Circ4207 regulates vasculogenic mimicry formation in colorectal cancer through the miR-20b-5p/VEGFA axis

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Abstract

Background

Colorectal cancer (CRC) is highly prone to metastasis, leading to a continual increase in the number of deaths each year. One of the commonly used clinical treatments for CRC metastasis is anti-angiogenesis, and vasculogenic mimicry (VM) is considered to be one of the important reasons for the unsatisfactory effect of anti-vascular therapy. Circular RNA (CircRNA) may have an essential regulatory effect during the development of VM and appears to be an ideal marker for fluid biopsy. Therefore, exploring the role of circular RNA in the formation of VM is of great value to the diagnosis and treatment of CRC.

Methods

The differentially expressed circRNAs in CRC were obtained by full transcriptome sequencing. Then the back splice site and its good stability were verified by Sanger sequencing, RNase R and Actinomycin D experiments. Then, the effects of Circ4207 on the growth, invasion and VM of CRC were investigated in vitro and in vivo. The regulatory mechanism between Circ4207 and miR-20b-5p/VEGFA was further confirmed by bioinformatics, fluorescence in situ hybridization (FISH) and dual luciferase reporting experiments. Finally, the serum levels of Circ4207 in patients with colorectal cancer were detected to evaluate its clinical diagnostic value.

Results

Circ4207 is highly expressed in CRC. Circ407 promotes the proliferation and invasion of colorectal cancer and also facilitates the formation of vascular mimicry. Further studies revealed that Circ4207 primarily enhances the formation of vasculogenic mimicry through the miR-20b-5p/VEGFA axis. The study found higher levels of Circ4207 in the serum of patients with colorectal cancer ($P < 0.001$).

Conclusions

Circ4207 promotes the formation of vasculogenic mimicry in colorectal cancer through the miR-20b-5p/VEGFA axis. It is suggested that it can be used as a potential liquid biopsy marker and a novel therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) remains a leading cause of cancer-related mortality worldwide, ranking second in mortality rates among all cancers.[1, 2]. Despite advances in medical science, the annual incidence of CRC continues to rise globally [3]. On the other hand, because the exact etiology of CRC is unknown and
the early symptoms are not obvious, distant metastasis often occurs at the time of diagnosis[1]. For now, comprehensive treatments for advanced colorectal cancer include traditional chemotherapy, immunotherapy and anti-vascular therapy[4]. However, the diagnosis and treatment of advanced colorectal cancer are not ideal due to the absence of effective diagnostic markers and therapeutic targets. Therefore, it is imperative to conduct further investigation into the molecular mechanism of CRC in order to identify a more suitable target for diagnosis and treatment.

Vasculogenic mimicry (VM) is a microvascular channel formed by tumor cells in order to transport nutrients and oxygen to maintain the continuous division and proliferation of tumor cells under the condition of hunger and hypoxia[5–7]. VM’s presence in various malignancies, including melanoma[5], breast cancer[8], colorectal cancer[9] and prostate cancer[10], correlates with a heightened metastatic potential and diminished efficacy of anti-vascular therapies [11, 12]. The underlying mechanisms of VM formation remain obscure, though they are known to involve complex signaling pathways[13–15] and the regulation by non-coding RNAs, such as long noncoding RNAs (IncRNAs) and microRNAs (miRNAs)[15]. However, the regulatory role of circular RNAs (CircRNAs) in VM formation is less explored and understood.

CircRNAs are a recently discovered type of non-coding RNA[16]that have emerged as significant players in cancer biology, influencing tumor stemness, proliferation, invasion, apoptosis, and angiogenesis by acting as miRNA sponge[17], protein bait[18] and translating into regulatory peptide[19, 20]. CircRNAs are characterized by high abundance and stability, which is its unique advantage as a diagnostic and prognostic marker[21, 22]. Despite numerous circRNAs being implicated in CRC angiogenesis[17, 23–25], their involvement in VM formation is scarcely reported.

In our research, we identified Circ4207 as a novel CircRNA through sequencing and various approaches. At the same time, we explored the biological functions of Circ4207 in colorectal cancer through in vitro and in vivo experiments, especially its role in VM formation and its molecular mechanism. Our findings reveal that Circ4207 enhances VEGFA expression and activates receptors such as VEGFR1 by sponging miR-20b-5p, thereby facilitating VM formation in CRC. This process underscores the potential of Circ4207 as a pivotal factor in CRC progression, offering new insights into CRC pathogenesis and indicating its utility as a novel diagnostic marker and therapeutic target.

**Materials and methods**

**Source of serum specimen**

Serum samples were acquired from the Affiliated Hospital of Chongqing Medical University. Serum samples were taken in 5ml enzyme-free tubes and stored in a -80°C refrigerator. RNA was extracted according to the kit instructions, followed by subsequent detection immediately, and stored at -80°C.

**Cell culture**
The CRC cell lines SW480 (Cat No.: CL-0223B), Caco2 (CL-0050), HCT116 (CL-0096), Lovo (CL-0144), SW620 (CL-0225B) and human normal colon epithelial cell NCM460 (CL-0393) were acquired from the Key Laboratory of Laboratory Medical Diagnostics designated by Chinese Ministry of Education, Chongqing Medical University. All CRC cell lines were cultured in DMEM (Gibco, Thermo Fisher, USA) with 10% serum (LONSA SCIENCE SRL, Uruguay), only NCM460 was cultured in RPM1640 medium. All cells were free of mycoplasma contamination and cultured in an incubator at 37°C and 5% CO2.

RNA extraction, reverse transcription (RT-PCR) and real-time quantitative PCR (qRT-PCR) detection

The SteadyPure Quick Extraction Kit (Accurate Biotechnology, Hunan, China) was used to extract total RNA from cells. Serum RNA was extracted by SteadyPure Blood, Serum and Plasma Small RNA Extraction (Accurate Biotechnology, Hunan, China). The ultra-ultraviolet spectrophotometer NanoDrop One (Thermo Fisher Scientific, USA) was used to determine the concentration and purity of total RNA. RT Premix (Accurate Biotechnology, Hunan, China) was used to reverse transcribed mRNA into cDNA. The TAKARA PrimeScript TM RT-PCR Kit (Takara, Dalian, China) was used to reverse microRNA. qRT-PCR was performed on Bio-Rad CFX96™ (Bio Rad, USA) using SYBR Green. The sequence of qRT-PCR primers was shown in Table S1. Using GAPDH/U6 as internal reference, the relative quantitative values of RNA were calculated by $2^{-\Delta\Delta Ct}$ method.

Vector construction and cell transfection

Circ4207 knockdown and miR-20b-5p modulation were achieved using siRNA and miRNA mimics/inhibitors, respectively (Tsing ke, Beijing, China). GP-transfect-Mate (Gene Pharma, Shanghai, China) was used for transfections. The sequences of SiRNA, mimics and inhibitors were provided in Table S2.

Western blot

BCA method was used to determine the concentration of total proteins extracted from each group. Equal amounts of protein were separated by SDS-PAGE gel at 90 V for 30 min, 120V,60 min, and transferred to PVDF membrane (Biosharp, Beijing, China), which was sealed with 5%BSA in TBST. Then the strips were incubated with the corresponding primary antibodies VEGFA (1:1000) (Immunoway, TX, USA), VEGFR1(1:500), VEGFR2(1:1000) and GAPDH (1:1000) (Proteintech, Wuhan, China) at 4°C overnight. Finally, the strips were then immersed in a solution of secondary antibody (1:5000) (ZSGB-BIO, Beijing, China) and incubated at 37°C for 1 hour. The band intensity was analyzed with ECL chemiluminescence kit (Biosharp, Beijing, China).

RNase R digestion and Actinomycin D tolerance assay

Total RNA of SW480 and Caco2 cells (2 µg/group) was incubated with 10 U/µg RNase R (Solarbio, Beijing, China) at 37°C for 0, 10, 20, 30 min. SW480 and Caco2 cells were subcultured and exposed to actinomycin D (MCE, USA) at a final concentration of 100 ng/ml for 0, 4, 8, 12, and 24 hours the next day, respectively. The levels of Circ4207, ATP9A and GAPDH were detected by qRT-PCR.
Fluorescence in situ Hybridization (FISH)

The cell slides were placed in a 24-well plate in advance, cells were added (4 \( \times 10^4 \) / well) and incubated overnight. The localization of Circ4207 and miR-20b-5p was observed by FISH SA Biotin kit (Gene Pharma, Shanghai, China). The procedure can be simply described as fixation, permeabilization, blocking, pre-hybridization, hybridization at 37 °C overnight, DAPI staining, and microscopic observation. The probe sequence is in table S3. Confocal laser scanning microscope (Leica, Germany) was used for observation.

CCK8, clone formation, transwell and wound healing experiments

CCK8 kit (MCE, USA) and clonal formation assay were used to detect cells proliferation. To assess the migratory and invasive capabilities of the cells, the Transwell and the wound healing experiments were employed. In the wound healing experiment, the cells of each well were crossed after they were full, then washed twice with PBS and replaced with serum-free medium. To perform Transwell invasion experiments, the upper chamber was pretreated by adding 50µl of dilution matrix gel (Corning, NY, USA) and incubating it for 1 hour. 6\( \times 10^4 \) cells were added to the upper chamber while 500µl of complete medium was added to the lower chamber.

In vitro vasculogenic mimicry formation experiment

Add 50µl of matrix gel to the 96-well plate in advance, and then 45 min was placed in the incubator at 37°C to be solidified. The cells in each group were digested and then re-suspended and counted with serum-free medium. A mixture of 150µl containing 3\( \times 10^4 \) cells was slowly added to the matrix glue. The cell formation was observed 6-8h later and photographed with inverted fluorescence microscope (Nikon, Japan). The Angiogenesis plug was used to perform tube branch and length analysis.

Xenograft model in nude mice

In xenograft tumor models, 4\( \times 10^6 \) SW480 cells transfected with Si Circ, Angomir, and NC alone were suspended in 100µL serum-free DMEM and injected subcutaneously into the groin. After 25 days, the mice were euthanised and the tumors used for further experiments.

Bioinformatics prediction of Circ4207/miR-20b-5p/VEGFA axis

We used two human circRNA databases, CircBank(http://www.circbank.cn/) and Circular RNA Interactome (https://circinteractome.nia.nih.gov/index.html) to predict the potential target of Circ4207 miRNAs. We used the intersection of two predictive miRNA target gene databases miRDB (https://mirdb.org/) and miRTarbase (https://mirtarbase.cuhk.edu.cn/) with known VM-related genes to screen possible targets of miR-20b-5p mRNA. It is worth mentioning that when predicting possible targeted molecules, we always select the intersection of 2–3 data sets for subsequent verification.
Dual luciferase reporter assay

To confirm that Circ4207 sponging miR-20b-5p, miR-20b-5p directly targeted VEGFA; We designed wild and mutant plasmids of Circ4207 and VEGFA in company (Tsing ke, Beijing, China) and inserted them into dual luciferase reporter vectors, named Circ4207-WT, Circ4207-MUT, VEGFA-WT, and VEGFA-MUT, respectively. We transfected WT and MUT double luciferase reporter plasmids into SW480 and Caco2 cells, respectively, and transfected miR-NC and miR-mimics simultaneously. After 48h, the luciferase activity was determined by the double luciferase kit (YEASEN, Shanghai, China).

Statistical analysis

All results were expressed as mean ± SD and plotted using GraphPad Prism 9.5. The difference between the two groups was analyzed by Student’s t test, and */P< 0.05, statistically significant differences.

Results

High expression of Circ4207 in CRC and validation of circular structure

To investigate the expression characteristics of circRNA in CRC, we performed whole transcriptome sequencing on 6 pairs of CRC and adjacent tissues as described previously[26]. As shown in the volcano map (Fig. 1A), a total of 573 differentially expressed circRNAs (LogFC ≥ 2.0, P< 0.05) were detected, of which 40 were significantly overexpressed and 533 were significantly down-regulated. It can be found that Circ4207 is highly expressed in CRC (Fig. 1A, B). Then, we selected Circ4207 for the subsequent research. Circ4207 was found to be significantly higher in colorectal cancer (CRC) cell lines (SW480, Caco2, HCT116, Lovo, SW620) compared to human normal colon epithelial cells (NCM460) (Fig. 1C). Based on the annotation results from circBase(http://www.circbase.org/), Circ4207(hsa_circ_0004207) is spliced from pre mRNA of ATP9A located at chr20:50290691–50292747 and eventually formed a circular transcript of 238 nt (Fig. 1D).

Next, we will demonstrate Circ4207’s circular RNA properties through a series of experiments. Figure 1D illustrates the splicing process of Circ4207. The amplification products of Circ4207 were subjected to sanger sequencing, which was consistent with RNA seq and circBase annotation (Fig. 1D, E). In CRC cells, Circ4207 can only be amplified by cDNA and not by gDNA (Fig. 1F). In addition, Circ4207 was more stable than the linear RNAs GAPDH and ATP9A during treatment of CRC cells with Act D and RNase R (Fig. 1G, H). These results indicate that Circ4207 was indeed a circular RNA formed by reverse splicing of ATP9A. FISH experiment revealed that Circ4207 (red) was primary localized in the cytoplasm (Fig. 1I). Then, we investigated the causes of Circ4207 elevation in CRC. We found that exogenous addition of TNF-α (inflammation) and Cocl2 (hypoxia) promoted the expression of Circ4207(Fig. 1J).
Effects of Circ4207 on the proliferation, apoptosis, invasion ability and vasculogenic mimicry formation

To investigate the biological characteristics of Circ4207 in colorectal cancer cells, we constructed SiRNA targeting Circ4207. As shown in Fig. 2A, the expression level of Circ4207 in SW480 and Caco2 was effectively knocked down by siRNA. The CCK8 growth curves revealed that the knockdown of Circ4207 had a significant inhibitory effect on the proliferation ability of SW480 and Caco2 cells (Fig. 2B). The results of the colony formation assay (Fig. 2C) further confirmed the previous results. The above results indicated that Circ4207 knockdown could significantly inhibit the proliferation of CRC cells. Flow analysis showed that knocking down Circ4207 significantly increased the proportion of apoptotic cells (Fig. 2D). As shown in Fig. 2E, F, the migration and invasion abilities of SW480 and Caco2 were significantly weakened after knocking down Circ4207. In addition to classical angiogenesis, solid tumors can also form microvascular channels themselves to transport nutrients and oxygen to maintain the needs of malignant proliferation. Therefore, we investigated whether knocking down Circ4207 affects the formation of VM in CRC cells. As expected, the formation of VM in SW480 and Caco2 cells was significantly reduced after Circ4207 knockdown (Fig. 2G). We detected the expression of some key molecules related to the formation of VM in SW480 and Caco2 cells, and found that reducing Circ4207 inhibited the production of FN, E-cadherin, and increased the level of E-cadherin (Fig. S1A).

Circ4207 acts as a sponge RNA for miR-20b-5p

Circular RNA exerts its regulatory function mainly through sponging miRNA. Considering that Circ4207 was mainly distributed in the cytoplasm, we first predicted potential targets for Circ4207 using the human CircRNA databases CircBank and Circular RNA Interactome. The target molecules predicted by the two databases were intersected, and 3 candidate miRNAs were screened (Fig. 3A). Next, the GEO dataset was used to verify whether the above three candidate molecules were low expressed in CRC. MiR-520h was highly expressed in GSE49246 (Fig. 3B), miR-1827 and miR-20b-5p were low expressed, but miR-20b-5p was even lower expressed in CRC (Fig. 3C, D). In other datasets GSE18392 and GSE125961, miR-20b-5p was also down-regulated in CRC (Fig. 3E, F). MiR-20b-5p in CRC cell lines (SW480, Caco2, HCT116, Lovo, SW620) was lower compare to NCM460 (Fig. 3G). Knockdown of Circ4207 in SW480 and Caco2 resulted in increased expression of miR-20b-5p (Fig. 3H). Above results indicate that Circ4207 can regulate miR-20b-5p in CRC. Next, we demonstrated their co-localization in the cytoplasm through RNA FISH experiments (Fig. 3I). Thus, we propose that Circ4207 may sponge miR-20b-5p. To validate this prediction, we carried out a dual luciferase reporting experiment. Full-length Circ4207-WT and the Circ4207-MUT were subcloned into pmirGLO (Fig. 3J). The result showed that miR-20b-5p mimics reduced the luciferase activity of the WT group but not the MUT group, and after adding mimics, the luciferase activity of the MUT was higher than WT (Fig. 3K). This suggests that Circ4207 may directly act on miR-20b-5p.
MiR-20b-5p suppresses the formation of vasculogenic mimicry in colorectal cancer

Previous research determined that miR-20b-5p, the target molecule of Circ4207, was low expressed in CRC. Next, we verified the biological characteristics of miR-20b-5p in CRC, and conducted a preliminary investigation on whether it affected VM formation. We used microRNA mimics and inhibitors to up-and down-regulate miR-20b-5p expression. The results demonstrated that we successfully regulated the expression of miR-20b-5p (Fig. 4A, B). CCK8 and colony-formation experiments proved that overexpression of miR-20b-5p in CRC cells inhibited their proliferative ability (Fig. 4C, D, Fig. S1 B, C). Flow analysis showed that up-regulation of miR-20b-5p promoted apoptosis in SW480 and Caco2 cells (Fig. S1D). Up-regulation of miR-20b-5p inhibited the migration and invasion ability of CRC cells (Fig. S1E, F). Next, we want to explore whether miR-20b-5p can affect the formation of VM in CRC cells. In vasculogenic mimicry forming experiments showed that raise the miR-20b-5p inhibited the formation of VM in SW480 and Caco2 cells, whereas down-regulation of miR-20b-5p showed more tube branching and longer tube formation (Fig. 4E). Next, we found that the addition of miR inhibitor reversed the down-regulation of Circ4207 on VM inhibition (Fig. 4F). The above results proved that Circ4207 affected VM formation in colorectal cancer through miR-20b-5p.

MiR-20b-5p suppresses the expression of VEGFA

To find the downstream target molecules of miR-20b-5p, we successfully screened out 5 candidate target genes (STAT3, HIF-1A, Smad4, Smad5, VEGFA) by intersecting two miRNA target gene databases, miRDB and miRTarbase, with known VM-related genes (Fig. 5A). Next, we discovered that overexpression of miR-20b-5p in CRC cells suppressed VEGFA and STAT3 expression (Fig. 5B). VEGFA was identified as being significantly upregulated in all colorectal cancer cell lines through qRT-PCR. (Fig. 5C). Subsequently, the data set GSE146587 was used to verify that VEGFA was significantly overexpressed in CRC (Fig. 5D), while STAT3 was not differentially expressed in CRC and its adjacent tissues (Fig. 5E). FISH experiment indicated a high degree of colocalization between miR-20b-5p and VEGFA in colorectal cancer cells. (Fig. 5F). Full-length VEGFA-WT and mutant versions were inserted into pmirGLO. (Fig. 5G). It was found that miR-20b-5p mimics markedly decreased the luciferase activity in the WT, but had no effect on the MUT (Fig. 5H). This proves that miR-20b-5p can directly bind to VEGFA.

Circ4207 regulates vasculogenic mimicry through the miR-20b-5p/VEGFA axis

VEGFA is a major promoter of angiogenesis, which mainly combines with its receptors VEGFR1 and VEGFR2 to continuously activate the downstream signaling pathway to promote angiogenesis. We found that down-regulation of miR-20b-5p increased mRNA levels of VEGFA, VEGFR1, and VEGFR2. Simultaneously, the abundance of VEGFR1 was higher than VEGFR2 according to cycle threshold values, and we hypothesized that miR-20b-5p regulates VM formation mainly through the VEGFA-VEGFR1 axis
(Fig. 6A). Similarly, knocking down Circ4207 expression in CRC cells decreased the expression of VEGFA, VEGFR1, and VEGFR2 (Fig. 6B). Western blot results showed that down-regulating Circ4207 lessened the abundance of VEGFA and VEGFR1 protein (Fig. 6C). The addition of miR-20b-5p mimics inhibited the levels of VEGFA and related receptors, while the addition of inhibitors promoted the expression of VEGFA and its receptors, and it was found that the abundance of VEGFR1 was higher than that of VEGFR2, which also confirmed the previous experimental results (Fig. 6D). We added miR-20b-5p inhibitors to reverse the inhibitory effect of Circ4207 knockdown on VEGFA (Fig. 6E). Next, to explore whether miR-20b-5p regulates VM formation via VEGFA, we designed rescue experiments. It was found that the suppression effect of mimics on VM could be reversed by adding VEGF165 (Fig. 6F). These results indicate that Circ4207 regulates the formation of VM in CRC cells through the miR-20b-5p/VEGFA axis.

**Knockdown of Circ4207 and up-regulation of miR-20b-5p inhibited tumor growth and vascular mimicry in vivo**

To elucidate the effects of Circ4207 and miR-20b-5p on tumor growth and VM formation in vivo. We constructed SW480 cells that knocked down Circ4207 and overexpressed miR-20b-5p (Angomir) and injected them into the groin of female nude mice (Fig. 7A). At 25 days, the mice were treated, sampled, photographed, weighed, and immobilized. Tumor growth was significantly inhibited in Si Circ4207 group and Angomir group (Fig. 7B), and the tumor weight was significantly lighter than that of normal control group (Fig. 7C). The results of immunohistochemical staining showed that the proliferation of Circ4207 and Angomir groups was inhibited (Fig. 7D). PAS+/CD31− is the most commonly used marker for VM. The staining results showed that the normal control group had more areas of vascular mimicry (black arrow - purple red) (Fig. 7E). Immunohistochemical results of VEGFA and its receptors also confirmed the previous experimental results. Knockdown of Circ4207 and overexpression of miR-20b-5p suppressed VEGFA and VEGFR1 production (Fig. 7F), Due to the low expression of VEGFR2 in tumor cells, many non-specific stains were generated during immunohistochemistry (Fig. S1G). Therefore, we conclude that Circ4207 knockdown and up-regulation of miR-20b-5p inhibit tumor growth and VM formation, and Circ4207 regulates VM formation via the miR-20b-5p/VEGFA axis.

Considering the many advantages of Circ4207 as a serum biomarker, we detected Circ4207 expression levels in sera of 23 colorectal cancer patients and 23 healthy controls, and found that Circ4207 expression was significantly elevated (Fig. 7G). ROC curve and statistical analysis were drawn based on serum Circ4207 levels, and AUC value was 0.8091, 95% confidence interval was 0.6779–0.9402, \( P = 0.0003 \) (Fig. 7H). These results indicate that Circ4207 is a sensitive serum diagnostic marker for CRC.

**Discussion**

The global mortality rate of colorectal cancer (CRC) has been significantly decreased by the use of early screening techniques like colonoscopy. However, new requirements and directions for research on mechanisms related to CRC have emerged due to the younger age of new cases and the growing number of patients diagnosed at advanced stages[1]. Anti-vascular therapy is an important part of
comprehensive treatment for advanced CRC. Current anti-angiogenic medications primarily target VEGFA and its receptor, which decrease tumor vascular density and induce hypoxic necrosis in tumors by preventing vascular endothelial cell proliferation or triggering death[27, 28]. Nevertheless, some research has demonstrated that hypoxia during anti-vascular medication may facilitate the formation of tumor VM, which results in drug resistance. Extensive evidence also shows that VM formation is associated with poor survival in tumor patients[29, 30]. Therefore, exploring the formation mechanism of tumor VM may provide new ideas for CRC diagnosis and treatment. Existing studies have shown that non-coding RNAs play a very important role in VM formation, but mainly focus on IncRNAs and miRNAs, and there are few studies on the influence of circRNA on the VM formation of CRC. In order to determine circRNA's potential as a diagnostic for CRC liquid biopsy, this research screened out high-expression circRNA in colorectal cancer and investigated its involvement in CRC tumor biology, including VM development.

We first obtained 573 differentially expressed circRNAs (fold change ≥ 2.0 and $P < 0.05$) through RNA-seq of CRC and adjacent tissues, of which 40 were up-regulated and 533 were down-regulated. We have chosen to investigate Circ4207, an entirely novel circRNA that is markedly up-regulated in colorectal cancer. Circ4207 is a 238 nt circular transcript spliced from exons 10 and 11 of the ATP9A (ATPase phospholipid transporting 9A, NM_006044.3) gene on chromosome 20. ATP9A is a phospholipid-flipping enzyme of class II P4-ATPase, which is involved in vesicular transport[31, 32] and neuronal development[33]. The splice site was confirmed by Sanger sequencing, and divergent primer was used to confirm the loop characteristics. In parallel, we demonstrated its strong stability by Act D and RNase R digestion studies. On the other hand, knocking down Circ4207 increased the percentage of apoptotic cells while inhibiting the proliferation, migration, and invasion of CRC cells, according to in vitro functional studies. Hypoxia and inflammation may stimulate the expression of Circ4207. We noted that Circ4207 affected the formation of VM, and after the down-regulation of Circ4207, the expressions of FN, VE-cadherin, and Vimentin decreased, while the expressions of E-cadherin increased. So, how does Circ4207 regulate VM formation in CRC?

It is generally believed that circRNA can act as an RNA sponge to regulate miRNA levels and indirectly regulate the expression of miRNA target genes after transcription[34–38]. We hypothesize that Circ4207 may play a regulatory function by sponging a certain miRNA. We used two human circRNA databases (CircBank and Circular RNA Interactome) to screen out three miRNAs (has-miR-520h, has-miR-1827 and has-miR-20b-5p). Further analysis revealed that only miR-20b-5p was notably downregulated in several colorectal cancer miRNA sequencing datasets. (GSE49246, GSE18392, and GSE125961), and was also down-regulated in five CRC cell lines. Research has demonstrated that miR-20b-5p is capable of regulating various biological functions of CRC cells, including stemness, migration, invasion, and proliferation[39–41]. In a clinical study of anti-vascular therapy, serum levels of miR-20b-5p in colorectal cancer patients were positively linked to survival[42], suggesting that miR-20b-5p may be related to CRC angiogenesis. Yuan et al also proposed that miR-20b-5p might associated with diabetic angiogenesis[43, 44]. To assess the impact of Circ4207 on miR-20b-5p. Firstly, we confirmed the negative correlation between Circ4207 and miR-20b-5p in colorectal cells, and the co-localization of Circ4207 and miR-20b-
5p in CRC cytoplasm was demonstrated by FISH experiment. The dual luciferase reporter experiment showed that Circ4207 could directly bind to miR-20b-5p. We verified the biogenic function of miR-20b-5p in colorectal cancer using CCK8, colony formation, transwell and wound healing assays, and the results were consistent with the conclusions of Tang et al[39–41]. Simultaneously, it was found that miR-20b-5p regulates the development of CRC VM, and that down-regulating miR-20b-5p can undo the decrease in VM formation brought on by down-regulating Circ4207. Thus, which pathway does miR-20b-5p regulate to prevent CRC VM from forming?

We used miRDB and miRTarbase to predict miR-20b-5p target genes and intersected with known VM-related genes to identify five candidate target genes (STAT3, HIF-1A, Smad4, Smad5, and VEGFA). MiR-20b-5p regulates only STA3 and VEGFA, and only VEGFA is highly expressed in all five CRC cell lines. Further FISH experiments confirmed the co-localization of miR-20b-5p and VEGFA in the cytoplasm of colorectal cancer cells, and the dual-luciferase experiments revealed that miR-20b-5p and VEGFA bind directly. Next, we confirmed that VEGFA is regulated by Circ4207 and miR-20b-5p: Down-regulation of Circ4207 in CRC cells can reduce the expression of VEGFA, VEGFR1 and VEGFR2, and overexpress of miR-20b-5p can obtain the same results, which is agreement with the findings of Feng et al.[45, 46] who believe that VEGFA can activate VEGFR1 and 2. Further studies showed that VEGFA recombinant protein (VEGF165) could reverse the inhibitory effect of miR-20b-5p on VM formation. The down-regulation of miR-20b-5p was found to effectively reverse the inhibitory impact of Circ4207 knockdown on VEGFA, thereby providing support for the regulatory role of Circ4207 in vasculogenic mimicry through the miR-20b-5p/VEGFA axis. These results have been confirmed in animal experiments. Lastly, we examined Circ4207 in the serum of 23 CRC patients who were not undergoing treatment, and we found that its expression was noticeably elevated compared to controls (AUC = 0.8090, \( P = 0.0003 \)).

**Conclusions**

In summary, we found that the novel circular RNA Circ4207 was significantly highly expressed in colorectal cancer tissues, cells and serum of patients. It may directly bind to miR-20b-5p, up-regulate VEGFA expression and activate VEGFR1 and other receptors, thus promoting the VM formation of colorectal cancer (Fig. 8). Our study suggests that Circ4207 is a potential liquid biopsy marker for CRC, providing new targets and ideas for the diagnosis and treatment of colorectal cancer.

**Abbreviations**

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<td>VM</td>
<td>Vasculogenic mimicry</td>
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<tr>
<td>CircRNA</td>
<td>Circular RNA</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<td>Act D</td>
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Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

This study using human serum samples was approved by the Ethics Committee of Chongqing Medical University (approved number: 2023-087) and conducted in accordance with the Declaration of Helsinki. Animal experiments were approved by Institutional Animal Care and Use of Chongqing Medical University (approved number: IACUC-CQMU-2024-0013) and were performed in accordance with the Basel Declaration.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. The CircRNA sequencing results can be found in Additional file 3 of the Supplementary Information.

Competing interests

The authors declare no competing interests.

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Authors’ contributions

MT and ZH conceived and designed the study. ZH, YW, TY and CX performed the experiments. ZH, YW, TY, ML, ZO and YC performed the data analyses. ZH, CX and ZO collected serum samples. ZH and MT wrote the manuscript. All authors have read and approved of the final manuscript.

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Not applicable.

References


Figures
Figure 1

Circ4207 is highly expressed in CRC, identification of the ring structure. A Volcanic map showed differentially expressed CircRNAs in colorectal cancer and adjacent tissues (LogFC ≥ 2.0, P ≤ 0.05). B Expression of Circ4207 in colorectal cancer and adjacent tissues. C Circ4207 was tested by qRT-PCR. D The reverse splicing site of Circ4207 was verified by Sanger sequencing. E The PCR product of Circ4207 was confirmed by agarose gel electrophoresis. F The closed ring structure was verified using Divergent...
and Convergent primers. G, H The abundance of Circ4207, GAPDH, ATP9A were detected when RNase R and Act D treated CRC cells at specific times. I The localization of Circ4207 was observed by FISH. J Changes of Circ4207 levels after adding TNFα and Cocl2.

Figure 2

Knockdown of Circ4207 promotes apoptosis of CRC cells, inhibits proliferation, migration, invasion and VM formation. A Verify the knockdown effect of Circ4207. B, C Using CCK8 and clone formation
experiments to measure cell proliferation after Circ4207 knockdown. D Apoptosis was determined by flow cytometry. E, F Transwell and wound healing experiments were used to determine migration and invasion. G The formation of VM was predicted by matrix glue tube formation in vitro.

**Figure 3**

Circ4207 sponged miR-20b-5p. A Prediction of Circ4207 targeted miRNAs. B, C, D GSE49246 for prediction of miRNA levels in CRC. E, F GSE18392 and GSE125961 datasets found that miR-20b-5p was
still lowly expressed in colorectal cancer. G miR-20b-5p was detected by qRT-PCR. H Knockdown of Circ4207 in SW480 and Caco2 cells increased the level of miR-20b-5p. I The co-localization of Circ4207 and miR-20b-5p in CRC cells was observed by FISH. J Schematic of dual luciferase reporter vectors for Circ4207-WT and Circ4207-MUT. K Circ4207-WT, Circ4207-MUT and miR mimics were transfected into SW480 and Caco2 together, and the relative fluorescence activity was detected 48 hours later.
Biological characteristics of miR-20b-5p in CRC. **A, B** Verify the effects of miR mimics and miR inhibitors. **C, D** CCK8 and clonal formation assay to detect proliferation. **E** In vitro matrix vasculogenic mimicry forming assay was performed to determine VM formation after addition of miR mimics and miR inhibitor. **F** Mir-inhibitor can reverse the inhibition of down-regulation Circ4207 on VM.

**Figure 5**
MiR-20b-5p directly targets VEGFA. A Prediction of miR-20b-5p target gene. B The level changes of 5 candidate target genes were detected after adding mimics. C The expression of STAT3 and VEGFA was determined through qRT-PCR. D, E The GSE146587 dataset verifies the abundance of VEGFA and STAT3 in CRC. F The co-localization of miR-20b-5p and VEGFA was observed by FISH experiment. G Schematic diagram of double luciferase reporter vector. H VEGFA-WT, VEGFA-MUT and miR-20b-5p mimics were simultaneously added to SW480 and Caco2 cells, and phase luciferase activity was detected 48 hours later.
Figure 6

Circ4207 regulates VM through the miR-20b-5p/VEGFA axis. **A, B** qRT-PCR was used to detect VEGFA, VEGFR1 and VEGFR2. **C, D** Changes in protein levels of VEGFA, VEGFR1 and VEGFR2. **E** Addition of miR-20b-5p inhibitor can reverse the inhibition on VEGFA of Circ4207 knockdown. **F** Rescue experiment to explore whether the addition of VEGF165 can restore the suppressive effect of the addition of mimics on VM formation.
Figure 7

Down-regulation of Circ4207 and up-regulation of miR-20b-5p suppressed tumor growth and VM. A Schematic diagram of establishing subcutaneous transplanted tumors in nude mice. B, C Tumor body photos of each group and weight of each group were taken. D Cell cycle was observed by immunohistochemical staining Ki67. E PAS/CD31 staining was used to observe the VM (purple red =PAS+/CD31). F Immunohistochemical staining of VEGFA and VEGFR1 were used to detect the expression of each group. G Circ4207 levels in the serum of 23 colorectal cancer patients and a control group were measured by qRT-PCR. H ROC curve based on Circ4207 expression in serum.

Figure 8

Schematic diagram of the mechanism. Circ4207 is highly expressed in CRC, and it directly binds to miR-20b-5p, then upregulates the expression of VEGFA and activates receptors such as VEGFR1, and finally
promotes the VM formation of colorectal cancer.

**Supplementary Files**

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