

# Hsa\_circ\_001659 Serves as a Diagnostic and Prognostic Biomarker for Colorectal Cancer

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## Research

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# Abstract

**Background:** Colorectal cancer (CRC) is prevalent worldwide and novel diagnostic and prognostic biomarkers are needed to improve precision medicine. Circulating circular RNAs (circRNAs) are currently being considered as emerging tumor biomarkers.

**Methods:** Candidate circRNA was selected by integrating analysis of Gene Expression Omnibus (GEO) database using GEO2R program. The expression data of serum circ\_001659 were obtained from the quantitative real-time polymerase chain reaction (qRT-PCR). Receiver operating characteristic curves were applied to evaluate the clinical applications of circ\_001659. Finally, biological functions and potential mechanisms of circ\_001659 in tumor progression were investigated in CRC progression.

**Results:** The results showed that the diagnostic performance of serum circ\_001659 were excellent for CRC detection. The predictive values and likelihood ratios were satisfactory for the diagnosis of CRC, including patients in early-stage disease or patients with carcinoembryonic antigen (CEA)-negative status. Serum levels of circ\_001659 declined significantly 3 months after surgery in both preoperative CEA-positive and CEA-negative patients. Functionally, circ\_001659 influenced tumor cell metastasis via regulating epithelial-mesenchymal transition (EMT) and cytoskeleton organization.

**Conclusion:** Our findings demonstrate that circ\_001659 could be a useful serum biomarker for CRC diagnosis and prognosis. Targeting circ\_001659 and its pathway may be meaningful for treating patients with CRC.

## Background

Colorectal cancer (CRC) is one of the most common gastrointestinal tumors. Studies have shown that the 5-year survival rates of stage I, II, III and IV of CRC are 82.4%, 74.3%, 56.5% and 13.3%, respectively [1,2]. Early diagnosis of colorectal cancer is of great importance in reducing the incidence rate and mortality [3]. However, only 30%-40% of the patients are diagnosed in the early stage because of the occult onset and the obscure early symptoms [1]. Moreover, the high relapse rate reduces the therapeutic effects and 5-year survival rate of CRC patients [4]. At present, there is still a lack of a non-invasive, reliable and economical methods for reliable and economical methods for early diagnosis and assessments of treatment effectiveness.

Carcinoembryonic antigen (CEA) is the most commonly used tumor marker of CRC [5]. CEA levels are elevated in 70%-90% of patients with stage III and IV CRC patients, compared with less than 40% in patients with stage I and II. The sensitivity of CEA is low (35%-75%) at the commonly used cutoff of 3.08 µg/L, particularly in detection of early-stage CRC (20%-55%) [6,7]. CEA is also considered as one of the markers associated with the stage of tumor progression. After surgery, patients with high CEA level will be closely examined and followed up by doctors [8]. However, for CRC patients with normal preoperative CEA levels, postoperative monitoring of CEA level is meaningless. Therefore, novel and reliable diagnostic and prognostic biomarkers to complement CEA are urgently needed to improve clinical outcomes.

Globally, great progress has been made in the identification of molecular markers based on DNA, RNA, protein or metabolite, so as to develop a new and non-invasive detection method for CRC [9]. Circular RNA (circRNA) is a type of single stranded closed RNA formed by reverse cleavage of exons of precursor mRNA. CircRNA forms a complete ring structure by reverse splicing of exons or introns [10]. CircRNAs are widespread, specifically expressed and have stable circular structures [11]. Recent studies have confirmed that circRNA plays an important role in tumorigenesis and malignant progression [9,10]. Importantly, circRNA has the characteristics of rich abundance, stable structure, space-time specificity and tissue-cell specificity, and is detectable in saliva, serum and exosomes [9,12]. Because of these characteristics, circRNA as a tumor biomarker has gradually become a research hotspot.

In this study, we combine the bioinformatic analysis and laboratory test to obtain a novel circRNA biomarker, and identify one highly expressed circRNA, hsa\_circ\_001659 (Affymetrix probe set: 265886\_at), which had not been reported to date. After stepwise screening and validation, we demonstrate that circ\_001659 may serve as a diagnostic and prognostic biomarker complementary for CEA in CRC early diagnosis and therapeutic monitoring. Subsequently, The biological roles of circ\_001659 and the underlying mechanism in tumor progression were determined. Future studies based on these findings may lead to discover novel CRC biomarkers and targeted therapies.

## Materials And Methods

### Study population

A total of 140 CRC patients, 66 patients with BID and 118 HCs were enrolled from the Fourth Affiliated Hospital of Guangxi Medical University from May, 2015 to June, 2017. Consecutive patients who were diagnosed with CRC based on pathological findings, colonoscopic and abdominal ultrasound findings were recruited into the study. None of CRC patients had received radiotherapy or chemotherapy prior to surgery. Patients being in I or II tumor stage and without lymphatic metastasis and vascular metastasis (Dukes stage A/B) were considered as early-stage CRC patients. We also recruited consecutive patients with acute/chronic enteritis, intestinal polyps, intestinal obstruction, and crohn's disease who were considered as BID group. The HCs were recruited from disease-free healthy volunteers. The collected peripheral blood was centrifuged at low speed (3000 rpm, 5 min) to obtain serum and stored at - 80 °C until testing. We matched the subjects in the CRC patients and controls for age and sex as far as possible. Additional file 1: Table S1 listed the clinical characteristics and pathological information of the subjects.

### CEA concentration measurement

CEA concentrations were measured using Roche Cobas E601 electrochemiluminescence automatic immunoassay analyzer (Roche, Switzerland), according to the manufacturer's recommendations. When the concentration of CEA was less than 10.0 ng/mL (maximum limit of normal reference range), the result was considered as negative. Otherwise, it will be regarded as positive. All measurements were done in duplicate.

## RNA extraction and quantitative RT-PCR

RNA was extracted from 200  $\mu$ L serum sample using RNAiso Blood reagent (TaKaRa, Cat# 9108) according to the manufacturer's instructions. pGL3 plasmid was used as the reference gene. Briefly, 1 ng (approximately  $2 \times 10^8$  copies) of pGL3 was added to 200  $\mu$ L serum samples. PrimeScript<sup>TM</sup> RT Reagent Kit (TaKaRa, Cat# RR037A) was applied for reverse transcription. Quantitative polymerase chain reaction (qPCR) using SYBR (TaKaRa, Cat# RR820A) was carried out with iTaq Universal SYBR Green Supermix. QPCR using TaqMan (TaKaRa, Cat# R10T1) was performed with iTaq universal probes Supermix. Fold changes of circ\_001659 levels were calculated using the formula  $2^{-\Delta\Delta C_t}$ . Primers for circRNAs and genes were listed in the Additional file 2: Table S2 and Additional file 3: Table S3, respectively.

## Cell lines and transfection

Human colorectal cancer cells SW620 and LS174T were grown in RPMI 1640 (Gibco) supplemented with 10% of fetal bovine serum (Gibco). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. All cell lines were tested routinely to avoid *Mycoplasma* contamination (Yeasen, cat # 40601ES20). The siRNAs targeting circ\_001659 and the scramble siRNA control were purchased from TAKARA Company and transfected using Lipofectamine 3000 reagent (Invitrogen). Cells were harvested 72 h post transfection for various assays. The sequences of the circ\_001659 siRNA1 and siRNA2 were 5'-AUT CAU UGC TTA ACA GAC GTG AC-3' and 5'-GAT UCC TUA TTU AAT UUT GCG C-3' respectively.

## RNase R and actinomycin D treatment

Ten micrograms of total RNA were incubated with 1  $\mu$ L RNase R (Epicentre, 20 U/ $\mu$ L, #RNR07250) at 37 °C for 30 min, and the control group was treated without RNase R. Then, RNA was left at 65 °C for 10 min to inactivate RNase R and then reverse-transcribed for qRT-PCR detection. Actinomycin D (Sigma, #SBR00013) was added to the culture medium to block RNA transcription. Cells treated with actinomycin D for 0, 12, 24, 36, and 48 h were harvested, and their RNA was extracted and reverse-transcribed for qRT-PCR detection.

## Gene expression profiling and data analysis

Total RNA samples isolated from SW620 cells transfected with circ\_001659 siRNAs or negative siRNA control using TRIzol (Invitrogen) and further purified with an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. cRNAs were synthesized with T7 RNA polymerase and labeled utilizing Cy3-CTP, after which they were hybridized to the RNA sequencing. RNA-Seq experiments were entrusted to Omicstudio Inc. (Shanghai, China) for establishing an Illumina high-throughput sequencing library, and for high-throughput and coverage RNA-Seq analysis. Differential expression of genes (control group vs circ\_001659 siRNA, fold change  $\geq 2$  or  $\leq 0.5$ ) was analyzed using the GeneSpring GX v.11.0 software package (Agilent Technologies).

## Transwell migration assay and invasion assay

Transwell migration assay was performed using 24-well transwells (8- $\mu$ m pore size; Millipore). Transwell invasion assay was performed using 24-well transwells precoated with Matrigel (BD Biosciences). Cells at logarithmic phase were transfected with circ\_001659 siRNA or control siRNA. Cells suspended in serum-free medium were seeded in the upper chamber and were allowed to transmigrate towards the bottom chamber, containing medium with 15% FBS for 48 h. Cells on the upper surface of filters were removed, and those on the undersurface were fixed, stained, and counted.

## Animal experiments

In order to clarify the effect of circ\_001659 on tumor metastasis *in vivo*, 4-week-old male BALB/c nude mice were used. LS174T cells were transiently transfected with circ\_001659 siRNA or control siRNA for 48 h. Cells were trypsinized and counted. We injected  $1.0 \times 10^6$  LS174T cells into nude mice ( $n = 5$ , per group) through the tail vein. The mice were all killed 12 weeks later, at which time individual organs were removed.

## Immunofluorescence

SW620 cells transfected with circ\_001659 siRNA or negative siRNA control were seeded on coverslips in 6-well plate. After incubated for 48 h, cells were fixed in 4% paraformaldehyde for 30 min, and blocked in 10% goat serum for 1 h at room temperature. The cells were then incubated with  $\alpha$ -tubulin antibodies (CST, #2125) in 10% goat serum at 4°C overnight, followed by incubation with secondary fluorochrome-labelled antibodies for 1 h at 37 °C. After incubation with DAPI for 3 min at room temperature to stain the nucleus, cells were washed three times with PBS and imaged with confocal laser scanning microscope (UltraView Vox; PerkinElmer, Waltham, MA, USA).

## Western blot

Western blot analysis was performed using standard techniques [30]. An anti-GAPDH antibody was used as a control. Primary antibodies against E-cadherin (CST, #3195), ZO-1 (Proteintech, #21773-1-AP), N-cadherin (Abcam, #ab18203), ZEB1 (Proteintech, #21544-1-AP), MMP2 (Abcam, #ab37150), MMP9 (Abcam, #ab38898), SMAD3 (CST, #9523) pSMAD3 S423/S425 (CST, #9520), Talin1 (Abcam, #ab71333), RHOA (Proteintech, #10749-1-AP), RHOC (Proteintech, #10632-1-AP), RAC1 (Sigma, #05-389), RAC2 (Proteintech, #10749-1-AP), Vimentin (Santa Cruz, #sc6260), LASP1 (CST, #8636), RAB10 (Proteintech, #11808-1-AP) and GAPDH (CST, #2118) were used in this study. The signal was visualized using an ECL detection reagent and quantified by densitometry using Image J software (<http://rsb.info.nih.gov/ij>).

## Statistical analysis

Statistical analyses were undertaken using SPSS 13.0 and GraphpadPrism 5.0 software. Student's *t* tests were used to evaluate differences of serum circ\_001659 levels in CRC patients and control groups. The Chi-square test was utilized to assess the relationship between serum circ\_001659 levels and

clinicopathological factors. We used receiver operating characteristics (ROC) to calculate diagnostic accuracy. We used binary logistic regression to evaluate the diagnostic efficacy of the combined markers. We compared serum circ\_001659 levels before and after surgical resection of CRC patients with the paired Student's *t* test. Data collection and analyses were undertaken by three independent researchers (D Luo, S Huang and J Yang). All statistical tests were repeated twice. Results with p-values of < 0.05 were considered statistically significant.

## Results

### **Serum circ\_001659 was significantly upregulated in sera of early-stage CRC patients with remarkable diagnostic value**

To find potential complementary biomarker of CEA for CRC, we first analyzed the abundance and differential expression of circRNAs in GSE100206 (HC, n = 32) and GSE100063 (CRC, n = 12) datasets (Fig. 1a). Fifty-three circRNAs (26 upregulated and 27 downregulated) with fold change  $\geq 2.0$  and FDR < 0.01 were identified as differentially expressed circRNAs (DEcircRNAs) (Fig. 1a-c, Additional file 2: Table S2). Considering that down-regulated circRNAs are not readily associated with tumor progression, we then evaluated the expression levels and diagnostic potentials of these up-regulated circRNAs with serum samples from 20 pairs of age and sex matched CEA-negative early-stage CRC patients and healthy controls (screen cohort). The criteria of screen-level investigation for selected candidates were: (i) the quantification cycle values of each serum sample should be less than 30; (ii) The detectable rate should be more than 95%; (iii) fold change  $\geq 2.0$  and p-value < 0.01. Eventually, one circRNA (hsa\_circ\_001659) remained distinct was selected as candidate biomarker (Fig. 1d, Additional file 4: Table S4).

We further verified the expression level of circulating circ\_001659 using TaqMan-qPCR method. The results were consistent with the data from SYBR-qPCR method ( $p < 0.0001$ ; Fig. 1e). ROC analysis showed that circ\_001659 was a reliable biomarker in CRC early diagnosis (Fig. 1f, AUC = 0.885 using SYBR-qPCR method; AUC = 0.868 using TaqMan-qPCR method). In addition, a positive correlation of circ\_001659 level from SYBR-qPCR method and TaqMan-qPCR method was found which verified the reliability of the experimental data ( $r = 0.982$ ,  $p < 0.0001$ ; Fig. 1g). Those data suggest that circ\_001659 could be a potential biomarker for diagnosis of early-stage CRC patients.

Finally, we assessed the structure of circ\_001659. Sanger sequencing revealed that circ\_001659 was 645 bp in length and is spliced by 5-9 exons of the SOX13 gene (RefSeq: NM\_005686.3) (Fig. 2a). To determine the stability of circ\_001659, total RNA was extracted from two CRC cells SW620 and LS174T and treated with RNase R at 37 °C for 30 min. Real-time PCR detected the expression levels of circ\_001659 and the host gene SOX13. As shown in Fig. 2b, circ\_001659 was more stable than the linear mRNA of the SOX13 gene. Next, we treated SW620 and LS174T cells by actinomycin D (an inhibitor of transcription) for 0, 12, 24, 36, and 48 h. Real-time PCR analysis showed that circ\_001659 transcript was stable in comparison to SOX13 mRNA (Fig. 2c), indicating that circ\_001659 had a bona fide circRNA structure and possessed the potential as a reliable biomarker.

## **Serum circ\_001659 served as a diagnostic biomarker complementary for CEA**

To confirm the diagnostic potential of circ\_001659 for early-stage CRC patients, serum samples of a new cohort (validation cohort) were collected. The validation cohort included 98 HC, 66 BID, and 120 CRC. First, we observed that expression level of circ\_001659 was similar in BID and HC group ( $p = 0.1535$ , Fig. 3a); while circ\_001659 was highly expressed in serum of CRC patients compared with BID and HC group ( $p < 0.0001$ , Fig. 3a). We then evaluated differential diagnostic accuracy of circ\_001659 and CEA for CRC patients. As we expected, circ\_001659 and CEA showed similar diagnostic values for CRC patients compared with control groups (Fig. 3b, Table 1).

Then we tried to demonstrate whether circ\_001659 showed better diagnostic potential than CEA in CRC early diagnosis. There are 51 of 120 CRC patients in validation cohort with early-stage disease (Dukes stage A/B). Results showed that circ\_001659 had greater AUC, sensitivity, and specificity values than did CEA in CRC early diagnosis (Fig. 3c, Table 1). When we combined two biomarkers together, the diagnostic values were greatly improved compared with either one of them (Table 1).

Further, we divided 51 early-stage CRC patients of validation cohort into CEA-positive and CEA-negative groups to determine the diagnostic value of circ\_001659. The ROC curves indicated that circ\_001659 showed a compelling diagnostic potential in both CEA-positive and CEA-negative early-stage CRC (Fig. 3d, Table 2). In other words, circ\_001659 could be an effective diagnostic biomarker of CRC early detection irrespective of CEA status. Altogether, serum circ\_001659 could be used as an independent biomarker for CRC diagnosis or in combination with CEA to improve the diagnostic efficacy of early-stage CRC.

## **Serum circ\_001659 served as a prognostic biomarker for CRC**

To evaluate the pathologic and clinical significance of serum circ\_001659, we compared the different histologic stages, positive or negative vascular invasion, high or poor pathologic differentiation and AJCC stages between circ\_001659 high-expression and circ\_001659 low-expression tumors based on the median of serum circ\_001659 level. Table 3 listed the association of different clinical characters with circ\_001659 high- and low-expression cohorts. There is a tendency that patients with advanced pathological stages have higher levels of circ\_001659 in their sera, suggesting that circ\_001659 is associated with tumor progression. Additionally, higher level of serum circ\_001659 was also correlated with tumor size and metastatic progression.

In patients with preoperative elevated CEA, CEA level will generally return to normal within half a year after surgery [8,13]. If CEA remains high, it indicates that there may be residual tumor or cancer recurrence [13]. For patients with CEA-negative status before operation, the monitoring of CEA level after operation has no significance for evaluation of clinical outcomes. We have demonstrated that serum circ\_001659 was increased in CEA-negative CRC patients compared with controls. Thus, we tried to investigate whether circ\_001659 can be used as an auxiliary monitoring indicator after treatment in CEA-negative patients. Ninety-eight CRC patients underwent surgical resection were divided into CEA-positive and CEA-negative groups. We compared the levels of circ\_001659 in pre- and post-therapeutic serum samples of

these cancer patients. Three months after surgery, levels of circ\_001659 in serum decreased rapidly in the majority of patients from both CEA-positive (5/72) and CEA-negative group (1/26) (Fig. 4). Interestingly, there were 6 cases showed a similar or elevated trend of serum circ\_001659 after operation (Fig. 4). Clinical examination reported that these cases either suffered relapse or had residual tumor in lymph nodes or metastasis during follow-up (Additional file 5: Table S5). Taken together, we conclude that serum circ\_001659 may be a novel biomarker in the assessment of successful treatment and remission of cancer tracking. High-level serum circ\_001659 after therapy is associated with poor prognosis for CRC patients.

### **Circ\_001659 influenced tumor cell metastasis *in vitro* and *in vivo***

To elucidate the potential functions and pathways of circ\_001659 involved in tumor progression, RNA sequencing (RNA-seq) analysis was performed to compare the gene expression profiles of circ\_001659 siRNA and control siRNA transfectants in SW620 and LS174T cells. A total of 1,985 downregulated genes and 2,258 upregulated genes in SW60 cell and 2,233 downregulated genes and 1,545 upregulated genes in LS174T cell were detected after knockdown of circ\_001659 (Fig. 5a-b). The intersection of two gene lists was performed as described in Fig. 5a. Potential functions and pathways that circ\_001659 may regulated were pooled using the overlapped gene signature (Additional file 6: Table S6). Gene ontology term enrichment (GO) analysis revealed that the main functions of circ\_001659 were tumor metastasis, negative regulation of cell shape, and reorganization of cytoskeleton (Fig. 5c). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis demonstrated that circ\_001659 mainly involved in the epithelial-mesenchymal transition (EMT) signaling pathway, small GTPase Rho/Rac signaling pathway, TGF- $\beta$  signaling pathways, and hypoxia-mediated signaling pathway (Fig. 5d).

To functionally validate the pathway findings, we first detected cell migration and invasion using transwell assays *in vitro*. The circ\_001659 siRNA significantly reduced the migratory activity of CRC cells in transwell assays (Fig. 5e) in both SW620 and LS174T cells. Similarly, in matrigel-coated transwell assays, we showed that downregulation of circ\_001659, but not the control siRNA, significantly reduced the invasive ability in colorectal cancer cells (Fig. 5f). To confirm the effect of circ\_001659 *in vivo*, LS174T cells transfected with circ\_001659 siRNA or scrambled control siRNA were inoculated into the tail veins of nude mice and assessed the number of metastasized tumor nodules in the lung (Fig. 5g). We found tumor nodules on the lung surface in 100% (5/5) of mice inoculated with LS174T control-siRNA cells, with an average of  $10.6 \pm 3.53$  nodules per lung. However, there were fewer metastatic foci in the lungs of nude mice at 12 weeks after injection of LS174T circ\_001659-siRNA cells, with an average of  $3.6 \pm 2.64$  nodules per mouse (Fig. 5h). The weight of mouse lungs were measured and reflected the decreased metastatic tumor foci size in circ\_001659 siRNA group compared with control (Fig. 5i). The data suggest that circ\_001659 may promote colorectal cancer progression by regulating cell invasion and metastasis.

### **Circ\_001659 regulated cytoskeleton organization**

Microtubules are tightly associated with cell movement and tumor metastasis. Considering the cytoskeleton-related functions of circ\_001659 involved in RNA-seq data, we attempted to investigate whether circ\_001659 regulated tubulin polymerization and maintained cytoskeleton stability. Immunofluorescence analysis demonstrated that unlike scrambled control-treated SW620 cells,  $\alpha$ -tubulin was unable to decorate microtubules in cells after circ\_001659 knockdown (Fig. 6), suggesting circ\_001659 influenced the content of stable microtubules by regulating tubulin organization.

### **Circ\_001659 regulated EMT, metastasis and cytoskeleton-related genes expression**

In order to examine the molecular mechanism in detail of circ\_001659, we examined whether circ\_001659 influenced the expression of EMT and metastasis-associated genes appeared in the differentially expressed gene signature. Real-time PCR and western blotting results demonstrated that circ\_001659 knockdown upregulated expression of the epithelial marker CDH1 (encoding E-cadherin), ZO-1 and downregulated the mesenchymal marker CDH2 (encoding N-cadherin), ZEB1 and metastasis-associated genes MMP2 and MMP9 (Fig. 7a-b). To better understand the relationship between circ\_001659 and EMT, we further detected the activation of TGF- $\beta$  signalling, which is known to be a master regulator of EMT [14]. Expectedly, circ\_001659 siRNAs indeed inhibited the activation of TGF- $\beta$  signaling by reducing SMAD3 protein phosphorylation (Fig. 7b), suggesting circ\_001659 may regulate EMT via influencing TGF- $\beta$  signaling.

Small GTPase Rho/Rac signaling pathway is a well-established regulator of the cytoskeleton reorganization [14-16]. Many GTPase family members, including TALN1 (encoding Talin1), RAC1, RHOC, RHOA, RAC2, Vimentin, LASP1 and RAB10 were significantly enriched in transcriptome sequencing data. We confirmed these results and found that Talin1, RAC1, RHOC, LASP1 and Vimentin were significantly downregulated, whereas RAC2, RHOA and RAB11B were upregulated upon circ\_001659 siRNAs treatment (Fig. 7a-b). These data suggest that circ\_001659 relies on the regulation of GTPase family members to influence cytoskeleton reorganization and cell migration.

## **Discussion**

Identification of novel serum biomarkers is an important goal in the diagnosis of cancer, especially for detection and screening in early-stage cancer [2,17,18]. Currently, the repertoire of well-documented biomarkers for CRC early diagnosis is quite limited. CEA is the most commonly used tumor marker in CRC patients and is increased in approximately 60% to 85% of the patients [19]. CEA has a specificity for colorectal cancer of 70% to 90%, but a sensitivity of only 35% to 75%, suggesting that CEA alone lacks the discriminative capacity for CRC early diagnosis [7]. In order to find complementary biomarkers of CEA for CRC early diagnosis, we set a special criteria in the screen stage: circRNA with high and stable level in sera of patients with CEA-negative early-stage CRC was considered as candidate biomarker. Then, we measured the candidate circRNA in two independent cohorts to verify its diagnostic efficacy for early-stage CRC, especially for CEA-negative early-stage CRC. Here, ROC analysis demonstrated that serum circ\_001659 could serve as an independent biomarker for CRC diagnosis or in combination with CEA to

improve the diagnosis of CEA-negative early-stage CRC. Interestingly, we found a similar level of serum circ\_001659 in CEA-positive and CEA-negative CRC patients. One possible reason for the similarity is that circ\_001659 and CEA may not interact or regulate each other in tumor cells, which may be an advantage of circ\_001659 used as a complementary biomarker of CEA. In our study, the sample size and the proportion of patients with CRC were smaller, thus further investigation will be warranted to validate the performance of circ\_001659 together with CEA, particularly in early CRC diagnosis with a large-scale and multicentre cohort.

Because the recurrence and metastasis are frequently observed in CRC, it is very important to have biomarkers for estimation and monitoring of disease remission after therapy [4,20]. The striking decrease of circ\_001659 level in serum after surgery suggests that this circRNA will be a useful surveillance biomarker to assess the therapeutic response of CRC patients. Additionally, high expression of circ\_001659 is associated with metastasis and poor prognosis in CRC patients (Table 3). Therefore, it might be feasible for circ\_001659 to be a prognostic factor. The serum samples used in this study were obtained from patients 3 months after surgical resection. Our findings showed that circ\_001659 can provide a timely evaluation of therapeutic outcome. Serum CEA is routinely used in the clinical setting for monitoring therapy outcomes in patients with advanced disease to predict prognosis [5]. It will be important to evaluate if these two different biomarkers may be used together for assessment of CRC therapy and monitoring of disease during remission. To further explore this potential role, we are undertaking long-term follow-up of the CRC patients who underwent surgery in this study.

As a novel circRNA, we preliminarily investigated the biological function of circ\_001659 in tumor progression. We performed gene expression profiling analysis using transcriptome sequencing technology as it is a fast and reliable approach to obtain differentially expressed genes (DEGs) [21]. Analysis of function and signaling enrichment based on DEGs indicated that circ\_001659 correlated significantly with EMT, metastasis, and cytoskeleton-associated gene signatures. The bioinformatic analyses were functionally validated using *in vitro* and *in vivo* experimental models. Downregulation of circ\_001659 markedly suppressed the migratory and invasive capacity in CRC cells. It has been clear that EMT process promotes tumor migration and invasion [22]. To date, whether and how circRNAs contribute to EMT in CRC remains elusive. In present study, we found that circ\_001659 could activate the TGF- $\beta$  pathway and subsequently promote EMT in CRC. Therefore, we speculate that circ\_001659 exert its oncogenic effect in TGF- $\beta$ /EMT axis. These data suggest that circ\_001659 has potential function in TGF- $\beta$ -induced EMT and CRC metastasis. However, it is indeterminate how circ\_001659 regulates signaling pathways to promote TGF- $\beta$ -induced EMT and enhance cancer cell invasion and migration.

Cytoskeleton, which connects the nucleus, cytoplasm and membrane, is involved in maintaining the specific shape of cells, coordinating the system for cell movement, and providing support for cell migration [23,24]. When cancer cells invade into surrounding tissues, they need to get rid of intercellular adhesion and push away the barrier of other cells. This process requires cancer cells to maintain a strong ability of movement, and the realization of this ability requires a complete cytoskeleton [25-27]. Emerging evidence suggests that the dynamic changes of cell cytoskeleton may affect the invasion and migration

of tumor cells. We detected more than 300 differentially expressed genes to be simultaneously regulated by circ\_001659 in SW620 and LS174T cells, including Rho/Rac GTPase family members, an important signal pathway involved in the regulation of tubulin and cytoskeleton reorganization. In addition, the GTPase-related proteins are also closely associated with actin cytoskeleton, as highlighted by the RHOC-induced stress-fiber formation and RAC1-dependent membrane ruffling [28]. Interestingly, our cytoskeleton staining data demonstrated that circ\_001659 knockdown led to a loss of tubulin formation. This suggests that circ\_001659 might promote tumor metastasis through regulation of cytoskeleton stability and cell mobility. Mechanistically, circ\_001659 may regulate the expression of cytoskeleton-related genes, including Talin1, RAC1, RHOC, LASP1, Vimentin, RAC2, RHOA and RAB11B, resulting in dysregulation of the Rho/Rac1 signaling pathway and cytoskeleton reorganization of CRC cells. Therefore, future studies should investigate whether this molecule can be targeted as part of a strategy to control tumor metastasis.

## Conclusion

In conclusion, our present validation of circ\_001659 as a circulating biomarker for CRC early diagnosis provides a rationale for further studies into the development of a blood-based biomarker approach using circ\_001659 that complements for current CRC screening modalities. Our report provides first line of comprehensive evidences that circ\_001659 is a novel oncogenic circRNA, as well as a prognostic biomarker in CRC. The current study provide novel insights into the potential applications of circ\_001659 as an effective diagnostic biomarker and target for the treatment of CRC patients.

## Abbreviations

CRC, colorectal cancer; CircRNA, circular RNA; GEO, Gene Expression Omnibus; ROC, receiver operating characteristic; CEA, carcinoembryonic antigen; BID, benign intestinal diseases; HC, healthy controls; qRT-PCR, quantitative real time polymerase chain reaction; FDR, false discovery rate; AUC, area under curve, SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio; EMT, epithelial-mesenchymal transition.

## Declarations

### Ethics approval and consent to participate

The study was reviewed and approved by the Research Ethics Committee of the Fourth Affiliated Hospital of Guangxi Medical University and the Renji Hospital, Shanghai JiaoTong University School of Medicine. The study was conducted in accordance with the Declaration of Helsinki. Informed consent Informed consent was obtained from all participants, according to the committee' regulations.

The animal experimental protocols were approved by the Medical Experimental Animal Care Commission of the Fourth Affiliated Hospital of Guangxi Medical University and the Renji Hospital, Shanghai JiaoTong

University School of Medicine, and performed in accordance with the institutional ethical guidelines for animal experiments.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### **Author Contributions**

B He, W Chao, Z Huang and J Zeng provided patients' samples and clinical data, performed the experiments. B He, W Chao and Z Huang wrote the manuscript. D Luo, S Huang and J Yang analysed and interpreted the data. H Pan and Y Hao conceptualised and designed the study and revised the paper. All authors vouched for the respective data and analysis, approved the final version and agreed to publish the manuscript.

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## Tables

**Table 1. Diagnostic performances of serum circ\_001659, CEA, or both in validation cohort**

	AUC (95%CI)	SEN	SPE	PPV	NPV	LR+	LR-
CRC vs HC and BID							
Circ_001659	0.872 (0.798-0.945)	0.925	0.793	0.884	0.863	4.469	0.095
CEA	0.792 (0.713-0.871)	0.545	0.799	0.775	0.822	2.711	0.569
Circ_001659+CEA	0.950 (0.910-0.990)	0.889	0.981	0.957	0.947	48.894	0.072
Early-stage CRC vs HC and BID							
Circ_001659	0.831 (0.758-0.902)	0.677	0.915	0.857	0.785	7.965	0.353
CEA	0.657 (0.521-0.793)	0.532	0.675	0.729	0.707	1.637	0.693
Circ_001659+CEA	0.874 (0.799-0.949)	0.781	0.700	0.913	0.901	10.013	0.237

Notes: SEN: sensitivity, SPE: specificity, PPV: positive predictive value, NPV: negative predictive value, LR: likelihood ratio.

**Table 2. Diagnostic performances of serum circ\_001659 in CEA-positive or CEA-negative early CRC detection**

	AUC (95%CI)	SEN	SPE	PPV	NPV	LR+	LR-
CEA <sup>+</sup> early-CRC vs HC and BID	0.862 (0.767-0.957)	0.807	0.862	0.581	0.974	5.848	0.224
CEA <sup>-</sup> early-CRC vs HC and BID	0.871 (0.768-0.975)	0.800	0.909	0.516	0.974	8.791	0.220

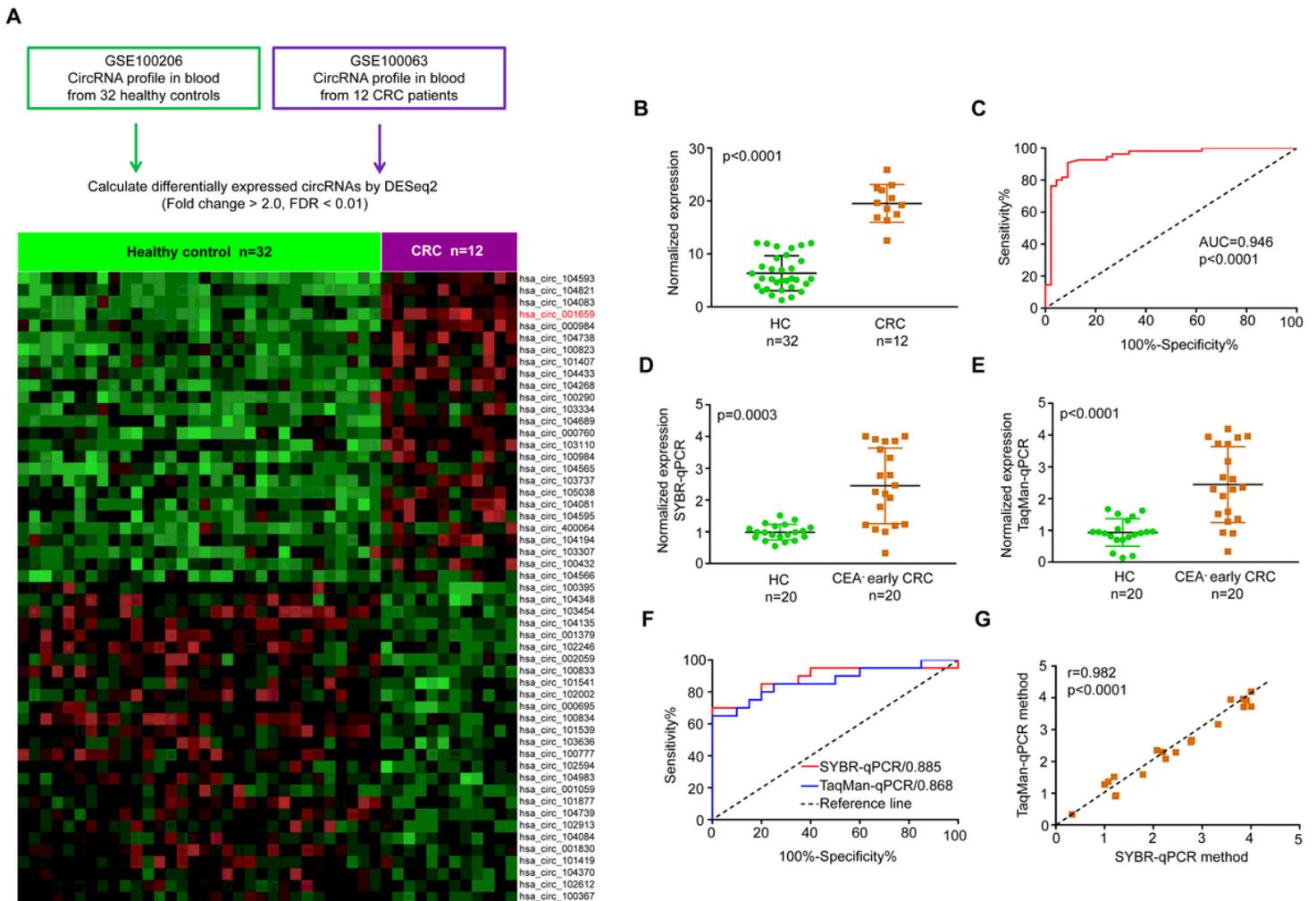
Notes: CEA<sup>+</sup>: CEA-positive, CEA<sup>-</sup>: CEA-negative.

**Table 3. Pooled analysis of the correlation between serum hsa\_circ\_001659 levels and clinicopathologic features in CRC patients from screen cohort and validation cohort**

Clinicopathologic features	Total (n=140)	Serum circ_001659 level		p-value
		Low (n=70)	High (n=70)	
Sex				0.4305
Male	92	47	45	
Female	48	24	24	
Age				0.7189
< 60	46	24	22	
≥ 60	94	46	48	
Differentiation				0.6037
Well	55	29	26	
Poor	85	41	44	
Size (cm)				0.0422*
< 4	66	39	27	
≥ 4	74	31	43	
Tumor stage (AJCC)				0.0035**
I-II	83	50	33	
III-IV	57	20	37	
Lymphatic metastasis				0.0176*
Negative	86	54	32	
Positive	54	20	34	
Vascular metastasis				0.0005***
Negative	122	79	43	
Positive	18	6	12	

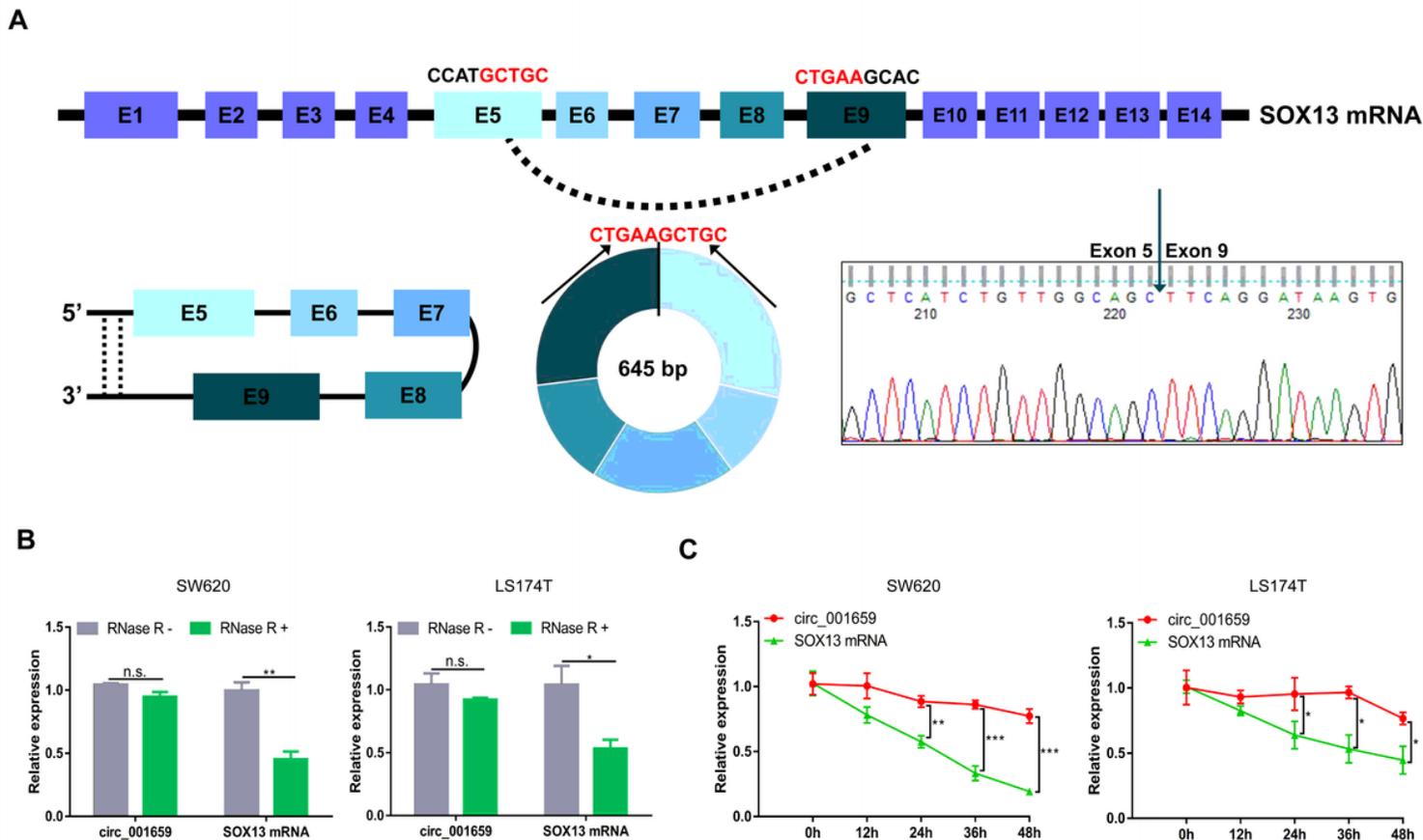
Note: \* was analyzed using Chi-square test. \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

## Figures



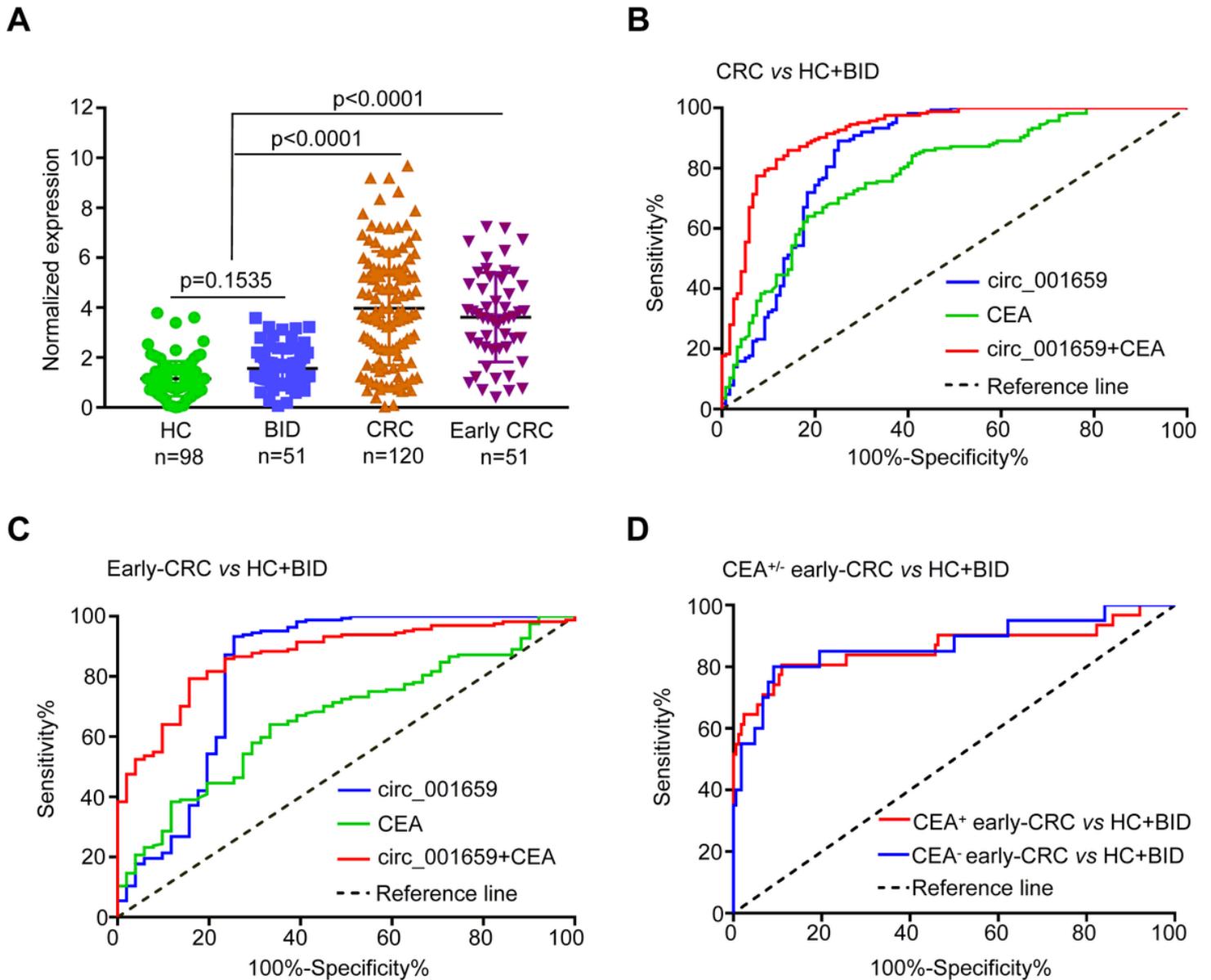
**Figure 1**

Serum circ\_001659 was significantly upregulated in sera of early-stage CRC patients with remarkable diagnostic value. a Heat maps of DEcircRNAs mined from two independent GEO datasets. b-c Expression and diagnostic values of circ\_001659 in GEO datasets. d-e Serum circ\_001659 was detected using SYBR-qPCR method and TaqMan-qPCR method in the screen cohort. f Diagnostic values of serum circ\_001659 with two different methods in the screen cohort. g Correlation analysis between SYBR-qPCR method and TaqMan-qPCR method.



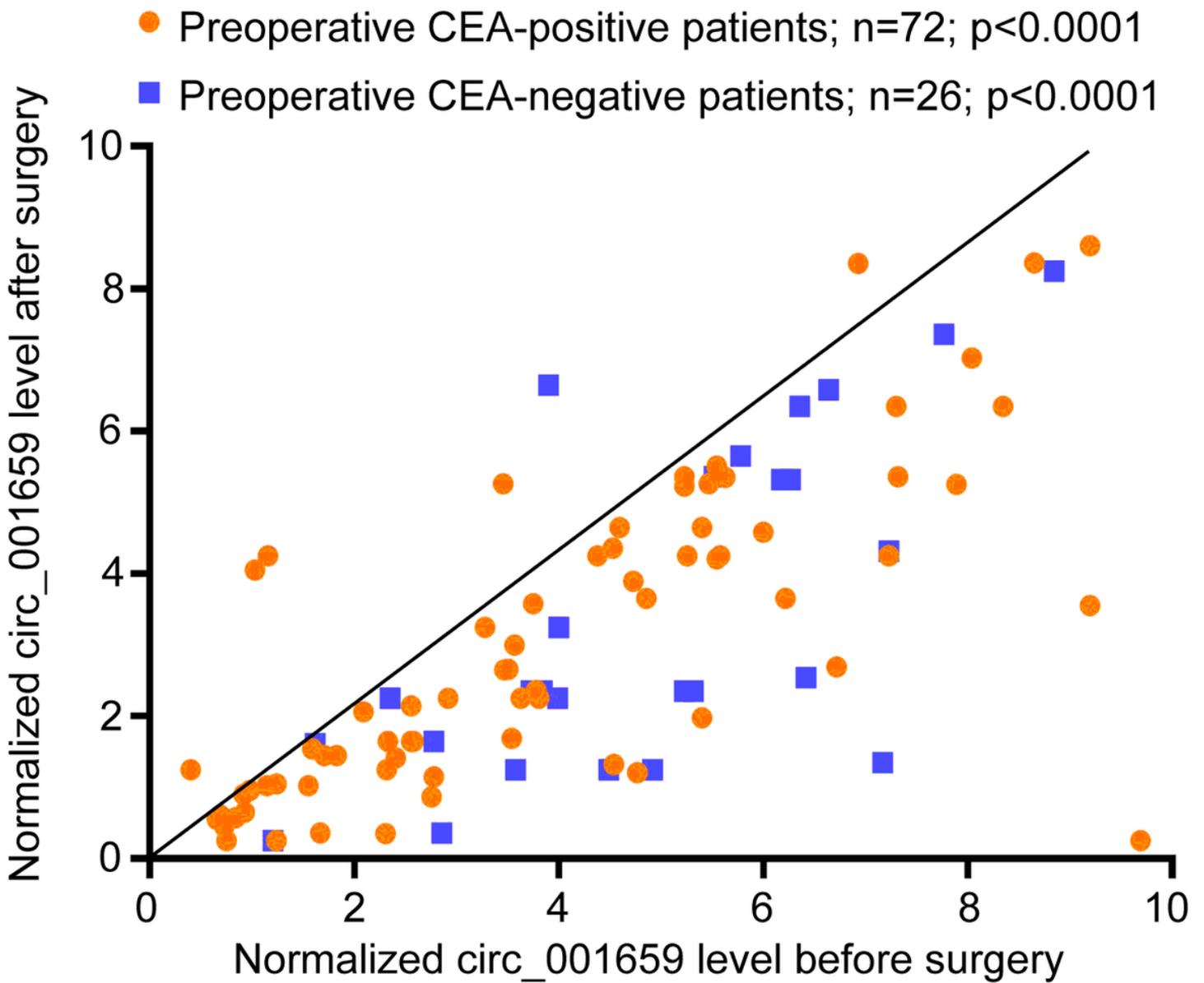
**Figure 2**

Characterization of circ\_001659. a We confirmed circ\_001659 was formed by circularization of exons 5, 6, 7, 8, 9 in the SOX13 mRNA by sanger sequencing, and determined the genomic size and full-length sequence. b-c The relative expression of circ\_001659 and SOX13 mRNA in CRC cells were detected by qRT-PCR after RNase R treatment for 30 min (b) or actinomycin D treatment for 0 h, 12 h, 24 h, 36, and 48 h (c). n.s., not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , Student's t test.



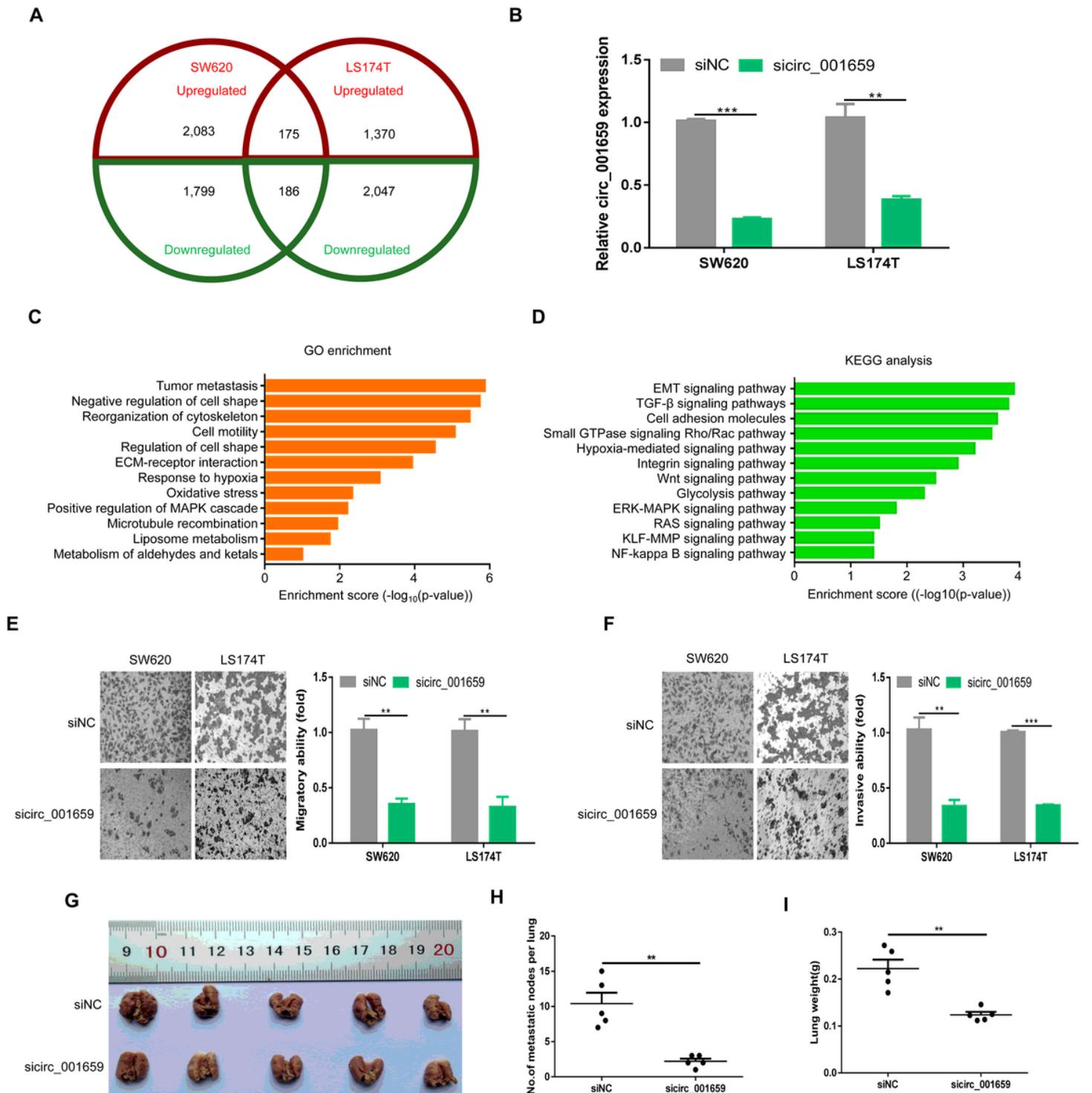
**Figure 3**

Serum circ\_001659 served as a diagnostic biomarker complementary for CEA. a Serum circ\_001659 levels in validation cohort. b ROC curves of circ\_001659, CEA or both for CRC patients versus controls in validation cohort. c ROC curves of circ\_001659, CEA or both for patients with early-stage CRC versus controls in validation cohort. d ROC curves of circ\_001659 for CEA-positive or CEA-negative early-stage CRC patients versus controls in validation cohort.



**Figure 4**

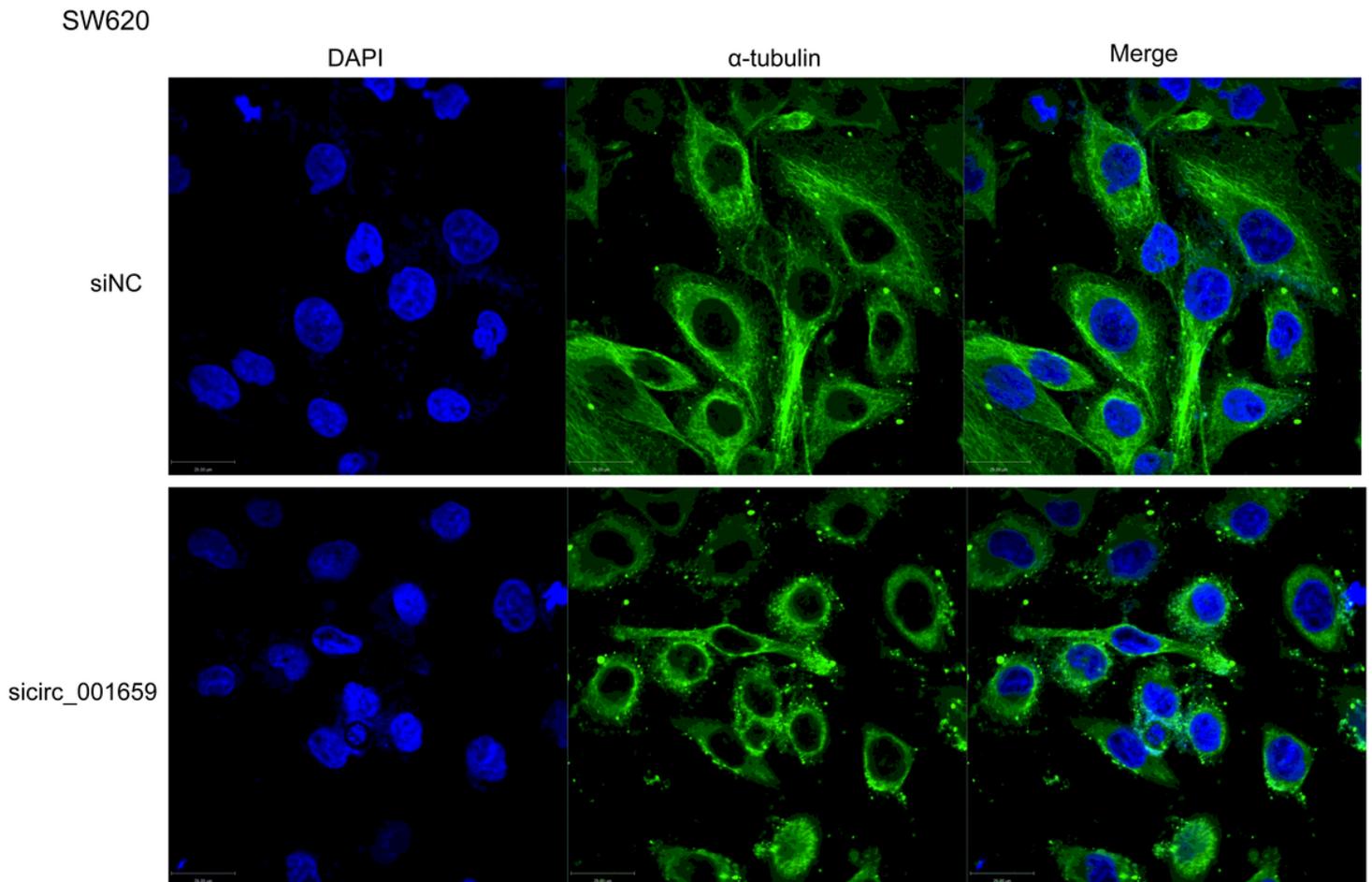
Serum circ\_001659 served as a prognostic biomarker for CRC. Scatter plot of serum circ\_001659 levels in paired serum samples from before and 3 months after surgery from the same patients with CRC. Red dots represent preoperative CEA-positive patients; Blue dots represent preoperative CEA-negative patients, paired Student's t test.



**Figure 5**

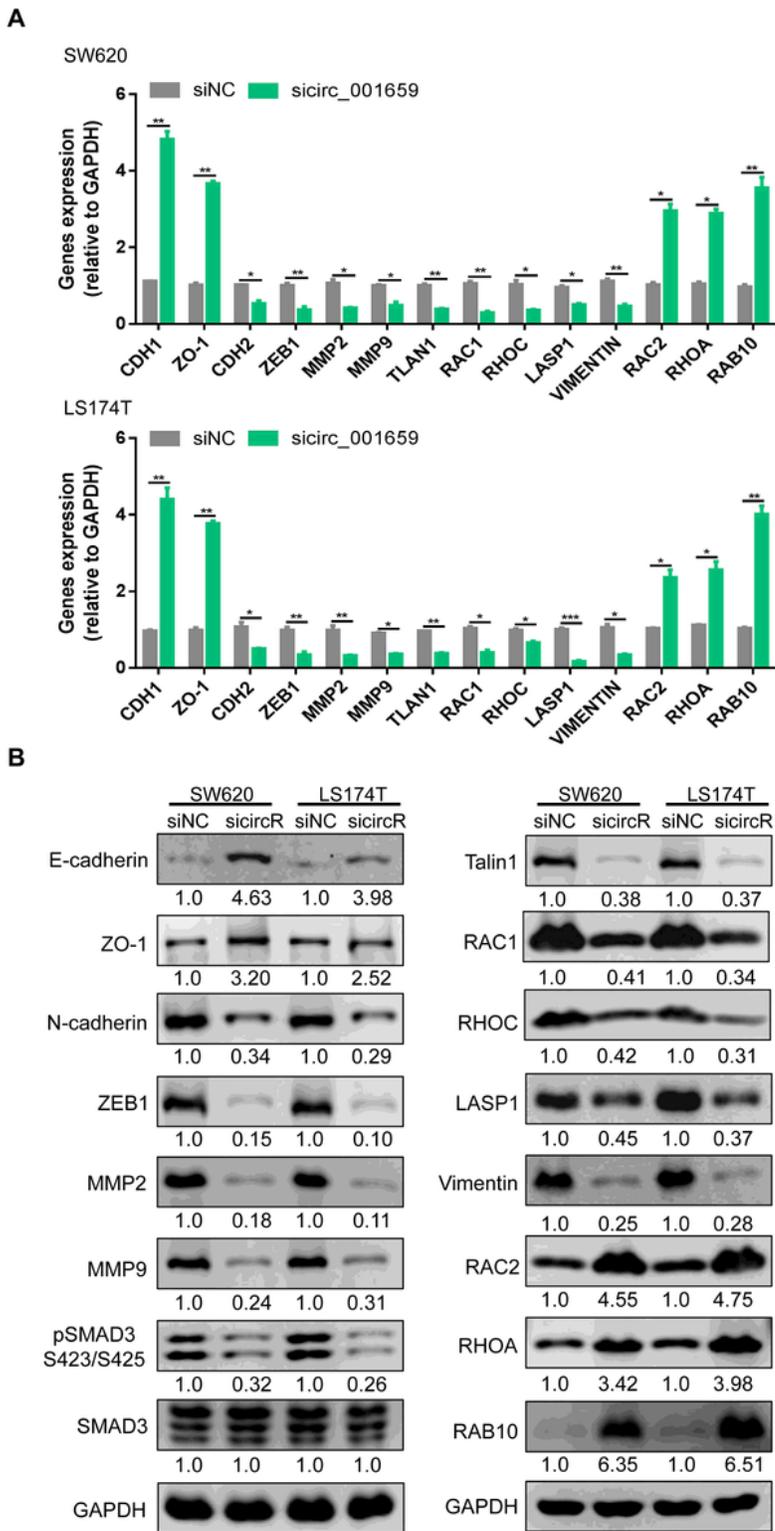
Circ\_001659 knockdown inhibited cancer cell migration and invasion. a The intersections of gene lists from DEGs (Differential expression genes) of gene expression profiling in SW620 and LS174T cells. b The efficiency of circ\_001659 knockdown was measured by examining circ\_001659 expression in SW620 and LS174T cells 48 h after transfection with the circ\_001659 siRNA. c Gene ontology (GO) enrichment analysis for circ\_001659 using overlapped genes. P-value < 0.05 was considered statistically significant. The vertical axis represents the types of biological functions, and the horizontal axis represents the -log<sub>10</sub>

(p-value) of these significant biological functions. d KEGG analysis for circ\_001659 using overlapped genes. P-values < 0.05 were defined as statistically significant. The vertical axis represents the categories of pathways regulated by circ\_001659, and the horizontal axis represents the  $-\log_{10}$  (p-value) of these pathways. e-f Circ\_001659 knockdown inhibited CRC cell migratory and invasive abilities as measured by transwell migration assay (e) and matrigel invasion assay (f). g Four-week-old male BALB/c nude mice were injected with  $1.0 \times 10^6$  LS174T cells transfected with circ\_001659 siRNA or scrambled control into the tail vein. The mice were killed 12 weeks later, and their lungs were removed for assessment of the metastasized tumor nodules. h Numbers of metastatic tumor nodules in the lung were counted. i Mouse lung weights were detected and reflected the metastasized tumor foci size. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , Student's t test.



**Figure 6**

Circ\_001659 knockdown destroyed microtubule formation in SW620 cells. SW620 cells were transfected with circ\_001659 siRNA or scrambled control (siNC), and 48 h after transfection, cells were fixed and stained for microtubule by  $\alpha$ -tubulin (green), and DAPI was used to stain nuclei (blue). A clear loss of microtubule formation was observed in circ\_001659 siRNA-transfected cells. Images were acquired at  $\times 400$ . Scale bar=20  $\mu\text{m}$ .



**Figure 7**

Circ\_001659 regulated EMT, metastasis and cytoskeleton-related genes expression. a SW620 or LS174T cells were transfected with circ\_001659 siRNA, 48 hours after transfection, cells were harvested and mRNA expression levels of EMT-related genes CDH1 (encoding E-cadherin protein), ZO-1, CDH2 (encoding N-cadherin), ZEB1, metastasis-related genes MMP2, MMP9, cytoskeleton-related genes TLAN1 (encoding Talin1), Vimentin, RAC1, RHOC, LASP1, RAC2, RHOA, RAB10 were measured by real-time PCR. b Protein

expression levels of EMT, metastasis and cytoskeleton-related genes were further measured by western blot 48 h after transfection with circ\_001659 siRNA in SW620 and LS174T cells. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, Student's t test.

## Supplementary Files

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