circRAE1 promotes colorectal cancer cell migration and invasion through modulating miR-338-3p/TYRO3 axis

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Abstract

Background: Growing evidences have revealed that long non-coding RNAs (IncRNAs) including circular RNAs (circRNAs) involve in numerous carcinogenesis. However, the roles of circRNAs in the cancer biology of colorectal cancer (CRC) remain vague.

Methods: qRT-PCR and western-blot were used to detecte the circRAE1 levels in CRC tissues and CRC cell lines. Cell proliferation, migration and invasion were detected using wound healing assays, and transwell assays. The interaction between circRAE1 and miR-338-3p and TRY03 was confirmed by dual-luciferase reporter assays.

Results: We uncovered that a novel circRNA Hsa_circ_0060967 (also known as circRAE1) was remarkably increased in CRC tissues, and high circRAE1 level was positively associated with advanced tumor stage, lymph node metastasis, and tumor size. Loss-of-function assay indicated that circRAE1 accelerated cell proliferation, migration and invasion. Besides, miR-338-3p, lowly expressed in CRC tissues and CRC cell lines. dual-luciferase reporter assays showed that circRAE1 could sponge miR-338-3p, which targeted TRY03 in CRC cells. Furthermore, overexpression of circRAE1 could recue the impaired migration and invasion triggered by miR-338-3p mimics or si-TYRO3 in CRC cells and vice versa.

Conclusion: We figured out the network of circRAE1, miR-338-3p, and TYRO3 in CRC cells and revealed that increased circRAE1 served as an oncogene through sponging miR-338-3p, resulting in upregulated TYRO3 expression, which suggested that circRAE1 would be a potential therapeutic target and diagnostic marker for CRC treatment.

Background

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide, leading to more than 600,000 deaths each year [1,2]. More importantly, the incidence of CRC in people under 50 years of age is increasing every year according to statistics[1]. Nearly 25% patients have metastatic diseases, which are considered to be the biggest challenge in the treatment for CRC, resulting in about 90 % of deaths [3]. With the advances in diagnosis and treatment for CRCs, patients with stage I or II disease, 5-year relative survival rates are 91% and 82%, respectively [4]. However, 5-year
survival rate decreased to 12% for patients suffering distant metastasis [4]. Therefore, to improve the treatment efficiency for CRC patients, further researches to figure out the molecular mechanisms of CRC tumorigenesis and identify novel targets are clinically important.

Circular RNAs (circRNAs) are noncoding RNAs, characterized for its stable circularized shape, pervasively expressed in various cell types [5,6]. CircRNAs are resistant to exonuclease-mediated degradation, and usually serve as competing endogenous RNAs (ceRNAs) to reduce downstream microRNA levels and function[7,8]. Recently, growing evidences have shown that circRNAs involve in regulating tumorigenesis. Su et al. uncovered that hsa_circ_0070269 could suppress hepatocellular carcinoma (HCC) cell proliferation and invasion and inhibited HCC tumor growth in vivo by sponging miR-182 to promote NPTX1 translation in HCC cells [9]. Li et al. revealed that circ-ITCH, decreased in esophageal squamous cell carcinoma, plays an antitumor role and acts as a ceRNA by sponging miR-17, miR-214, and miR-7 to increase ITCH mRNA [10]. Yu et al. revealed that CDR1 could promote HCC through down regulating miR-7 expression [11]. Moreover, emerging studies have also revealed that circRNAs invovle in the tumorigenesis, and metastasis of CRC and identified as latent therapeutic targets for CRC treatment [12]. However, the detailed roles of circRNAs in CRC tumorigenesis and metastasis are just beginning to be revealed.

Herein, we identifed a novel circRNA (hsa_circ_0060967, we renamed it as circRAE1) which was remarkably increased in CRC tissues, and high circRAE1 level was positively associated with advanced tumor stage, tumor size, and lymph node metastasis. Additionally, we confirmed that circRAE1 played an oncogenic role in the progression of CRC by serving as a ceRNA for miR-338-3p to increase TYRO3 expression. These findings suggested circRAE1 as a novel potential therapeutic target to intervenes CRC treatment.

Materials And Methods

CRC patients and clinical tumor tissues

Eighty pairs of CRC tissues and the adjacent non-tumor tissues were collected in the Second Affiliated Hospital of Fujian Medical University during Jan. 2019 to Dec. 2019. The collection was approved by the Ethical Committee of the Second Affiliated Hospital of Fujian Medical University and all patients
were informed of details before admission. No additional treatment was conducted in the collected patients before surgery. The tissues were snap-frozen in liquid nitrogen and stored at -80°C.

Cell culture and transfection

Human colorectal cancer cells HCT116, SW620, HT29, and SW480, normal colonic epithelial cell line NM460 and HEK293T were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in DMEM-High glucose (Gibco) containing 10% fetal bovine serum (PAN biotech) and 100 u/mL penicillin/streptomycin at 37 °C under 5% CO₂ in a humidified chamber.

The miR-338-3p mimics, miR-negative control (miR-NC), siRNAs for circRAE1(si-circRAE1#1,2 and 3) and TYRO3 (si-TYRO3), and siRNA-negative control (si-NC) were from Shanghai Gene-Pharma. circRAE1-overexpression plasmid was constructed using the Pcd-ciR vector. All the recombinant plasmids were sequenced confirmed by Fuzhou Biosune. circRAE1-overexpression plasmid was then co-transfected with RRE, REV, and VSV/G into HEK293T cells to produce lentivirus-circRAE1 (lv-circRAE1). si-circRAE1 (100 nM), si-TYRO3 (100 nM) and miR-338 mimics (50 nM) were transfected through Lipofectamine 2000 reagent (Life) according to product specification. Lentivirus-circRAE1 infected CRC cells with 10 μg/mL polybrene. Cell, RNA and protein samples were collected 24 h or 48 h after transfection.

Quantitative RT-PCR assays (qRT-PCR)

Total RNA was isolated with the TRIzol reagent as per the product specification. cDNA was synthesized using reverse transcription kit (Sangon, China) for circRNA and mRNA analysis, while RiboBio microRNA reverse transcription kit (Guangzhou, China) was used for miRNA. Quantitative PCR was conducted using GoTaq® qPCR Master Mix (Promega, USA). Relative expression levels of circRNAs, mRNAs and miRNAs were calculated as the method of 2^{-ΔΔCt}, and normalized to GAPDH for circRNAs and mRNAs or U6 for miRNAs. Each experiment was in triplicate. Primer sequences are showed in Table 1.

Western blot assay

Western blot assays were conducted as previously described [13]. Briefly, equal amount of proteins
were separated on 10-12% SDS-PAGE, transferred onto PVDF membranes (0.45 μM), blocked with 5% skim milk. The membranes were then incubated with various primary antibodies at 4 °C for more than 10 h. Afterwards, the membranes were blotted with HRP-conjugated secondary antibodies. The signal was visualized with ECL (Beyotime Biotechnology, Jiangsu, China) using the LiCor C-DiGit Blot scanner with LiCor Biosciences Image Studio Software. Anti-E Cadherin antibody [HECD-1], Anti-Vimentin antibody [RV202], and Anti-TYRO3 antibody [EPR4308] were from Abcam.

Stability analysis of circRAE1

SW620 and HT29 were treated by Actinomycin D (2 μg/mL) and then total RNA was extracted after 0, 4, 8, 12 and 24 h. Levels of circRAE1 and its linear subtype were detected by qRT-PCR. As for RNase digestion assay, total RNA was co-incubated with 3 units of RNase R per 1μg RNA for 30 min at 37 °C. Then re-purified RNA was subjected to qRT-PCR for circRAE1 and its linear subtype.

Fluorescence in situ hybridization

In situ hybridization was employed to explore the intracellular location of circRAE1 and miR-338-3p in HT29 and SW620. Cells were grown on cover slips and fixed with 4% paraformaldehyde. The procedure was carried out as previously reported [14]. Briefly, the slides were treated with CSK Buffer (0.5% TritonX-100, 10 mM VRC) for 10-12 min and then treated with 70% alcohol for 10 min at 4 °C, followed by incubating with series alcohol to dehydration. After air drying, the slides were prehybridized for 1 h at 55 °C in prehybridization solution. Cy3-circRAE1 and/or Digoxin-labeled miR-338-3p (DIG-miR338-3p) probe in hybridization buffer were denatured at 76°C for 10 min, and then added to each slide and hybridized overnight at 37 °C in dark humidified chamber. After washing, the anti-DIG [21H8]-FITC (Abcam, USA) was added to the slides and the slides were incubated at 37 °C for 1 h in the humidified chamber. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) after washing.

Luciferase reporter assay

PCR method was used to amplificate the wild-type (WT) and mutant (MUT) 3’-UTR of human TYRO3, and the WT and MUT of circRAE1 (shown as follow), which were then cloned into the psiCHECKTM-2-luciferase reporter plasmid. HEK293T cells were co-transfected with WT or MUT psiCHECKTM-2-
circRAE1 plasmids or psiCHECKTM-2-TYRO3 (3′-UTR), and miR-338-3p mimics or miR-NC using Lipofectamine 2000 reagent. 48 h later, cells were collected and subjected to the commercial Dual-Luciferase reporter assay system (Promega) following the manufacturer's instructions for measuring both firefly and Renilla luciferase activity.

Wound-healing assay

A wound migration model for in vitro assay was used as previously described [15]. Culture-Inserts (Ibidi) were used in the wound-healing assay. Briefly, HT29 and SW620 cells was seeded to each well of the Culture-Inserts. Cells were incubated at 37°C for 24 h, a cell-free gap of 500 μm was created after removal of the Culture-Insert. An inverted phase-contrast microscope was employed to capture images at 0, 24 and 48 h. Five randomly chosen fields were used to calculate the percent of wound closure using the ImageJ software.

Transwell migration assay

Transwell chambers (Corning, USA) was used in the transwell migration assay as previously described[9]. SW620 and HT29 cells were seeded into the upper chambers with serum-free DMEM, and the lower wells were filled with DMEM containing 20% FBS. 24 h later, cells in the top chamber were removed with cotton swabs and those on the lower surface were fixed using 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet for 15 min. After washing twice with PBS, cell numbers in five randomly chosen fields were calculated under the microscope (Olympus, Japan).

Transwell invasion assay

Transwell invasion assay was detected as previously described [16]. Briefly, the upper chambers (Corning, USA) were coated with diluted Matrigel (BD, USA). Afterwards, SW620 and HT29 cells were seeded into the upper chambers with serum-free DMEM, and then the transwell chambers were incubated in wells filled with DMEM containing 10% FBS. Following 24 h incubation at 37 °C, cells inner the chambers and the remaining Matrigel were removed with cotton swabs and the cells on the outside surface of the lower chambers were fixed using 4% PFA, stained with 0.1% crystal violet for 15 min. After washing twice with PBS, cell numbers in five randomly chosen fields were calculated under the microscope.
Statistical analysis

SPSS 22.0 statistical software was employed for the statistical analysis. Data was shown as mean ± the standard deviation (SD). The significant differences between groups were estimated by One-Way ANOVA. P < 0.05 was considered as statistically significant.

Results
circRAE1 was up-regulated in CRC tissues and CRC cell lines

The previous study (CapitalBio microarray data) has showed that numerous circRNAs were differentially expressed in CRC tissues compared with their normal adjacent tissues [17], among of which hsa_circ_0060967 (the corresponding Gene Symbol is RAE1, so we named it as circRAE1) was significantly up-regulated. To validate the expression properties of circRAE1 in CRC, circRAE1 expression in 80 paired CRC tissues was detected using qRT-PCR, showing that circRAE1 expression was observably upregulated in CRC tissues (Fig. 1A). Moreover, through analyzing the clinical data and the expression levels of circRAE1, we concluded that high circRAE1 expression was positively related to advanced TNM stage, large tumor size, and lymph node metastasis (Table 2).

We then explored circRAE1 expression in CRC cells, which showed that circRAE1 expression levels were significantly higher in CRC cell lines (HCT116, SW620, HT29 and SW480) than normal colonic epithelial cell line NM460 (Fig. 1B), especially for SW620 and HT29. Next we explored the stability of circRAE1 by adding Actinomycin D which could block new transcription and detecting the levels of circRAE1 and its linear control by qRT-PCR. These data indicated that circRAE1 was more stable than linear mRNA (Fig. 1C). Moreover, we observed that circRAE1 was resistant to RNase R, whereas its linear subtype was not (Fig. 1D). In addition, fluorescence in situ hybridization results showed that circRAE1 was dominate localized in the cytoplasm (Fig. 1E).

circRAE1 increased CRC cells migration and invasion ability

Herein, we intended to select SW620 and HT29 for the following functional assays, considering the high expression level of circRAE1 in the two cell lines (Fig. 1B). We transfected cells with various siRNAs targeting circRAE1, showing that all the three siRNAs could effectively knockdown circRAE1 expression with si-circRAE1#1 having the best interference efficient (Fig. 2A). Thus we chose si-
circRAE1#1 for the following circRAE1 knockdown assays and named it as si-CircRAE1. Scratch tests showed that downregulation of circRAE1 significantly reduced SW620 and HT29 cells wound healing efficiency (Fig. 2B). Subsequently, transwell assays without Martrigel also showed that downregulation of circRAE1 significantly inhibited SW620 and HT29 cells migration and invasion activity in vitro (Fig. 2C). Furthermore, we explored whether circRAE1 would involve in epithelial-mesenchymal transition (EMT) process by detecting the expression of EMT marker genes (E-cadherin and Vimentin) following silencing of circRAE1 expression (Fig. 2D). Both the mRNA and protein levels of E-cadherin was remarkably up-regulated by circRAE1 knocking down, whereas Vimentin was just the reverse (Fig. 2E). These results revealed that circRAE1 might serve as an oncogene in CRC progression by regulating EMT process.

circRAE1 targeted miR-338-3p

Competing endogenous RNA (ceRNA) is a newly proposed mechanism that indicates a crosstalk among IncRNAs including circRNA, mRNAs and their shared miRNAs. Using online tool (CircInteractome), we speculated the potential miRNAs that might be bound by CircRAE1. Among of them was miR-338-3p which was dramatically reduced in CRC tissues (Fig. 3A) and showed a negative relation with CircRAE1 in expression (Fig. 3B). In addition, using si-CircRAE1 we determined that downregulation of CircRAE1 could observably increase miR-338-3p expression in SW620 and HT29 cells (Fig. 3C). Furthermore, fluorescence in situ hybridization results indicated that CircRAE1 and miR-338-3p was colocalized in the cytoplasm (Fig. 3D). To further confirm the direct binding of miR-338-3p to circRAE1, we constructed circRAE1 WT and circRAE1 MUT luciferase reporter plasmids, and the luciferase reporter assay revealed that co-transfection with miR-338-3p mimics repressed the luciferase activity of circRAE1 WT, while circRAE1 MUT was not affected (Fig. 3E), indicating that circRAE1 could directly sponge miR-338-3p.

circRAE1 functions through targeting miR-338-3p

To explore whether circRAE1 functioned through binding miR-338-3p, firstly we determined whether circRAE1 overexpression would alter miR-338-3p expression or miR-338-3p mimic might affect circRAE1 expression. The results showed that miR-338-3p mimic had no effect on circRAE1 expression
while circRAE1 overexpression observably reduced miR-338-3p (Fig. 4A and 4B), indicating that circRAE1 do control miR-338-3p expression. Additionally, we employed would healing assay, transwell migration and invasion experiments to figure out if miR-338-3p influenced CRC cell migration and invasion through miR-338-3p. Shown in Fig. 4C, miR-338-3p mimic suppressed the wound closure in SW620 and HT29 cells and circRAE1 overexpression partially reversed the effects caused by miR-338-3p mimics on both cells. Meanwhile, the migration and invasion capacity of SW620 and HT29 cells determined by transwell assays was also depressed by miR-338-3p mimic and rescued by circRAE1 overexpression (Fig. 4D).

TYRO3 was directly targeted by miR-338-3p
Bioinformatics analysis using Targetscans (http://www.targetscan.org/) indicated that TYRO3 was a putative target of miR-338-3p. Binding sites between them was displayed in Fig. 5A. Then, luciferase reporter plasmids of TYRO3-WT and TYRO3-MUT were constructed in our study (Fig. 5A). Luciferase reporter assay showed that adding miR-338-3p mimic could dramatically inhibit the reporter activity of TYRO3-WT rather than TYRO3-MUT (Fig. 5A), confirming that miR-338-3p could directly bind to the 3'-UTR of TYRO3. Moreover, the expression level of TYRO3 was also observably upregulated in CRC tissues (Fig. 5B) and showed a negative relation with CircRAE1 in expression (Fig. 5C).

circRAE1 functions through regulating TYRO3 expression
Thereafter, we disclosed that TYRO3 protein level was upregulated by LV-CircRAE1 and significantly reduced by si-TYRO3 (Fig. 6A). Functionally, we revealed that si-TYRO3 suppressed the wound closure in SW620 and HT29 cells and circRAE1 overexpression partially blocked the effects of si-TYRO3 on both cells (Fig. 6B). Furthermore, the transwell migration and invasion experiments uncovered that si-TYRO3 remarkably reduced the migration and invasion capacity of SW620 and HT29 cells and circRAE1 overexpression could rescue this effect (Fig. 6C). Thess results suggested that circRAE1 could alter TYRO3 expression to regulate CRC cells migration and invasion.

Discussion
In this paper, we validated that circRAE1 was upregulated in CRC tissues and positively associated with higher TNM stage, larger tumor size, and more lymph node metastasis in CRC patients. In
addition, loss-of-function assays indicated that circRAE1 silence obviously reduced CRC cells wound
closure efficiency, migration and invasion in vitro. Moreover, gain-of-function assays revealed that
circRAE1 overexpression promoted CRC cell migration and invasion via acting as a ceRNA for miR-
388-3p to regulate TYRO3 levels. These data indicated that circRAE1 serve as an oncogene in CRC
tumorigenesis.

In accordance with the remarkable advance in microarray and RNA sequencing technology, growing
numbers of circRNAs have been revealed to play vital roles in numerous disease processes, especially
in cancers including CRC [18]. For instance, circ_0026344 could suppress CRC cells growth and
invasion in vitro and reduce CRC growth in vivo by sponging miR-21 and miR-31[19].

Hsa_circ_0000069, dramatically upregulated in CRC tissues, could promote proliferation, migration,
and invasion of CRC cells [20]. Circular RNA hsa_circ_000984 was also found to promote colon cancer
growth and metastasis by binging miR-106b to increase CDK6 expression [21]. In this context, by
analyzing the circular RNA chips of four paired CRC tissues and their adjacent non-tumor control from
Chen et al [17]., we discovered a higher expression of circRAE1 in CRC tissue samples and qRT-PCR
analysis confirmed a similar pattern in CRC cell lines. High circRAE1 expression was concluded to
indicate bad prognosis of patients with CRC. Through the wound healing tests and transwell assays,
we recognized circRAE1 as an oncogene to promote CRC cell migration and invasion.

Recently, more researches have revealed that circRNAs preferred to function as ceRNAs to sponge
various miRNAs, thus leading to the increase of miRNA targets expression[22]. Hence, we conducted
bioinformatics analyses online (CircInteractome) and uncovered that miR-338-3p could be bound by
CircRAE1. Previous studies revealed that miR-338 could suppress tumor progression in various human
cancers. For example, Zhang et al. uncovered that miR-338 inhibited bladder cancer cell proliferation,
and invasion by reducing ETS1 [23]. He et al. confirmed that miR-338 inhibited cell proliferation and
EMT process of non-small cell lung cancer (NSCLC) cells through directly down-regulating NFATc1
expression [24]. By targeting MACC1, miR-338-3p could inhibit sw480 proliferation and migration, and
induce apoptosis [25]. Sun et al. illustrated that miR-338-3p was remarkably down-regulated in CRC
compared to those adjacent non-tumor tissues [26], which was in accordance with our current results.
In the present study, our data indicated that miR-338-3p presented inverse impacts on wound healing efficiency, migration and invasion in contrast to circRAE1 in CRC cells. Overexpression of circRAE1 could reverse the effect on the CRC cells induced by miR-338-3p mimics and vice versa. To further figure out the mechanism of circRNA-miRNA-mRNA network, we identified TYRO3 as one direct target of miR-338-3p in CRC cells. TYRO3 is a protein tyrosine kinase and previous literatures have revealed that TYRO3 is significantly increased in various cancers, promoting cancer cell proliferation and metastasis and enhancing the drug resistance, making it a potential therapeutic target [27,28]. In addition, TYRO3 was also confirmed to play vital roles in the regulation of EMT process, chemical resistance, liver metastasis, cell proliferation and apoptosis in colorectal cancer as an oncogene [29]. Herein, we revealed that knocking down TYRO3 expression could rescue the increased cell wound healing efficiency, migration and invasion induced by overexpression of circRAE1 and vice reverse, which were also mediated by miR-338 mimics. Of course, more in vivo assays are needed to further confirm the effectiveness in clinical.

Conclusion
We figured out the network consist of circRAE1, miR-338-3p, and NPTX1 in CRC cells and revealed that increased circRAE1 served as an oncogene in CRC cells through functioning as a ceRNA to sponge miR-338-3p, resulting in upregulated TYRO3 expression. These results provide a novel potential therapeutic strategy targeting circRAE1/miR-388-3p/TYRO3 axis.

Abbreviations
LncRNAs: long non-coding RNAs; CircRNAs: circular RNAs; CRC: colorectal cancer; EMT: epithelial-mesenchymal transition; qRT-PCR: Quantitative RT-PCR assays; WT: the wild-type; MUT:mutant; PFA: paraformaldehyde; ceRNA: Competing endogenous RNA;

Declarations
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Authors’ Contributions
Conception and design: Jiabin Du, Kai Ye and Jianhua Xu
Development of Methodology: Jiabin Du, Junxing Chen, Weinan Liu, Pengcheng Wang
Acquisition of data: Jiabin Du, Jianhua Xu, Junxing Chen, Weinan Liu, Pengcheng

Analysis and interpretation of data: Jiabin Du, Jianhua Xu,

Writing, review, and/or revision of the manuscript: Jiabin Du and Jianhua Xu wrote the initial draft and all authors reviewed the manuscript

Study Supervision: Kai Ye and Jiabin Du

**Availability of data and materials**

Not applicable.

**Ethics approval and consent to participate**

We were used for patients tissues experiments with the ethical approval of the Second Affiliated Hospital of Fujian Medical University.

**Consent for publication**

Authors involved in this paper all signed written consent for publishing in your journal.

**Competing interests**

The authors declare that they have no competing interests.

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Tables
Table 1. Sequences used for qRT-PCR and siRNA assays.
| Names                        | Sequences (5'—3') |
|------------------------------|------------------|
| **cirClRAE1**                |                  |
| Forward                      | AGAGAGGGGCCCTGAT |
| Reverse                      | AGTGTGCATCTGCTG |
| **E-cadherin**               |                  |
| Forward                      | GCTGGACCGAGAGA  |
| Reverse                      | CAAAATCCAAGCCCC|
| **Vimentin**                 |                  |
| Forward                      | GGACCAGCTAAACCA |
| Reverse                      | AAGGTCAGACGTG  |
| **Linear RAE1**              |                  |
| Forward                      | AGGCAGTACTTCTCC |
| Reverse                      | GGCAAGGTTGGTG  |
| **TYRO3**                    |                  |
| FORWARD                      | AGTTGGCTGTGGACC |
| REVERSE                      | AGGATGTGCGGCTGT |
| **GAPDH**                    |                  |
| Forward                      | ATGGGGAAGGTGAA  |
| Reverse                      | TTACTCCTTGGAGGC |
| **miR-338-3p**               |                  |
| Forward                      | CGCGTCCAGCATCA |
| Reverse                      | AGTGCAGGGTCCGA |
| RT Primer                    | GTCGTATCCAGTGCGGGTCCGAGACGCAACAA | |
| **U6**                       |                  |
| Forward                      | CTCGCTTCGGCAGCAC |
| Reverse                      | ACGCTTCAGAATTTTC |
| RT Primer                    | AAAATATGGAAAGCTTCC |
| si-circRAE1-Homo1            |                  |
| Sense                        | GGAUCAAAGCUUCA |
| Antisense                    | UAGUUUGGACUUUC |
| si-circRAE1-Homo2            |                  |
| Sense                        | GCAGUGCAACUACAC |
| Antisense                    | UUGUCUGUAGUUGC |
| si-circRAE1-Homo3            |                  |
| Sense                        | GGACCUCAGCAGUA |
| Antisense                    | UUGGUUACUUGCUGA |
| si-TYRO3                     |                  |
| Sense                        | GGUGGAGAGGAACU |
| Antisense                    | UUCGUAGUUCUCUC |
| Negative control             |                  |
| Sense                        | AUGGAAUUUGUUGL |
| Antisense                    | UUCACAACUUAAUC |
Table 2. The relationship analysis between relative expression levels of circRAE1 in CRC cancerous tissues and clinical characteristics.

| Clinical Characteristics | Total (n=80) | CircRAE1 expression | Counts | Low | High | P value |
|--------------------------|-------------|----------------------|--------|-----|------|---------|
| Gender                   |             |                      | Male   | 46  | 17   | 29      | 0.1143  |
|                          |             |                      | Female | 34  | 7    | 27      |         |
| Age                      |             |                      | ≤65    | 49  | 13   | 36      | 0.3946  |
|                          |             |                      | 65     | 31  | 11   | 20      |         |
| Tumor size               |             |                      | <5cm   | 50  | 19   | 31      | 0.0438  |
|                          |             |                      | ≥5cm   | 30  | 5    | 25      |         |
| Tumor site               |             |                      | Colon  | 25  | 9    | 16      | 0.4298  |
|                          |             |                      | Rectum | 55  | 15   | 40      |         |
| Depth of invasion        |             |                      | T1-T2  | 10  | 5    | 5       | 0.1401  |
|                          |             |                      | T3-T4  | 70  | 19   | 51      |         |
| Lymph node metastasis    |             |                      | N0     | 37  | 20   | 17      | 0.001   |
|                          |             |                      | N1-N2  | 43  | 4    | 39      |         |
| Distant metastasis       |             |                      | M0     | 78  | 24   | 54      | 0.3484  |
|                          |             |                      | M1     | 2   | 0    | 2       |         |
| TNM stage                |             |                      | I-II   | 37  | 20   | 17      | 0.001   |
|                          |             |                      | III-IV | 43  | 4    | 39      |         |

Figures
CircRAE1 was up-regulated in CRC. (A) The expression of CircRAE1 in paired CRC tissues from 80 CRC patients was measured by qRT-PCR. (B) Relative expression levels of CircRAE1 in normal cell line NM460 and CRC cell lines (HCT116, SW620, HT29, and SW480) by qRT-PCR. (C) Relative expression level of CircRAE1 and its linear types in SW620 and HT29 cells at various time points after treated by Actinomycin D. (D) Total RNA from SW620 or HT29 cells was treated with RNase R. CircRAE1 was stably expressed in both RNase R(-) and RNase R(+) total RNA samples. (E) RNA in situ hybridization for CircRAE1 (red) in SW620 and HT29 cells (×200). Nuclei were stained with DAPI (blue). *P < 0.05, **P < 0.01, ***P < 0.001.

Function of CircRAE1 in CRC cells. (A) After transfection with various siRNAs and negative control for 48 h in SW620 and HT29 cells, expression of CircRAE1 was detected by qRT-PCR. (B) Downregulation of CircRAE1 suppressed SW620 and HT29 cell migration ability as determined by wound healing tests. (C) Downregulation of CircRAE1 suppressed SW620 and HT29 cell migration and invasion ability as determined by transwell assays. (D) The expression of E-cadherin and Vimentin in SW620 and HT29 was detected by qRT-PCR after transfected with si-circRAE1 or si-NC. (E) The protein level of E-cadherin and Vimentin in SW620 and HT29 was detected by western-blot assay. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3

CircRAE1 serves as a competing endogenous RNA (ceRNA) for miR-338-3p in CRC cells. (A) The expression of miR-338-3p in paired CRC tissues from 80 CRC patients was measured by qRT-PCR. (B) CircRAE1 expression was negatively correlated with miR-338-3p expression in CRC tissues. (C) Downregulation of CircRAE1 promoted miR-338-3p expression in SW620 and HT29 cells as determined by qRT-PCR. (D) RNA in situ hybridization for miR-338-3p (green) and CircRAE1 (red) in SW620 cells (×200). Nuclei were stained with DAPI (blue). (E) miR-338-3p mimics significantly reduced the luciferase activity of CircRAE1 WT. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4

CircRAE1 serves as an oncogene by sponging miR-338-3p in CRC cells. (A) miR-338-3p mimics did not significantly alter the relative expression of miR-338-3p in SW620 and HT29 cells as determined by qRT-PCR. (B) Overexpression CircRAE1 with LV-CircRAE1 significantly reduced miR-338-3p expression in SW620 and HT29 cells as determined by qRT-PCR. (C) Overexpression CircRAE1 dramatically rescued the impaired wound healing ability of SW620 and HT29 cells induced by miR-338-3p mimics and vice versa. (D) Overexpression CircRAE1 dramatically rescued the impaired migration and invasion ability of SW620 and HT29 cells induced by miR-338-3p mimics and vice versa detected by transwell assays. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5

TYRO3 was a direct target gene of miR-338-3p. TYRO3 axis in CRC. (A) The binding site between miR-338-3p and the 3′-UTR of TYRO3. (B) miR-338-3p mimics significantly reduced the luciferase activity of TYRO3 WT. (C) The expression of TYRO3 in paired CRC tissues from 80 CRC patients was measured by qRT-PCR. **P < 0.01.
CircRAE1 functions through regulating miR-338-3p/TYRO3 axis in CRC. (A) miR-338-3p mimics rescued the effects of CircRAE1 on TYRO3 protein expression levels. (B) Si-TYRO3 reversed the effects of CircRAE1 upregulation on migration activities of SW620 and HT29 cells as determined by wound healing tests. (C) Si-TYRO3 reversed the effects of CircRAE1 upregulation on migration and invasion activities of SW620 and HT29 cells as determined by transwell assays. *P < 0.05, **P < 0.01, ***P < 0.001.