Original Article

Long Non-Coding RNA *CASC2* Functions as A Tumor Suppressor in Colorectal Cancer via Modulating The *miR-18a-5p/BTG3* Pathway

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Abstract

Objective: Reportedly, long non-coding RNA (lncRNA) cancer susceptibility candidate 2 (*CASC2*) is involved in regulating colorectal cancer (CRC) progression. However, the function and detailed downstream mechanism of *CASC2* in CRC progression are not fully elucidated. The aim of the study was to investigate the potential function and molecular mechanism of *CASC2* in CRC progression.

Materials and Methods: In this experimental study, quantitative real-time polymerase chain reaction (qRT-PCR) was adopted to probe *CASC2*, microRNA-18a-5p (*miR-18a-5p*) and B cell translocation gene 3 (*BTG3*) mRNA expression in CRC tissues and cell lines. After *CASC2* was overexpressed in Colo-678 and HCT116 cell lines, methylthiazol tetrazolium (MTT) and 5-bromo-2'-deoxyuridine (BrdU) assays were employed to examine the proliferation of CRC cells. Transwell migration and invasion assays were executed to evaluate the metastatic potential of CRC cells. The targeting relationships among *CASC2*, *miR-18a-5p* and *BTG3* were validated by dual luciferase reporter gene assay. Western blot assay was applied to examine the regulatory effects of CASC2 and miR-18a-5p on BTG3 protein expression.

Results: *CASC2* was decreased in CRC tissues and cell lines, and its low expression in CRC tissues was associated with larger tumor size and lymph node metastasis. *CASC2* overexpression restrained proliferative, migrative and invasive capabilities of CRC cells. *CASC2* could function as a molecular sponge for *miR-18a-5p* and repress the expression of *miR-18a-5p*. Furthermore, the inhibitory effects of *CASC2* on the malignant phenotypes of CRC cells was counteracted by *miR-18a-5p* mimics. Additionally, *CASC2* could positively regulate BTG3 expression via suppressing *miR-18a-5p*.

Conclusion: *CASC2* inhibits CRC development by suppressing *miR-18a-5p* and raising *BTG3* expression.

Keywords: B Cell Translocation Gene 3, Colorectal Cancer, lncRNA CASC2, miR-18a-5p

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Introduction

Colorectal cancer (CRC) brings about a huge health burden globally (1). In 2020, there were approximately 147,950 new cases of CRC and 53,200 deaths from CRC worldwide (2). In China, the incidence of CRC is on the rise (3). Currently, colonoscopy is the most reliable screening method for CRC screening, but it has high economic and physical burdens (4). In addition, relapse after the surgery, distant metastasis and chemoresistance contribute to the adverse prognosis of CRC patients (5). It is therefore pivotal to decipher the mechanism of CRC progression to identify potential therapy targets for treating CRC.

Long non-coding RNA (lncRNA) exceeding 200*nt*, is vital in imprinting, epigenetic regulation, transcription and translation regulation (6). Through modulating gene expression and the function of proteins, lncRNAs participate in regulating diverse biological processes (7). In these years, some lncRNAs emerge as either oncogenes or tumor suppressors in diverse cancers (8, 9). Notably, the expression of lncRNA cancer susceptibility candidate 2 (*CASC2*) is reduced in pancreatic cancer and oral squamous cell carcinoma (10, 11). Importantly, it is reported that *CASC2* can play a tumor suppressive role in CRC through sponging *miR-18a* (12). However, the mechanism of action of *CASC2* in CRC still awaits more investigation.

As reported, lncRNA can serve as a competing endogenous RNA (ceRNA) to regulate targeted gene expression via sponging microRNA (miRNA) (13). Besides, miRNA dysregulation is a feature of CRC (14, 15). However, the lncRNA-miRNA network in CRC is still partly covered and requires further research. MiR-18a-5p has been confirmed to be a promoter in many cancers including CRC (16). However, the interaction mechanism between *CASC2* and *miR-18a-5p* in CRC has not been clarified.

B cell translocation gene 3 (*BTG3*) belongs to B-cell translocation gene / Transducer of ERBB2 (BTG/TOB) family. *BTG3* is a crucial participant in regulating cell proliferation, differentiation and apoptosis (17, 18). It has been reported that *BTG3* overexpression suppresses the malignancy of CRC cells through regulating Wnt/β-

catenin signaling (19). But the hidden role and mechanism of *BTG3* relevant to the progression of CRC are inconclusive. Here we focused on the role and mechanism of *CASC2* in CRC. We investigated the regulatory effects of the *CASC2*/*miR-18a-5p/BTG3* axis on the proliferative, migrative and invasive abilities of CRC cells.

Materials and Methods

Tissue samples

In this experimental study, this work was endorsed by the Suzhou Science and Technology Town Hospital of Nanjing Medical University's Ethics Committee (20190712-006) and all experiments were performed according to "Declaration of Helsinki". Specifically, all subjects provided written informed consent. 65 pairs of cancerous tissues and adjacent non-cancerous tissues were available from CRC patients in the Suzhou Science and Technology Town Hospital of Nanjing Medical University. All samples were kept in liquid nitrogen immediately at -196°C after the surgery.

Cell culture and transfection

In this experimental study, the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) was the supplier of CRC cell lines HCT116, SW620, LoVo. Colo-678 cells were provided by Honsun Biological Technology Co., Ltd. (Shanghai, China). Normal human colorectal mucosal cell line FHC was acquired from American Type Culture Collection (ATCC). Notably, cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 at 37°C. *MiR-18a-5p* mimics (*miR-18a-5p*), and the mimics negative control (miR-NC) were available from GenePharma (Shanghai, China). *CASC2* sequences with full length were inserted into pcDNA3.0 plasmid to construct *CASC2* overexpression plasmid. Empty pcDNA3.0 vector was adopted as negative control (NC). Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) was employed for transfections as protocols.

Quantitative real-time polymerase chain reaction

Total RNA of tissues and cells isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were reversely transcribed into complementary DNA (cDNA) by a PrimeScript™ RT Reagent kit (Invitrogen, Carlsbad, CA, USA). Next, qRT-PCR was conducted with SYBR®Premix-Ex-Taq™ (Takara, Tokyo, Japan) on the ABI PRISM 7000 Fluorescent Quantitative PCR System. The data were analyzed with $2^{-\Delta\Delta Ct}$ method, with Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *U6* as the endogenous references. The sequences of the primers are:

CASC2-

F: 5′-GCACATTGGACGGTGTTTCC-3′
R: 5′-CCCAGTCCTTCACAGGTCAC-3′

miR-18a-5p-

F: 5′-TCCGAGATAGACGTGATCTA-3′
R: 5′-GTGCAGGGTCCGAGGT-3′

BTG3-

F: 5′-ATGAAGAAAATGAAATTGCTG-3’
R: 5′-TTAGTGAGGTGCTAACATGTG-3’

GAPDH-

F: 5′-GTCAACGGATTTGGTCGTATTG-3′
R: 5′-CCGTTCTCAGCCATGTAGTT-3′

U6-

F: 5′-CTCGCTTCGGCAGCACA-3′
R: 5′- AACGCTTCACGA ATTTGCGT-3′

Subcellular fractionation analysis

Cytoplasmic and nuclear RNA were isolated and extracted from Colo-678 and HCT116 cells by the Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Belmont, CA, USA). Then, the expression of *CASC2* in the cytoplasm and nucleus of Colo-678 and HCT116 cells was investigated by qRT-PCR, with *GAPDH* as a cytoplasmic control and *U6* as a nuclear control.

Methylthiazol tetrazolium (MTT) assay

Transfected Colo-678 and HCT116 cells were inoculated into 96-well plates (3×10^3 cells/well). The cells were cultured for different times. At different time points, 20 μL of MTT solution (5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was dripped into each well, and then the cells were incubated for 4 hours at room temperature. Next, the medium was discarded, and 150 μL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) was loaded into each well to dissolve the formazan. Finally, a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) was used for measuring the absorbance of the cells at 570 nm.

5-bromo-2'-deoxyuridine (BrdU) assay

The viability of transfected cells was examined by a BrdU incorporation assay kit (WuHan AmyJet Scientific Inc. Wuhan, China). Briefly, Colo-678 and HCT116 cells in different groups were respectively plated into a 96-well plate (1×10^3 cells/well). Next, 10 μL of BrdU solution was loaded into each well. Subsequently, 4% paraformaldehyde was added into each well to fix the cells. After the DNA was denatured, anti-BrdU antibody (Abcam, Cambridge, UK) was loaded into each well and cells were subsequently incubated at 4°C overnight. Cells were thoroughly washed by phosphate buffered solution (PBS), and cell nuclei were counterstained by Hoechst 33258 staining solution (Beyotime Institute of Biotechnology, Haimen, China) at ambient temperature for 30 minutes. Immersed in PBS again, cells were accordingly observed under a fluorescent microscope (Olympus BX51, Tokyo, Japan). The number of cells was counted using Image J. The percentage of BrdU positive

cells = the number of red fluorescent cells/the number of blue fluorescent cells × 100%.

Transwell assay

Transwell assays were accomplished with Transwell chambers (pore size: 8 μm; Corning, NY, USA). In the invasion assay, the filter was specifically covered with a layer of Matrigel (30 μg/well; BD, San Jose, CA, USA); in the migration assay, Matrigel was not used. For each well, 1×10^5 cells suspended in serum-free medium was loaded into the upper chamber, with 700 μL of medium containing 20% FBS in the lower chamber as a chemoattractant. Next, cells were cultured for 36 hours, and then cells on the upper surface of the filter were wiped off, and those on the lower were subsequently fixed with ethanol and stained with 0.2% crystal violet solution. Subsequently, the cells were meticulously observed and photographed under an inverted microscope (Nikon TE2000-S, Tokyo, Japan). Specifically, the number of stained cells after migration or invasion was counted with the Image J software.

Dual-luciferase reporter gene assay

Through StarBase software, we found putative binding sites between *miR-18a-5p* and *CASC2* or BTG2. Also, bioinformatics analysis predicted a binding site between *miR-18a-5p* and *CASC2*. Then the wild type (WT) and mutant type (MUT) predicted binding sequences of *CASC2* were subsequently synthesized and cloned into pmirGLO vector (Promega, Madison, WI, USA) to construct *CASC2*-WT and *CASC2*-MUT reporter plasmids. Next, the CRC cells were co-transfected with *CASC2*-WT or *CASC2*-MUT and *miR-18a-5p* or miR-NC. 48 hours later, the relative luciferase activity of the cells in each group was examined by the dual-luciferase assay system (Promega). The targeting relationship between *miR-18a-5p* and *BTG3* was validated in the same way.

RNA immunoprecipitation assay

RNA immunoprecipitation (RIP) assay was accomplished employing the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Colo-678 and HCT116 cells (2×10^7 cells/mL) were collected and subsequently lysed in RIPA buffer containing a protease inhibitor cocktail and RNase inhibitor. Notably, cell lysates were specifically incubated with magnetic beads containing immunoglobin G antibody (IgG, ab172730, Abcam, Cambridge, UK) or argonaute-2 antibody (Ago2, ab186733, Abcam, Cambridge, UK) at 4°C overnight. The samples were accordingly incubated with proteinase K along with shaking at 55°C. Subsequently, the immunoprecipitated RNA was isolated. Ultimately, the enrichment of *CASC2* and *miR-18a-5p* was probed by qRT-PCR. Specifically, the group without antibody was the positive control (Input), the group with IgG antibody was the negative control (anti-IgG), and the group with Ago2 antibody was the experimental one (anti-Ago2).

Western blot

CRC cells harvested were lysed in RIPA lysis buffer (Pierce, Rockford, IL, USA) on ice for 30 minutes to extract the total protein. Notably, the total protein in each sample was respectively quantified by a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Then the protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), which was blocked with 5% skimmed milk for 2 hours at 37°C and incubated with primary antibodies against *BTG3* (1:1000, ab197399, Abcam, Cambridge, UK) or *GAPDH* (1:1000, ab181602, Abcam) overnight at 4°C. Next, the membranes were rinsed three times with Tris-buffered saline containing Tween-20 (TBST) and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hours at ambient temperature. At last, the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, UK) was adopted for observing the protein band. *GAPDH* was utilized as the endogenous reference.

Statistical analysis

The analysis was fulfilled by SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Each experiment was independently replicated 3 times, with data expressed as mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used to the for normality and equal variance of the data. For data that were skewed distributed, comparisons between two groups were performed by the Mann-Whitney test. For normally distributed data, comparisons between two groups, among multiple groups were respectively accomplished by *t* test and one-way analysis of variance. Overall survival analysis was operated with Kaplan-Meier plots and log rank tests. Pearson's correlation analysis was wielded to delve into the relationships among *CASC2*, *miR-18a-5p* and *BTG3* mRNA. Chi-square ($\chi2$) test was executed to analyze the relations between *CASC2* expressions and the clinicopathological features of CRC patients. $P<0.05$ denoted meaningful difference.

Results

CASC2 expression characteristics in CRC and its clinical significance

In the beginning, we evaluated *CASC2* expression characteristics in cancer tissues and tissues adjacent to cancer from 65 patients with CRC by qRT-PCR. As against normal tissues, *CASC2* expression in CRC tissues was remarkably down-regulated (Fig.1A). qRT-PCR was also employed to detect *CASC2* expressions in human normal colon epithelial cell line (FHC cells) and 4 human CRC cell lines (Colo-678, HCT116, SW620, and LoVo cells). It was found that compared with FHC cells, *CASC2* expression in all 4 CRC cell lines was down-regulated (Fig.1B). Subsequently, we analyzed the subcellular

distribution of *CASC2* and observed that *CASC2* was mainly located in the cytoplasm of CRC cells (Fig.1C). Chi-square test was used to analyze the relations between *CASC2* expressions and the clinicopathological indicators of CRC patients, and the results highlighted that *CASC2* low expression was in close association with the larger tumor size and lymph node metastasis of the patients (Table 1). Besides, Kaplan-Meier analysis indicated that low expression of *CASC2* was associated with poor overall survival of CRC patients (Fig.1D). These findings implied that *CASC2* could probably participate in repressing tumor progression in CRC.

Table 1: Correlations between *CASC2* expression and multiple clinicopathological characteristics in colorectal cancer patients

Characteristics	Number (n=65)	*CASC2* expression		χ2	P value
		Low (n=33)	High (n=32)		
Age (Y)				0.7753	0.3785
<60	35	16	19		
≥60	30	17	13		
Gender				0.1641	0.6854
Male	39	19	20		
Female	26	14	12		
Tumor grade				0.1269	0.7216
I~II	27	13	14		
III~IV	38	20	18		
Tumor size (cm)				6.9350	0.0085**
<5	26	8	18		
≥5	39	25	14		
Smoking history				0.7392	0.3899
Yes	38	21	17		
No	27	12	15		
Lymph node metastasis				4.5241	0.0334*
Present	39	24	15		
Absent	26	9	17		
Differentiation				3.4529	0.0631
Well/moderate	29	11	18		
Poor	36	22	14		

*; P<0.05 and **; P<0.01.

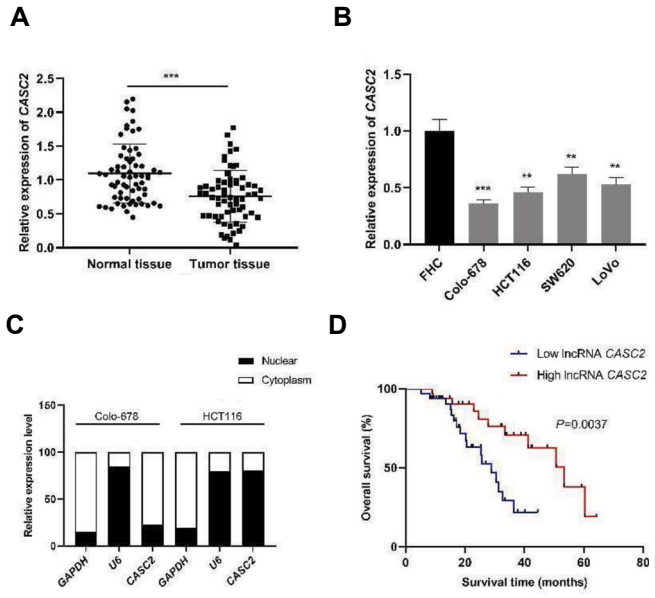

Fig.1: *CASC2* was down-regulated in CRC tissues and cells. **A.** qRT-PCR was used to detect the expression of *CASC2* in CRC tissues and adjacent normal tissues (n=65). **B.** qRT-PCR was adopted to detect the expression of *CASC2* in normal colorectal mucosal cell line (FHC) and CRC cell lines (Colo-678, HCT116, SW620, LoVo cells). **C.** Subcellular fractionation assay was used to measure the expression of *CASC2*, *GAPDH* and *U6* in the nuclei and cytoplasm of Colo-678 and HCT116 cells. **D.** Kaplan-Meier analysis showed a correlation between *CASC2* expression and overall survival of CRC patients. Data are represented as the mean ± SD (n=3). **; P<0.01, ***; P<0.001, CRC; Colorectal cancer, and qRT-PCR; Quantitative real-time polymerase chain reaction.

CASC2 overexpression impeded the malignant progression of CRC cells

To work out the biological function of *CASC2* in CRC cells, we transfected *CASC2* overexpression plasmids into Colo-678 and HCT-116 cells to construct cell models with *CASC2* overexpression (Fig.2A). MTT, BrdU and Transwell assays revealed that in comparison with the control group, *CASC2* overexpression greatly inhibited the proliferative, migrative and invasive abilities of Colo-678 and HCT-116 cells (Fig.2B-E).

CASC2 directly targeted miR-18a-5p in CRC cells

To expound the downstream mechanism of *CASC2*, bioinformatics analysis was performed, and a binding site between *CASC2* and *miR-18a-5p* was predicted (Fig.3A). Besides, *miR-18a-5p* expressions in 65 pairs of CRC tissues and tissues adjacent to cancer were then evaluated by qRT-PCR. As against normal tissues adjacent to cancer, *miR-18a-5p* was observed to be highly expressed in CRC tissue (Fig.3B). Additionally, *miR-18a-5p* expression was inhibited in Colo-678 and HCT116 cells with *CASC2* overexpression (Fig.3C). Besides, we observed a negative correlation between *CASC2* and *miR-185-5p* expression in CRC tissue (Fig.3D). In addition, dual-luciferase reporter gene assay revealed that *miR-18a-5p* mimics could demonstrably restrain the luciferase activity of wild-type *CASC2* reporter, but make no difference on that of mutant *CASC2* reporter (Fig.3E). RIP assay highlighted that *CASC2* and *miR-18a-5p* were significantly enriched in anti-Ago2 group of Colo-678 and HCT116 cells (Fig.3F). Collectively, *CASC2* could target *miR-18a-5p* and regulate its expression negatively.

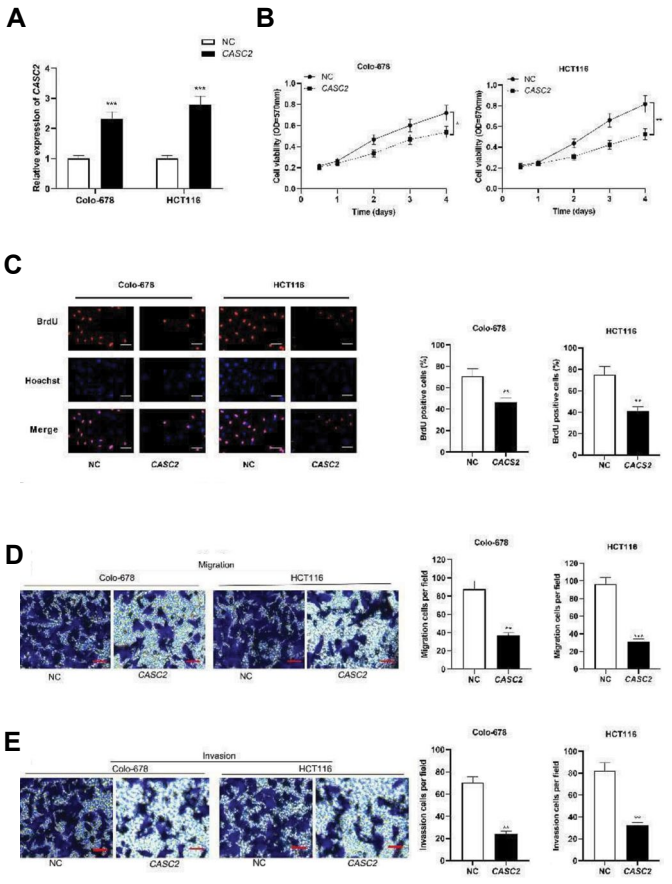

Fig.2: CASC2 overexpression inhibited CRC cell proliferation, migration and invasion**. A**. qRT-PCR was used to detect the expression of *CASC2* in Colo-678 and HCT116 cells transfected with the *CASC2* overexpression plasmid. **B, C.** MTT and BrdU assays were employed to detect the proliferation of CRC cells with *CASC2* overexpression (scale bar: 100 μm). **D, E.** Transwell assay was used to detect the migration and invasion of CRC cells with *CASC2* overexpression (scale bar: 250 μm). The data were analyzed by independent samples t test or one-way ANOVA. Data are represented as the mean ± SD (n=3). *; P<0.05, **; P<0.01, ***; P<0.001, NC; Negative control, CRC; Colorectal cancer, qRT-PCR; Quantitative real-time polymerase chain reaction, MTT; Methylthiazol tetrazolium, and BrdU; 5-bromo-2'-deoxyuridine.

MiR-18a-5p counteracted the biological functions of CASC2 in CRC cells

To expound the role of *CASC2/miR-18a-5p* axis in CRC, *miR-18a-5p* mimics was transfected into Colo-678 and HCT116 cells with CASC2 overexpression.

qRT-PCR showed that the transfection of *miR-18a-5p* mimics reversed the declination of *miR-18a-5p* expression caused by *CASC2* overexpression (Fig.4A). Next, MTT assay, wound healing assay and transwell assay revealed that *miR-18a-5p* restoration counteracted the inhibiting impacts of *CASC2* overexpression on the proliferative, migrative and invasive abilities of Colo-678 and HCT116 cells (Fig.4B-E). These results implied that *CASC2* inhibited the progression of CRC cells via targeting *miR-18a-5p*.

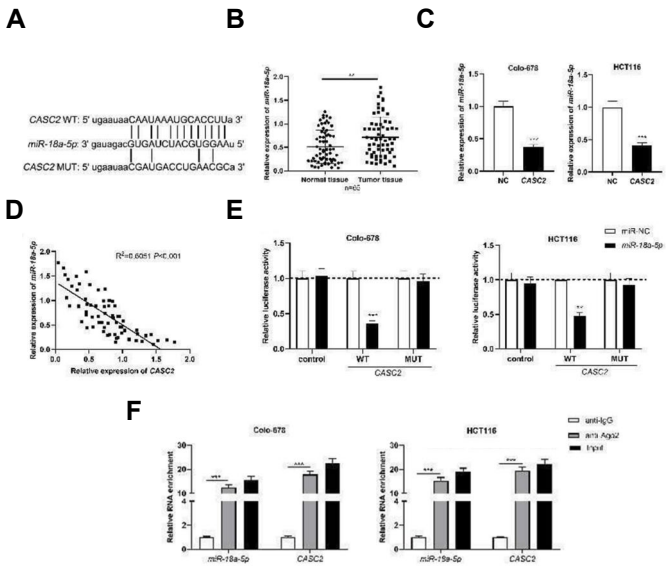

Fig.3: MiR-18a-5p was the target of CASC2 in CRC. **A.** StarBase software (http://starbase.sysu.edu.cn/) predicted a binding site between *CASC2* and *miR-18a-5p*. **B.** qRT-PCR was used to detect the expression of *miR-18a-5p* in CRC tissues and adjacent normal tissues (n=65). **C.** qRT-PCR was used to detect the expression of *miR-18a-5p* in CRC cells with *CASC2* overexpression. **D.** Pearson's correlation analysis was adopted to analyze the correlation between *CASC2* expression and *miR-18a-5p* expression in CRC tissue samples. **E.** Dual-luciferase reporter gene assay was employed to verify the targeting relationship between *miR-18a-5p* and *CASC2*. **F.** The interaction between *CASC2* and *miR-18a-5p* in Colo-678 and HCT116 cells was detected by RIP assay. The data were analyzed by Mann-Whitney test, one-way ANOVA or independent samples *t*-test. Data are represented as the mean ± SD (n=3). **; P<0.01, ***; P<0.001, CRC; Colorectal cancer, qRT-PCR; Quantitative real-time polymerase chain reaction, and RIP; RNA immunoprecipitation.

BTG3 was the target of miR-18a-5p

Next, we searched the targets of *miR-18a-5p* using the StarBase database, and the results showed that *miR-18a-5p* could probably bind to *BTG3* 3′-UTR (Fig.5A). Dual-luciferase reporter gene assay indicated that the transfection of miR-18a-5p mimics dramatically repressed the luciferase activity of wild-type *BTG3* reporter but could not repress that of the mutant type *BTG3* reporter (Fig.5B). qRT-PCR revealed that *BTG3* mRNA expression was markedly reduced in CRC tissues and cells (Fig.5C, D). Additionally, western blot assay showed that in comparison with FHC cells, BTG3 protein expression was declined in CRC cell lines (Fig.5E). Transfection of *miR-18a-5p* mimics reduced *BTG3* mRNA and protein expressions in CRC cells; *CASC2* overexpression induced the up-regulation of *BTG3* in CRC cells, and this effect could be diminished by *miR-18a-5p* overexpression (Fig.5F, G). Notably, *BTG3* mRNA expression was in negative correlation with *miR-18a-5p* in CRC tissue (Fig.5H), but in positive correlation with *CASC2* expression (Fig.5I). The aforementioned results confirmed that *BTG3* was a downstream target of *miR-18a-5p*, and its expression was negatively modulated by *miR-18a-5p* and positively modulated by *CASC2*.

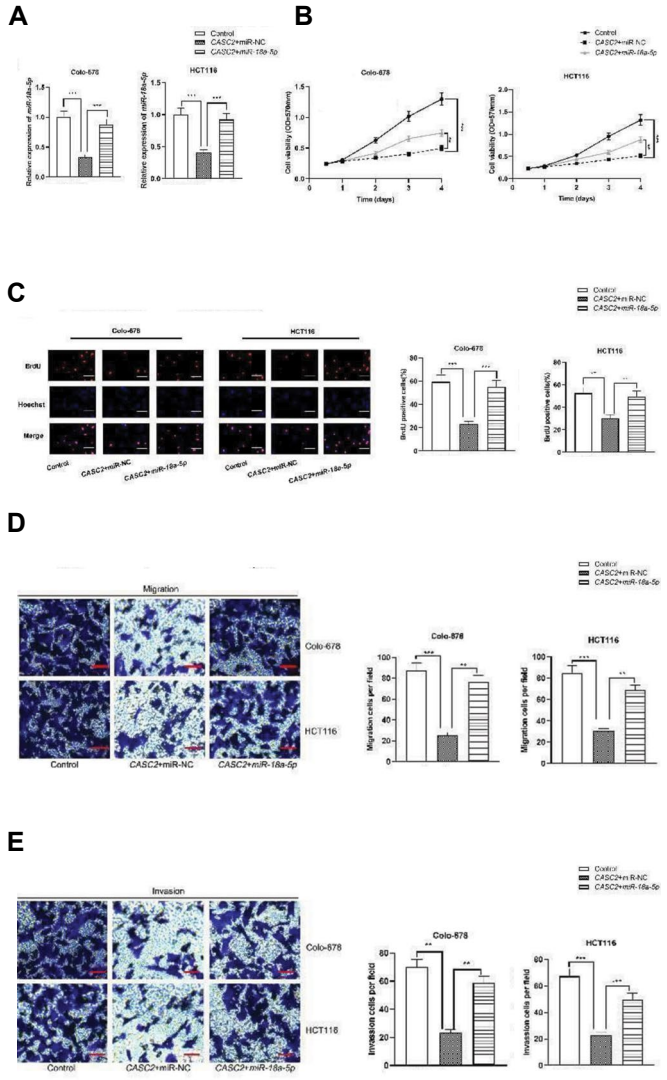

Fig.4: MiR-18a-5p partially reversed the inhibitory effects of CASC2 on CRC cells. **A.** *MiR-18a-5p* mimics were transfected into Colo-678 cells and HCT116 cells with *CASC2* overexpression, and the expression of *miR-18a-5p* in CRC cells was detected by qRT-PCR. **B, C.** MTT assay and BrdU assay were used to detect the proliferation of Colo-678 and HCT116 cells after the transfection (scale bar: 100 μm). **D, E.** Transwell assay was used to detect the migration and invasion of Colo-678 and HCT116 cells after the transfection (scale bar: 250 μm). Data are represented as the mean ± SD (n=3). The data were analyzed by one-way ANOVA. **; P<0.01, ***; P<0.001, CRC; Colorectal cancer, qRT-PCR; Quantitative real-time polymerase chain reaction, MTT; Methylthiazol tetrazolium, and BrdU; 5-bromo-2'-deoxyuridine.

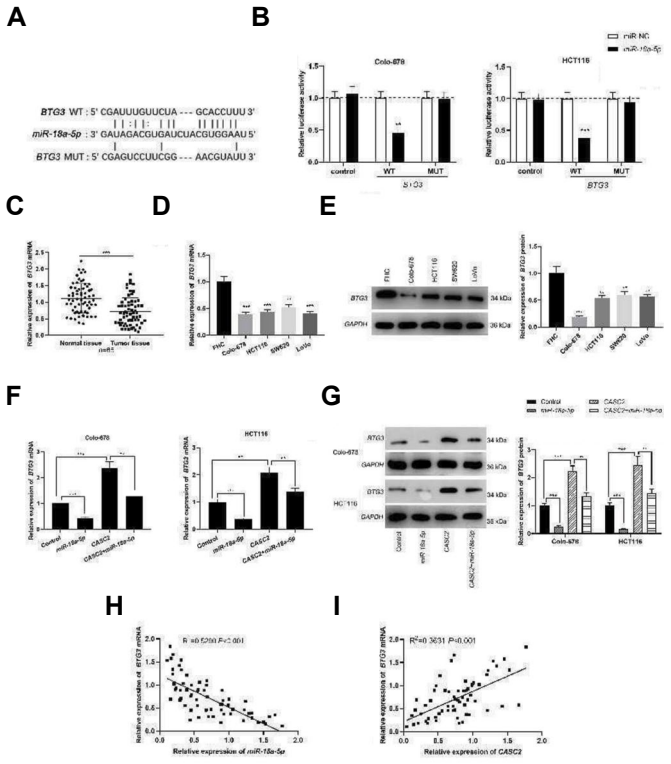

Fig.5: BTG3 was a target of miR-18a-5p in CRC. **A.** StarBase software (http://starbase.sysu.edu.cn/) predicted a binding site between *BTG3* 3´UTR and *miR-18a-5p*. Other potential targets of *miR-18a-5p* predicted on the StarBase website were detailed in Table S1 (See Supplementary Online Information at www.celljournal.org). **B.** Dual-luciferase reporter gene assay was used to verify the targeting relationship between *miR-18a-5p* and *BTG3*. **C.** qRT-PCR was used to detect the expression of *BTG3* mRNA in CRC tissues and adjacent normal tissues (n=65). **D.** The expression of *BTG3* mRNA in normal colorectal mucosal cell line (FHC) and CRC cell lines (Colo-678, HCT116, SW620, LoVo cells) was detected by qRT-PCR. **E.** Western blot assay was used to detect the expression of *BTG3* protein in CRC cell lines (Colo-678, HCT116, SW620, LoVo cells) and FHC cells. The original blot images were provided in Figures S1, S2 (See Supplementary Online Information at www.celljournal.org). **F, G.** qRT-PCR and western blot assays were used to detect the expression of *BTG3* mRNA and protein in CRC cells transfected with *miR-18a-5p* mimic, *CASC2* overexpression plasmid or co-transfected with *miR-18a-5p* and *CASC2*, respectively. The original blot images were provided in Figure S3 (See Supplementary Online Information at www.celljournal.org). **H, I.** Pearson's correlation analysis was used to detect the correlations between *BTG3* mRNA and *miR-18a-5p*, or *BTG3* mRNA and *CASC2* expression in CRC tissues. Data are represented as the mean ± SD (n=3). The data were analyzed by Mann-Whitney test or one-way ANOVA. **; P<0.01, ***; P<0.001, CRC; Colorectal cancer, and qRT-PCR; Quantitative real-time polymerase chain reaction.

Discussion

In recent years, multiple lncRNAs have been identified as a potential biomarkers and therapy targets for CRC prognosis and treatment (20). As reported, *CASC2* is frequently dysregulated in diverse cancers and modulates a wide range of biological processes (21-23). For instance, *CASC2* up-regulation inhibits the viability and metastasis of breast cancer cells by inhibiting the TGF-β signaling pathway (21); *CASC2* inhibits the malignant biological processes of hepatocellular carcinoma cells through the MAPK signaling pathway (23). Here, we validated that *CASC2* expression was reduced in CRC tissues and cell lines and demonstrated that *CASC2* low expression was associated with the unfavorable clinicopathological indexes; functionally, we also demonstrated that *CASC2* overexpression can suppress the malignant phenotypes of CRC cells. Our findings are consistent with the previous reports (12).

Reportedly, lncRNA can interact with miRNA as a molecular sponge of miRNA, thereby regulating the expression of the target genes of miRNA (24, 25). Besides, some studies have revealed several target miRNAs of *CASC2* in cancer. For example, in hepatocellular carcinoma, *CASC2* inhibits cell viability and induces apoptosis via modulating *miR-24-3p* (26). In addition, CASC2 inhibits the tumorigenesis of hepatocellular carcinoma, melanoma, nasopharyngeal carcinoma and other malignant tumors through sponging *miR-18a-5p* (27-29). However, there are few reports about the target the miRNA of *CASC2* in CRC. Here, we confirmed that *miR-18a-5p* was a direct target of *CASC2* in CRC cells.

As reported, *miR-18a-5p* is pivotal in the pathogenesis of many human diseases including cancers. For example, *miR-18a-5p* accelerates osteosarcoma cell migration and invasion via directly targeting *IRF2* (30); in renal cell carcinoma, *miR-18a-5p* up-regulation can promote cancer cells proliferation, migration, invasion and inhibit apoptosis (31). The present work proved that *miR-18a-5p* expression level was elevated in CRC tissues, which is consistent with previous reports (16). Additionally, we focused on the interactions between *CASC2* and *miR-18a-5p* in CRC. Our research proved that *miR-18a-5p* was a direct target of *CASC2*, and *CASC2* could negatively modulate *miR-18a-5p* expression. Moreover, up-regulation of *miR-18a-5p* could counteract the inhibitory effect of *CASC2* overexpression on the proliferative, migrative and invasive abilities of CRC cells.

To further understand the downstream mechanism of *CASC2*, we focused on studying the downstream target of *miR-18a-5p*. Interestingly, a hidden binding site between *miR-18a-5p* and *BTG3* was revealed by searching the StarBase database. Reportedly, *BTG3* is an important participant in regulating cell growth, differentiation, migration, and DNA damage repair (32, 33). In gastric cancer, *BTG3* down-regulation facilitates the proliferative, migrative and invasive abilities of gastric cancer cells (18). *BTG3* overexpression represses the multiplication and invasion of epithelial ovarian cancer cells via modulating AKT/GSK3β/β-Catenin signaling (34). In this work, we validated that *BTG3* was weakly expressed in CRC tissues and cells. Besides, we confirmed for the first time that there was a targeting relation between *miR-18a-5p* and *BTG3* in CRC cells. Moreover, we reported that *CASC2* could positively regulate *BTG3* expression, probably via suppressing *miR-18a-5p*. Collectively, *CASC2*, *miR-18a-5p* and *BTG3* could form a ceRNA network to regulate the CRC development.

Conclusion

According to our findings, *CASC2* expression is reduced in CRC tissues and cell lines. In addition, *CASC2* can

repress the proliferative, migrative and invasive abilities of CRC cells via targeting *miR-18a-5p* and increasing *BTG3* expression. This work provides a novel mechanism to explain the progression of CRC cells, and restoration of *CASC2* may be a novel therapy strategy for CRC treatment.

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Authors' Contributions

X.H.S.; Conceive and experiments design. L.M.K., J.S., J.L.; Experiments performing. F.X., Q.L.Z.; Data analysis. L.M.K., X.H.S.; Manuscript writing. All authors read and approved the final manuscript.

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