**Abstract**

Noncoding RNAs (ncRNAs) are the dominant product of eukaryotic transcription. These products range from short microRNAs (miRNAs) to long intergenic noncoding RNAs (lincRNAs). Circular RNAs composed of exonic sequences represent an understudied form of ncRNA that was discovered more than 20 years ago. Using a TaqMan-based reverse transcriptase polymerase chain reaction assay, we analyzed the relationship between cir-ITCH expression and colorectal cancer (CRC) in a total of 45 CRCs and paired adjacent non-tumor tissue samples. We found that cir-ITCH expression was typically down-regulated in CRC compared to the peritumoral tissue. This result, as well as several follow-up experiments, showed that cir-ITCH could increase the level of ITCH, which is involved in the inhibition of the Wnt/β-catenin pathway. Therefore, our results showed that cir-ITCH plays a role in CRC by regulating the Wnt/β-catenin pathway.

**Introduction**

Colorectal cancer (CRC) is a malignant neoplasm that is situated in the colon or rectum [1, 2]. CRC remains a major cause of cancer mortality in the developed world, largely due to its propensity to metastasize [3]. CRC poses a major public health problem, as it is the third most commonly diagnosed cancer in males and the second most common in females. There have been many comparative studies demonstrating epigenetic changes closely associated with the occurrence and development of CRC. Although the CRC research achievements are remarkable, the etiological factors and pathogenesis mechanisms underlying CRC development appear to be complex and heterogeneous.

Recently, circular RNA, a type of noncoding RNA that typically does not act by encoding a protein, was found to be closely related to tumor development [4]. Circular RNA is usually composed of more than one exon and is usually enriched with functional microRNA (miRNA) binding sites; for example, CDRI contains several conserved binding sites for miRNA-7 (miR-
Recently, one study reported that cir-ITCH is a circular RNA that spans several exons of Ubiquitin (Ub) protein ligase (E3) (ITCH) [5–7]. The article indicated that cir-ITCH harbors many miRNA binding sites that can bind to the 3'-UTR of ITCH, including those for miR-7, miR-17, miR-214, miR-128 and miR-216b [5, 8]. The study of miRNAs is more mature than that of circular RNA; miRNAs are 21-nucleotide-long non-coding RNAs that have important roles in numerous biological processes in both plants and animals [9]. Mature miRNAs play important regulatory roles in cell growth, proliferation, differentiation, and cell death.

cir-ITCH plays an important role in the development and progression of ESCC [7]. ITCH’s targets, including p63, p73, and Notch1, are usually associated with tumor formation and chemosensitivity, demonstrating the connection of ITCH to cancer biology [10]. Previous research discussed circular RNA anti-cancer activities in malignant melanoma cell lines [11]. However, there are no reported studies on the functional roles of circular RNA in CRC.

In this study, we hypothesized that cir-ITCH plays a similar role in CRC. To test this hypothesis, we developed a method to delineate the transcriptional differences in cir-ITCH between CRC and paired adjacent non-neoplastic tissues.

Materials and Methods

Study subjects

All subjects in this study were homogenous Han Chinese. Forty-five CRC and corresponding paracancerous tissue samples were obtained from patients at The First Affiliated Hospital of Wenzhou Medical College (Wenzhou). There were no restrictions on the age, stage of CRC, sex or histology. At recruitment, each subject gave written informed consent by scheduling an interview at which they received a structured questionnaire. This study was approved by the institutional review board of Wenzhou Medical College. The clinical characteristics of all the patients are listed in Table 1.

Cell culture

The human CRC cell lines HCT116 and SW480 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology and were passaged for less than 6 months.

Construction of the circular RNA plasmid

We constructed the circular RNA plasmid used in this study. The construct method was previously published [8]. The resulting construct (pcDNA3.1-cir-ITCH) was verified by direct sequencing.

RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was isolated from cells and tissues using the TRIzol reagent according to the manufacturer’s instructions. The relative gene expression of cir-ITCH was determined using qPCR, which is based on the TaqMan method. GAPDH was used as an internal standard control, and all reactions were performed in triplicate [12, 13]. The primers used for qPCR amplification is the forward GCAGAGGCCAACACTGGAA, the reverse TCCTTGAAGCTGACTACGCTGAG, the probe CCGTCCGGAACTATGAACAACAATGGCA.
RNase R digestion

The RNase R digestion reaction was performed following previously published procedures. The digestion and precipitation reactions were repeated twice with a ratio of 3 U of enzyme/mg of RNA [14].

Transient transfections and luciferase assays

HCT116 and SW480 cells were transfected with the reporter plasmids described above using Lipofectamine 2000. Cells were co-transfected with the miRNAs according to the manufacturer’s instructions [15]. Each group included 6 replicates, and triplicate independent experiments were performed.

Actinomycin D assay

HCT116 and SW480 cells were transiently transfected using Lipofectamine 2000 and were co-transfected with mRNAs as indicated for 24 h. The cells were then exposed to actinomycin D. The cells were harvested, and the stability of the cir-ITCH mRNA was analyzed using

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Table 1. Baseline demographic and clinical characteristics of study populations.

| Characteristics          | population |
|--------------------------|------------|
|                          | N (%)      |
| **Age(years)**           |            |
| ≤40                      | 4 (8.9)    |
| 40–60                    | 16 (35.5)  |
| ≥60                      | 25 (55.6)  |
| **Sex**                  |            |
| Male                     | 22 (48.9)  |
| Female                   | 23 (51.1)  |
| **Tumor invasion**       |            |
| T1                       | 5 (11.1)   |
| T2                       | 9 (20)     |
| T3                       | 31 (68.9)  |
| **Family history**       |            |
| Yes                      | 6 (13.3)   |
| No                       | 39 (86.7)  |
| **Smoking**              |            |
| Never                    | 30 (66.7)  |
| Ever                     | 15 (33.3)  |
| **Drinking**             |            |
| Never                    | 21 (46.7)  |
| Ever                     | 24 (53.3)  |
| **Pathological type**    |            |
| Highly                   | 23 (51.1)  |
| Moderately               | 12 (26.7)  |
| Low                      | 10 (22.2)  |
| **Stage**                |            |
| I                        | 9 (20)     |
| II                       | 16 (35.6)  |
| III                      | 20 (44.4)  |

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quantitative reverse transcription PCR (qRT-PCR). The assay was performed according to the previous article.

**Western blot**

Western blot analysis to assess Wnt3a, β-catenin and β-action expression was carried out as described previously[16].

**Cell viability assay**

The cell viability was measured using the Cell Counting Kit-8 (CCK-8) system according to the manufacturer’s instructions. There were 6 replicates for each group, and the experiments were repeated at least 3 times.

**Statistical analyses**

The correlation between the expression of cir-ITCH and the ITCH gene in CRC tissues was determined using one-way analysis of variance and linear regression models. Differences between the groups were assessed by a paired, 2-tailed Student’s t-test. A P-value of <0.05 was considered statistically significant.

**Results**

**Identification of the circular RNA**

Two sets of ITCH primers were designed for this study: a divergent set to amplify only the circular form and a convergent set to amplify the linear forms. We confirmed that the circular form was amplified using the divergent primers. cDNA and genomic DNA were used as the controls. As expected, no amplification was observed using the divergent primers on genomic DNA. GAPDH was used as the linear control (Fig 1A).

**Expression of cir-ITCH in CRC and the surrounding tissues**

We used the divergent set of primers and utilized a TaqMan-based qRT-PCR assay to validate the presence of the circular RNA in 45 CRC tissues and paired non-cancerous tissues. cir-ITCH was expressed at a higher level in approximately 75.6% of the CRC tissues compared to that of the matched non-cancerous samples (Fig 1B).

**Characterization of cir-ITCH in CRC cells**

We constructed a vector to express cir-ITCH in cells following the previous study and then performed experiments. The constructed plasmids were transiently transfected into HCT116 and SW480 cells.

In the reverse transcription experiments, we used random primers and oligo (dT) primers. The circular products were depleted in the polyA-enriched samples compared with the linear products. When using the oligo (dT) primers, the expression quantity (normalized to GAPDH) of linear ITCH was significantly higher than that of circular ITCH (Fig 1C) [11].

We used the enzyme RNase R, a highly processive 3’-5’ exoribonuclease, to test our predictions of the circular RNA characteristics. This exonuclease degrades linear RNA molecules in a 3’-5’ direction, but it does not work on circular RNAs [17, 18]. As expected, in contrast to the linear control RNAs, which were degraded, the predicted circular RNA was resistant to the RNase R treatment (Fig 1D).
Interaction between cir-ITCH and miRNA

Recently, circular RNA has been identified as an abundant class of regulatory transcripts that function as miRNA sponges \[5, 8\]. The miRanda software was used to forecast the target miRNA. The sequence of the predicted microRNAs binding sites were presented in Table 2. We found that miR-7, miR-20a, and miR-214 can bind to the 3'-untranslated region (UTR) of ITCH and cir-ITCH. Therefore, we inserted the ITCH binding sequence into psiCHECK-2 and constructed luciferase reporters for these 3 miRNAs by transiently co-transfecting them into HCT116 cells. The construct had significantly reduced luciferase activity for miR-7, miR-20a, and miR-214 in a concentration-dependent manner in the control HCT116 cells (1 pmol miRNA-7: 1.02 ± 0.028 versus 1.236 ± 0.068, \(P = 0.05\); 40 pmol miRNA-7: 0.808 ± 0.052 versus 1.236 ± 0.068, \(P = 0.02\); 1 pmol miRNA-20a: 2.09 ± 0.123 versus 2.498 ± 0.068, \(P = 0.02\); 40 pmol miRNA-20a: 1.887 ± 0.11 versus 2.498 ± 0.068, \(P = 0.006\); 1 pmol miRNA-214: 1.511 ± 0.059 versus 1.88 ± 0.043, \(P = 0.03\); 40 pmol miRNA-214: 1.38 ± 0.058 versus 1.88 ± 0.043, \(P = 0.006\)). Similar results were found when we repeated the same experiments in the control SW480 cells.

Fig 1. cir-ITCH is correlated with CRC. (A) Convergent primers can amplify circular RNAs and linear RNAs. Divergent primers amplify circular RNAs only in cDNA compared with genomic DNA (gDNA). GAPDH is linear control. (B) The cir-ITCH was expressed at a higher level in approximately 75.6% of the CRC adjacent tissues compared to match CRC tissues. The expression level of cir-ITCH was analyzed by qRT-PCR based on Taq-man and normalized to GAPDH. Data are represented as mean±SEM from three independent experiments. (C) Random primers and oligoT primers were used respectively in the reverse transcription experiments. The predicted cir-ITCH is absent in poly(A) enriched samples. (D) The predicted cir-ITCH is react against to RNase R treatment. 2-tailed student’s t-test were used in test the differences between groups *\(p < 0.05\) compared to control.

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| microRNA   | miRNA binding sites 3'-UTR | miRNA binding sites in cir-ITCH |
|------------|----------------------------|---------------------------------|
| miRNA-7    | guggccacaguauauagucuucc   | uagguuaguagguagguauaggu        |
| miRNA-214  | uguauaugucuccguccggu     | acagcagcagcagcagggcag         |
| miRNA-20a  | gauuggcgauaaucguggaaau    | uaaaggcuaauagucggag           |

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We performed the same experiment in cir-ITCH hyper-expression cells. The results showed that there were no significant differences in luciferase activity when the psiCHECK-2-ITCH-binding site with miRNAs were cotransfected into HCT116 and SW480 cells. Six replicates for each group and the experiment repeated at least three times. Data are means±SEM. (B) HCT116 and SW480 cells after transfected with cir-ITCH and Control cells were respectively transfected with miR-20a, miR-7 and inhibitor for 24 h and were then further exposed to actinomycin D for 1, 2 and 3 h. Cells were harvested and the stability of cir-ITCH mRNA was analyzed by qRT-PCR relative to time 0 after blocking new RNA synthesis with actinomycin D; data are means±SEM, normalized to GAPDH.

Association of cir-ITCH and ITCH in CRC

Recently, cir-ITCH was reported to regulate gene expression through its role as an miRNA sponge, thus protecting the miRNAs’ target genes. Therefore, we tested the correlation between cir-ITCH and ITCH in an additional 15 pairs of CRC adjuvant non-cancerous tissues. The results showed that patients with higher cir-ITCH expression levels in the CRC tissues had a substantial up-regulation of linear ITCH (R² = 0.32, P < 0.01; Fig 3A).

cir-ITCH is involved in the regulation of the Wnt/β-catenin signaling pathway in vivo

ITCH protein ubiquitinates the phosphorylated form of Dvl2 and promotes its degradation, thereby inhibiting canonical Wnt signaling [19]. To further verify whether cir-ITCH regulates
the Wnt/β-catenin signaling pathway in CRC cells, we used a β-catenin/TCF-responsive luciferase reporter assay. The results showed that overexpression of ITCH inhibited the TOP flash activity in a dose-dependent manner (Fig 3B). The β-catenin and Wnt3a levels were analysed using western blot in cells with ITCH hyperexpression, and as shown in Fig 3C, there was an obvious decrease in β-catenin levels while there were no change in Wnt3a expression. We then tested the expression of c-myc and cyclinD1, two target genes of the Wnt/β-catenin signaling pathway, in cells transfected with cir-ITCH. Their expression was also suppressed (Fig 3D).

**cir-ITCH modulates cell growth**

We performed CCK-8 assays to test the effects of cir-ITCH on cell proliferation in CRC cells. We observed a consistent decrease in the cell proliferation of HCT116 (28%-39% decrease) and SW480 cells (15%-33% decrease) when cir-ITCH was overexpressed at physiological levels through the CCK-8 assays compared to that of the control (Fig 3E).
Discussion

In this study, we identified *cir-ITCH* in CRC and found that it was dramatically down-regulated in CRC tissues using a TaqMan-based reverse transcriptase polymerase chain reaction assay, indicating the potential function of *cir-ITCH* in CRC. A series of experiments illustrated the correlation between *cir-ITCH* and miRNAs, demonstrating that *cir-ITCH* plays an important role as an miRNA sponge in the process of CRC development.

Thousands of lincRNAs have been identified in humans and mice, and many are closely related to the development of various types of cancer, such as esophageal squamous cell carcinoma (ESCC) and endometrial carcinoma [20, 21]. Circular RNA is one type of lincRNA that plays a role in tumor development. To our knowledge, *ITCH* is one of the E3 ubiquitin protein ligases that specifically targets p73 [22], Dvl2 [19], and Notch1 [23], all of which are usually associated with tumor formation and chemosensitivity.

Although the functions of miRNAs are far from being fully understood, it is predicted that approximately 30% of protein-encoding genes are controlled by miRNAs[24], and some studies have suggested that miRNA can bind to the 3’-UTR of *ITCH* to decrease its expression [25]. Memczak et al. (2013) and Hansen et al. (2011) proposed that the cerebellar degeneration-related protein 1 (CDR1) locus harbors 70 conserved matches to the miR-7 seed. This striking feature suggested that circular RNA has a possible function as an miRNA sponge [5, 8, 26]. Previous research has shown that *cir-ITCH* acts as a sponge for miR-7, miR-17, and miR-214, whereas in the present study, we found that in the CRC cell lines, miR-7 and miR-20a down-regulate *ITCH* expression by binding to its 3’-UTR. Further, these miRNAs are always harbored by *cir-ITCH*. In our study, *cir-ITCH* did not act as a sponge for miR-214. To our knowledge, ectopic expression of miR-214 suppresses proliferation, migration, and invasion in vitro, whereas miR-214 knockdown promotes proliferation, migration, and invasion in CRC cell lines [27].

miRNAs have long been associated with cancer. Elevated miR-7 expression has been described in a variety of tumor types, including CRC, and has been implicated in oncogenesis, classification, and cancer progression [28]. miR-20a contributes to the increased proliferation and invasiveness of CRC cells [29]. Based on this information, our data verify our predecessors’ work. From the above data, it was shown that *cir-ITCH* participates in the development of CRC through regulation of miRNA activity.

Aberrant regulation of the Wnt signaling pathway has emerged as a prevalent theme in cancer biology, and it is crucial for the onset and progression of human CRC. Approximately 90% of sporadic colon cancers show aberrant Wnt signaling [30]. *cir-ITCH* acts as an miRNA sponge and increases the level of *ITCH*, which is involved in the Wnt/β-catenin pathway. According to previous research, ITCH can promote the ubiquitination and degradation of phosphorylated Dvl2 and, therefore, inhibit canonical Wnt signaling [19]. A β-catenin/TCF-responsive luciferase reporter assay was used to examine whether a single gene regulates the Wnt/β-catenin signaling pathway. Our study and other previous results reported that overexpression of *cir-ITCH* significantly suppressed the relative TCF transcriptional activity.

The oncogenes *c-myc* and *cyclinD1* are effector proteins of the karyomitosis signal, which can trigger and regulate the transcription of genes related to proliferation. They are frequently overexpressed in several human tumors, including CRC [31]. Our results showed that in cells transfected with *cir-ITCH*, the expression of *c-myc* and *cyclinD1* was markedly decreased. Therefore, we suggest that *cir-ITCH* has an inhibitory effect on the canonical Wnt pathway. *cir-ITCH* plays an anti-tumor role by controlling miRNA activity, and it has an inhibitory role in the canonical Wnt pathway, inhibiting *c-myc* and *cyclinD1* expression.
In summary, our present study indicates that the miRNA sponge cir-ITCH represents a new generation of technology for miRNA inhibition. cir-ITCH is involved in the Wnt/β-catenin pathway, and by inhibiting this pathway, it plays a role in CRC.

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Author Contributions
Conceived and designed the experiments: XC GH. Performed the experiments: GH HZ. Analyzed the data: XC GH HC. Contributed reagents/materials/analysis tools: YS WW. Wrote the paper: GH XC. Recruited subjects: GH XC.

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