues (n = 3) was significantly different from that in adjacent normal tissues (n = 3) (Fig. 1). Compared with the adjacent normal tissues, our microarray data showed that 66 circRNAs were significantly upregulated while 77 circRNAs were significantly downregulated in CRC tissues (Supplementary Table 1). We verified the expression levels of two circRNAs (hsa_circ_0004104 and hsa_circ_0044556) in 10 pairs of CRC and adjacent normal tissue samples, and only hsa_circ_0044556 was determined to be upregulated in CRC tissues, and with statistical significance (P = 0.0304) [Supplementary Fig. 1]. The expression level of hsa_circ_0004104 was inconsistent with the microarray results, and there was no statistical significance (P = 0.9208) [Supplementary Fig. 2]. The above results indicate that validation of differently expressed circRNAs in microarray analysis is an important step in such a screening study. In addition, to verify the results above, we expanded the sample size to detect the expression of hsa_circ_0044556 in other tissue samples (n = 52). The results showed that hsa_circ_0044556 was significantly upregulated in 69.23% (36/52) of CRC tissues, with an average increase of 6.65-fold compared to the adjacent normal tissues. ROC analysis showed that hsa_circ_0044556 level had relatively high sensitivity and specificity, with an AUC of 0.7274 (Fig. 2). More importantly, considering the clinical pathological factors, we found that the high expression level of hsa_circ_0044556 in CRC was closely related to tumor stage and lymph node metastasis (Table 2), important factors in evaluating the prognosis of CRC. These results indicate that hsa_circ_0044556 might be involved in the development, progression, and metastasis of CRC and could be used as a potential biomarker and a new therapeutic target for CRC.

One of the most important things about circRNAs is that they act as miRNA sponges. Certain specific circRNAs can bind and negatively regulate miRNAs involved in the competitive endogenous RNA (ceRNA) network, thereby regulating linear RNA transcription and protein production. Thomas et al. found that circRNA ciRS-7 can strongly inhibit miR-7 activity, leading to increased miR-7 target expression levels (39, 42). Other functions may include gene expression regulation at the transcriptional or posttranscriptional level (43), and even encoding proteins (44, 45). In this study, to further understand the biological functions of hsa_circ_0044556, 5 miRNAs with the highest mirSVR scores were identified for each differentially expressed circRNA using miRNA target-prediction software (namely, hsa-mir-214-3p, hsa-mir-761, hsa-mir-194-3p, hsa-mir-412-3p, and hsa-mir-362-5p) [Supplementary Table 1]. We used TargetScan, miRDB, and miRTarBase to predict the hsa_circ_0044556-miRNA-mRNA network. In essence, this network diagram shows a cellular RNA network with hsa_circ_0044556 interacting with 5 miRNA nodes and 107 target genes (Fig. 5B). Through the retrieval of database ENCORI, it was found that hsa-mir-214-3p and hsa-mir-194-3p had low expression in colorectal adenocarcinoma. Furthermore, it has been found that hsa-mir-214-3p was significantly reduced in epithelial ovarian cancer cells and could affect epithelial ovarian cancer cell proliferation, invasion, increasing cisplatin chemosensitivity and inhibiting in vivo tumor growth proliferation by binding X-inactive specific transcript (XIST) (46). It have also been reported that the downregulated hsa-mir-145-5p and hsa-mir-214-3p may modulate the expression of both EMT and NGAL/MMP-9 pathways. Therefore, the decreased expression and inhibited function of hsa-mir-214-3p in cancer further support our hypothesis that hsa_circ_0044556 functions as a miRNA sponge to regulate the hsa_circ_0044556- hsa-mir-214-3p-mRNA network.
We found that a large number of mRNAs may participate in the above hsa_circ_0044556-miRNA-mRNA network, such as ARL2, MAPK1, and PTEN. Therefore, GO and KEGG pathway analysis was performed to detect the functions of these potential target genes. The results of GO enrichment analysis (Fig. 6A) showed that the target genes of hsa_circ_0044556 participated in the regulation of the cell cycle and angiogenesis, indicating that the regulation of these genes in the occurrence and development of CRC has importance in cell responses. The cancer pathway and the ErbB signaling pathway, two KEGG pathways correlated with hsa_circ_0044556 expression (Fig. 6B), may be related to the proliferation, migration, and invasion of CRC cells. Therefore, we speculate that the hsa_circ_0044556-miRNA-mRNA axis is a possible mechanism that promotes the development of CRC, and it is worthwhile to further study the overexpression of hsa_circ_0044556 as an inhibitor of miRNA and its possible mechanism of action.

In summary, this study revealed the expression profile of circRNAs in CRC tissues and demonstrated the abnormal expression of circRNAs in CRC. This study confirmed the significance of the upregulation of hsa_circ_0044556 and analyzed the relationship between hsa_circ_0044556 and the clinicopathological features of CRC patients, suggesting its potential role in the development and progression of CRC and its potential application as a CRC diagnostic biomarker. In the future, it will be necessary to explore the molecular mechanism of hsa_circ_0044556 as a miRNA sponge regulating the development and progression of CRC.

**Conclusion**

Hsa_circ_0044556 promoted the development and progression of CRC. It is possible that hsa_circ_0044556 will become a new biomarker or therapeutic target for CRC.

**Abbreviations**

ARL2: ADP ribosylation factor-like protein 2; ANOVA: Analysis of Variance; cDNA: Complementary DNA; CEA: Carcinoembryonic antigen; circRNAs: Circular RNAs; COL1A1: Collagen type 1 alpha 1; CRC: Colorectal cancer; DAVID: The Database for Annotation Visualization and Integrated Discovery; DMEM: Dulbecco’s Modified Eagle Medium; DNA: Deoxyribonucleic acid; ENCORI: The Encyclopedia of RNA Interactomes; FBS: Fetal bovine serum; GAPDH: Glyceraldehyde phosphate dehydrogenase; GEO: Gene expression omnibus; GO: Gene Ontology; ITCH: Itchy E3 ubiquitin protein ligase; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK1: Mitogen activated protein kinase 1; miRNA: MicroRNA; mRNA: Messenger RNA; NC: Negative control; ncRNAs: Noncoding RNAs; OB: Occult blood; ROC: Receiver operating characteristic; PBS: Phosphate buffered saline; PCR: Polymerase chain reaction; PTEN: Phosphatase and tensin homolog; RT-qPCR: Reverse transcription and real-time quantitative PCR; RNA: Ribonucleic acid; siRNA: Small interfer RNA; shRNA: Short hairpin RNA; SPSS: Statistical Product and Service Solutions; TCGA: The Cancer Genome Atlas.

**Declarations**

**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions**

Experiments were designed by CL and BT and were performed by LJ, MM, FL. XT analyzed data. JW interpreted results of experiments. The manuscript was written by LJ and JW and edited by CL and BT.

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Data Availability Statement

All datasets presented in this study are included in the article/ supplementary files.

Ethics approval and consent to participate

The study which including human and animal approval were acquired from the Ethics Committee of The Third Xiangya Hospital of Central South University [Lot 2016 - S086].

Consent for publication

We assure that the material is original and it has not been published elsewhere yet.

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Table 1

The top 10 co-upregulated circRNAs in our microarrays and CRC circRNA microarray GSE126094 from the GEO database.

| CircRNA ID       | Fold change | P-value      | CircRNA type | Chromosome | Best transcript | GeneSymbol |
|------------------|-------------|--------------|--------------|------------|-----------------|------------|
| hsa_circ_0004    | 8.8323548   | 0.009595155  | exonic       | chr5       | NM_003118       | SPARC      |
| 104              |             |              |              |            |                 |            |
| hsa_circ_0044    | 6.9246896   | 0.027427284  | exonic       | chr17      | NM_000088       | COL1A1     |
| 556              |             |              |              |            |                 |            |
| hsa_circ_0092    | 4.5172247   | 0.039069424  | intronic     | chr22      | ENST0000021618  | MYH9       |
| 283              |             |              |              |            |                 |            |
| hsa_circ_0004    | 3.5141603   | 0.045304817  | exonic       | chr16      | NM_018124       | RFWD3      |
| 519              |             |              |              |            |                 |            |
| hsa_circ_0028    | 3.3084192   | 0.04170796   | exonic       | chr12      | NM_025247       | ACAD10     |
| 299              |             |              |              |            |                 |            |
| hsa_circ_0000    | 3.1460597   | 0.010803855  | intronic     | chr15      | ENST00000558261 | RP11-351M8.1 |
| 644              |             |              |              |            |                 |            |
| hsa_circ_0004    | 3.0425366   | 0.025612242  | exonic       | chrX       | NM_024917       | TRMT2B     |
| 957              |             |              |              |            |                 |            |
| hsa_circ_0080    | 3.0098437   | 0.009922637  | exonic       | chr7       | NM_022479       | WBSCR17    |
| 425              |             |              |              |            |                 |            |
| hsa_circ_0070    | 2.7063551   | 0.013208653  | exonic       | chr4       | NM_005443       | PAPSS1     |
| 610              |             |              |              |            |                 |            |
| hsa_circ_0008    | 2.6635341   | 0.043991198  | exonic       | chr2       | NM_006216       | SERPINE2   |
| 365              |             |              |              |            |                 |            |
The Associations between the hsa_circ_0014130 expression level and clinicopathological characteristics of patients with CRC. *P < 0.05, compared among different groups. The expression level of hsa_circ_0044556 was significantly associated with tumor stage and lymphatic metastasis.

| Characteristic       | NO. of patients (%) | Hsa_circ_0044556 (Mean ± SD) | P value |
|----------------------|---------------------|------------------------------|---------|
| Gender               |                     |                              |         |
| Male                 | 32 (61.54)          | 6.992 ± 1.614                | 0.3548  |
| Female               | 20 (38.46)          | 4.934 ± 1.029                |         |
| Age (years)          |                     |                              |         |
| ≥ 60                 | 24 (46.15)          | 5.521 ± 1.596                | 0.562   |
| < 60                 | 28 (53.85)          | 6.783 ± 1.461                |         |
| Tumor diameter       |                     |                              |         |
| ≥ 4.5                | 28 (53.85)          | 6.217 ± 1.477                | 0.9865  |
| < 4.5                | 24 (46.15)          | 6.180 ± 1.587                |         |
| Tumor site           |                     |                              |         |
| Colon                | 25 (48.08)          | 6.388 ± 1.699                | 0.8677  |
| Rectum               | 27 (51.92)          | 6.026 ± 1.364                |         |
| Tumor stage          |                     |                              |         |
| I-II                 | 23 (44.23)          | 3.233 ± 0.6187               | 0.0121* |
| III-IV               | 29 (55.77)          | 8.553 ± 1.749                |         |
| Lymphatic metastasis |                     |                              |         |
| Yes                  | 27 (51.92)          | 9.054 ± 1.843                | 0.0045* |
| No                   | 25 (48.08)          | 3.118 ± 0.5761               |         |
Figure 1. Differences in circRNA expression profiles between CRC and normal tissues. (A) Box plots show the distribution of circRNAs for the six samples (CRC and normal tissues). The distributions were nearly the same after normalization. (B) Hierarchical clustering shows a distinguishable circRNA expression profile (C for CRC and N for adjacent normal tissues). Each column represents a tissue sample, and each row corresponds to a circRNA. "Red" indicates high expression level, and "green" indicates lower expression level. (C) Volcano plots showing differential expression between the two groups. The red points represent the differentially expressed genes with an expression change ≥ 2.0 and $P < 0.05$. 
Figure 2. Hsa_circ_0044556 significantly upregulated biomarker for CRC. (A). The expression levels of hsa higher than those in adjacent normal tissues. (B) Hsa-
higher than those in adjacent normal tissues. (B) Histoc in which hsa_circ_0044556 expression was upregulated green), or no change (13, 25.00%, gray). Log2(T/N exp which < -1 as lower expression, and between -1 and characteristic (ROC) curve of hsa_circ_0044556 was b area under curve was 0.7274, P < 0.0001. (D). The ex lines and NCM460.
Figure 3. Hsa_circ_0044556 is a circRNA. (A). Verification that h and convergent primers. Top, schematic illustration of hsa_circ_0044556 RT-PCR products with divergent primers showing circularization. Middle, Sanger sequencing result of hsa_circ_0044556. (B). RT-l circularization of hsa_circ_0044556. (C). The expression levels of or absence of RNase R treatment, respectively. (D) Schematic illu
of absence of RNase K treatment, respectively. (D) Schematic flu targets the back-splice junction of hsa_circ_0044556. (E) and (F). treated with three siRNAs. (G) The expression levels of COL1A1

Figure 3
Figure 4. Silencing hsa_circ_0044556 inhibited proliferation, migration, and invasion of CRC cells. (* P <
Figure 5. Bioinformatics prediction of hsa_circ_0044556 in CRC. (A) The miRNAs matched hsa_circ_0044556. (B) The co-expression network was software. Five miRNAs and their mRNA target genes were found with ov
Figure 6. Gene ontology (GO) enrichment analysis (A) The top 10 significantly enriched target genes respectively. (B) The top 9 significantly enriched the y-axis, respectively.