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RESEARCH ARTICLE

# Long noncoding RNA CASC2c inhibited cell proliferation in hepatocellular carcinoma by inactivated ERK1/2 and Wnt/β-catenin signaling pathway

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## Abstract

**Purpose** Long non-coding RNAs (lncRNAs) have been shown to play important roles in tumorigenesis, but their biological functions and the underlying molecular mechanisms remain unclear. Alternative splicing of five exons results in three transcript variants of cancer susceptibility 2 (CASC2): the lncRNAs CASC2a, CASC2b, and CASC2c. CASC2a/b have been found to have crucial regulatory functions in a number of malignancies, but few studies have examined the effects of CASC2c in cancers. The objective of the study was to investigate the role of CASC2c in the proliferation and apoptosis of hepatocellular carcinoma (HCC) cells.

**Methods** This study first investigated the expression levels of CASC2c in tumor tissues, corresponding non-tumor tissues and cells using quantitative real-time polymerase chain reaction. The function and underlying molecular mechanism of CASC2c in human HCC were investigated in QGY-7703 cell line, as well as in gastric cancer (GC) cell and colorectal cancer (CRC) cell.

**Results** In the present work, we observed that CASC2c was significantly down-regulated in HCC tissues and cells. Moreover, its overexpression remarkably inhibited the growth, migration, and invasion of HCC cells in vitro and promoted their apoptosis. Furthermore, we demonstrated that CASC2c overexpression decreased p-ERK1/2 levels in HCC, GC, and CRC cells. Interestingly, while overexpression of CASC2c decreased β-catenin expression in HCC and GC cells, it increased that in CRC cells.

**Conclusion** The lncRNA–CASC2c has a vital role in tumorigenesis and cancer progression, and may serve as a biomarker or therapeutic target in cancer treatment via down-regulation of the ERK1/2 and Wnt/β-catenin signaling pathways.

**Keywords** lncRNA · CASC2c · HCC · ERK1/2 · β-Catenin

## Introduction

Hepatocellular carcinoma (HCC) is among the most malignant tumors, and is the fourth leading cause of cancer-related death worldwide after lung cancer, colorectal cancer and stomach cancer in a recent study [1]. Hepatocarcinogenesis is influenced by many risk factors, including infection with hepatitis B and C viruses, alcohol abuse, autoimmune liver diseases, and drug toxicity [2–5]. For HCC patients, surgery remains the only effective therapy, assisted by chemotherapy or chemoradiotherapy [6–9]. HCC treatment remains a thorny issue, with multidrug resistance being a major obstacle.

Long noncoding RNAs (lncRNAs) are a class of RNA whose members are at least 200 nucleotides in length and do not encode proteins [10–13]. Genome-wide transcriptome studies (RNA-seq, microarrays, and tiling arrays) have led to the discovery of thousands of noncoding RNAs [14, 15]. LncRNAs are involved in numerous important biological phenomena, such as genomic imprinting [16], alteration of chromosome conformation, and allosteric regulation of enzyme activity [17, 18]. Specific patterns of lncRNA

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expression coordinate cell state, differentiation, and development. Importantly, overexpression, deficiency, and mutation of lncRNA genes have been implicated in numerous human diseases [10]. However, the functions of the majority of lncRNAs are unknown, and many may not in fact have appreciable functions.

The lncRNA gene cancer susceptibility 2(*CASC2*), located on chromosome 10q26, was first discovered in the context of endometrial cancer. Alternative splicing of the five exons of *CASC2* generates three transcript variants: CASC2a, CASC2b, and CASC2c [19]. Baldinu et al. found CASC2a to be down-regulated in endometrial cancer and suggested that it may play a tumor suppressive role [20]. In addition, He et al. recorded decreased CASC2a/b levels in non-small cell lung cancer and showed them to be associated with poor prognosis [11]. Consistent with this, Pei et al. reported that down-regulation of CASC2a/b can promote cell proliferation in bladder cancer [21]. Nevertheless, few studies of the function of CASC2c in cancer have been performed. Thus, the aim of the present work was to improve our understanding of the influence of CASC2c on this disease.

## Materials and methods

### Human tissue samples

Hepatocellular cancer tissues and adjacent normal tissue samples were obtained during surgery from 26 patients (16 men and 10 women) aged between 42 and 81 years (60.3 ± 9.8 years). These patients were admitted to hospital between April 2014 and March 2015, and none had received adjuvant treatment before surgery. The samples were frozen immediately and stored at − 80 °C prior to RNA extraction. The patients consented to the sampling process, which was performed according to the instructions of our institute and under the supervision of its ethics committee.

### Cell culture

SMMC-7721, HepG2, QGY-7703, and HuH-6 human hepatocellular cancer cells, L-02 normal hepatic cells, SGC-7901 human gastric cancer (GC) cells, GSE-1 normal gastric cells, SW-620 human colorectal cancer (CRC) cells, and normal human colonic epithelial cells (HCoEpiCs) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37 °C with 5% $CO_2$ in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Cromwell, CT, USA) supplemented with 10% fetal bovine serum (Biological Industries), and 1% penicillin/streptomycin (Solarbio, Beijing, China).

### Vector construction and transfection

The human CASC2c sequence was obtained from the National Center for Biotechnology Information (NCBI). Full-length CASC2c was amplified with HotStarTaq DNA Polymerase (QIAGEN, Valencia, CA, USA) by polymerase chain reaction (PCR), and inserted into the *Kpn*I and *Xba*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) to create the vector pcDNA3.1–CASC2c. The sequences of the primers for cloning were as follows: 5′-CGGGGTACCCCG GGAGAACAGGATGGCCATGT-3′ (forward) and 5′-TGC TCTAGAGCAGCCTTCTCCATGTTGGTCTC-3′ (reverse). Hepatocellular cancer, GC, and CRC cells were transfected when approximately 50–70% confluent (after 24 h of culture) using Opti-MEM and Lipofectamine3000 Reagent (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was tested by quantitative real-time PCR (qRT-PCR).

### qRT-PCR

Total RNA was extracted from tissues and cells with TRIzol (TaKaRa, Shiga, Japan) according to the manufacturer's instructions, before being reverse-transcribed into complementary DNA (cDNA) using PrimeScript Reverse Transcriptase (TaKaRa). qRT-PCR was performed on a LightCycler 480 (Roche, Penzberg, Germany) with 500 ng cDNA and primers specific for CASC2c, and GAPDH, the latter serving as an internal control. This experiment was repeated three times and data were analyzed with the $2^{-\Delta\Delta Ct}$ method. Sequences of the primers used in this procedure were as follows: CASC2c, 5′-TTCCTCTCCCCTTTGGAC TT-3′ (forward) and 5′-TCTGCTTCTGCTGCTGTTGT-3′ (reverse), GAPDH, 5′-TCATGGGTGTGAACCATGAGAA-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse).

### Western blotting

Cells were transfected with pcDNA3.1–CASC2c or the empty plasmid for 48 h for the western blotting experiment. The cells were lysed with RIPA lysis buffer containing fresh protease and phosphatase inhibitors, and the lysates incubated at 4 °C for 15 min. Equal quantities of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel, and transferred onto polyvinylidene fluoride membranes. Western blot was performed by wet transfer. The membranes were incubated overnight at 4 °C with primary antibodies, and subsequently exposed for 1 h to anti-rabbit secondary antibodies. Anti-p-ERK1/2, anti-ERK1/2, anti-β-catenin, anti-p-AKT, anti-AKT,

anti-p-STAT3, anti-STAT3 and anti-GAPDH antibodies (Cell Signaling Technology, Danvers, MA, USA) were used, the latter as an internal control. The phosphorylated antibodies "place/point" of phosphorylation are phospho-Stat3 (Tyr705), phospho-P44/42 (Erk1/2) (Thr202/Tyr204), and phospho-Akt (Ser473). Protein bands were scanned using a Vilber Lourmat (Eberhardzell, Germany) imaging system according to the manufacturer's instructions.

## Cell proliferation assay

Hepatocellular cancer, GC, and CRC cells transfected with pcDNA3.1–CASC2c or the negative control plasmid, as described above, were plated in 96-well plates (2000 cells per well). Cell proliferation at 37 °C in a 5% $CO_2$ incubator was assessed daily over five successive days (days 0, 1, 2, 3, and 4) using CCK-8 assays (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

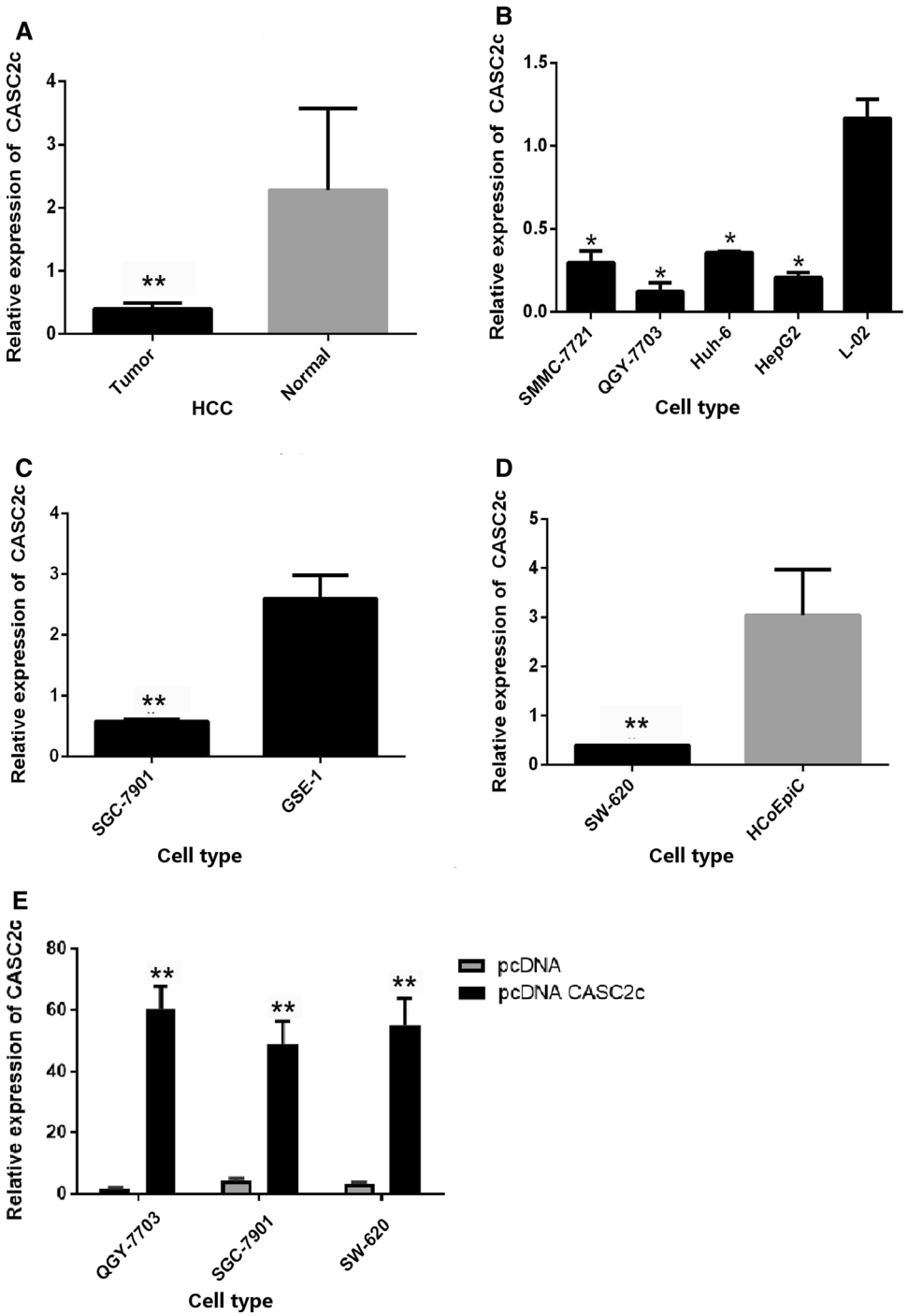


**Fig. 1** CASC2c expression is down-regulated in HCC, CRC, and GC. **a** Relative expression of CASC2c in 26 pairs of hepatocellular cancer tissues and adjacent healthy tissues measured by qRT-PCR and compared with the non-tumor control. **b** CASC2c expression was drastically lower in HCC cells than normal cells. **c** CASC2c expression was decreased in SGC-7901 GC cells compared with GSE-1 normal gastric cells. Expression of CASC2c was higher than that of CASC2a/b in HCC tissues. **d** Expression of CASC2c was lower in SW-620 CRC cells than normal human colonic epithelial cells (HCoEpiCs). **e** The pcDNA–CASC2c significantly up-regulated the expression level of CASC2c in QGY-7703, SGC-7901 and SW-620 cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

### Cell invasion and migration assays

Cell invasion and migration were evaluated using transwell and wound healing assays, respectively. For the transwell assay, $1 \times 10^5$ cells were suspended in serum-free medium and seeded in the upper chambers of the inserts and coated with gelatin (8-μm pore; BD Biosciences, San Jose, CA, USA). In the lower chambers, 500 μl high-glucose DMEM supplemented with 10% fetal bovine serum was placed as a chemoattractant. After incubation of 24 h, the cells on the upper surface of the membrane (i.e., those that had not invaded the lower chamber) were gently removed with a swab, and those having invaded the lower chamber were fixed with methanol and stained with 0.1% crystal violet. The number of invasive cells was calculated by counting them under a microscope (OLYMPUS IX73) at 200× magnification in five random fields of view. For the wound healing assay, $5 \times 10^4$ cells were seeded in 24-well plates and an artificial wound was created using a 200-μl pipette tip. Then, we washed the cells twice with PBS and observed cell migration to the wounded region under a microscope (OLYMPUS IX73) at 40× magnification at the time point we designed. The area beyond the original wound covered by cells was measured to determine the migration rate.

### Apoptosis assay

pcDNA3.1–CASC2c or empty pcDNA3.1 was transfected into cells. Apoptosis was then detected using an annexinV–fluorescein isothiocyanate (FITC) apoptosis kit (Sigma-Aldrich, St. Louis MO, USA) according to the manufacturer's instructions. Annexin V–FITC and propidium iodide (PI) fluorescence levels were measured by flow cytometry (FACSCalibur, BD Biosciences). Annexin V-positive cells (whether PI-negative or -positive) were considered apoptotic.

### Statistical analysis

All data are presented as the mean ± standard deviation (SD) from three independent experiments. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). One-way ANOVA and Student's *t* tests were used for statistical comparisons, as appropriate. Differences were considered to be significant when the associated *P* value was less than 0.05.

## Results

### CASC2 is down-regulated in HCC

To measure CASC2 expression in HCC tissues and cells by qRT-PCR. CASC2c levels were found to be lower in HCC tissues than normal tissues ($P < 0.05$, Fig. 1a). CASC2c was detected in cells of all of the hepatocellular cancer lines tested, and its expression was down-regulated in such cells compared with that in L-02 immortalized normal hepatic cells ($P < 0.01$, Fig. 1b). Levels of the lncRNA were also tested in SGC-7901 GC cells, GES-1 normal gastric cells, SW-620 CRC cells, and normal HCoEpiCs. Our results indicate that CASC2c is down-regulated in HCC, GC, and CRC, and we speculate that CASC2c plays a major role in HCC.

### CASC2c inhibits cell proliferation, migration, and invasion

To assess the influence of CASC2c on cell proliferation, migration, and invasion, pcDNA3.1–CASC2c or empty pcDNA3.1 was transfected according to the manufacturer's

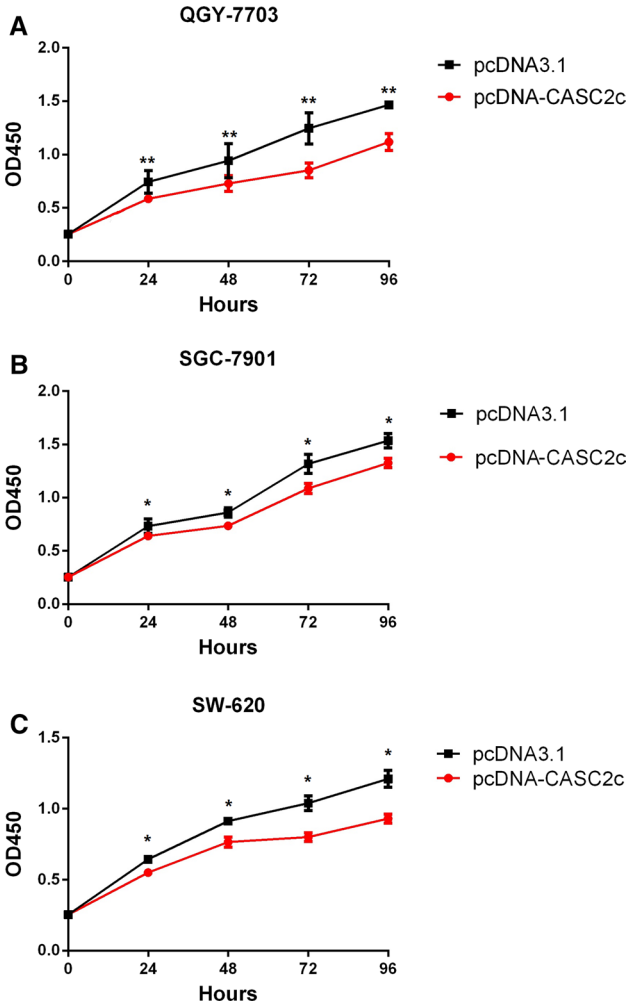


**Fig. 2** Effect of CASC2c overexpression on cell proliferation. **a** A CCK-8 assay revealed that CASC2c overexpression inhibited QGY-7703 cell proliferation. **b** Overexpression of CASC2c restricted growth of SGC-7901 cells. **c** CASC2c overexpression reduced proliferation of SW-620 cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

instructions into QGY-7703 cells cultured for 24 h. qRT-PCR was then performed to confirm the successful overexpression of CASC2c ($P < 0.01$, Fig. 1e). A CCK-8 assay indicated that CASC2c overexpression significantly inhibited QGY-7703 cell proliferation ($P < 0.01$, Fig. 2a). In addition, transwell and wound healing assays demonstrated that overexpression of CASC2c suppressed the migration and invasion of these cells ($P < 0.05$, Fig. 3). We also tested the effects of CASC2c on the regulation of proliferation, migration, and invasion in GC and CRC cells, finding that its overexpression inhibited these behaviors in SGC-7901 and SW-620 cells in an identical manner ($P < 0.05$, Figs. 2b, c, 3).

## CASC2c overexpression promotes apoptosis

To investigate whether overexpression of CASC2c induces apoptosis, pcDNA3.1–CASC2c or empty pcDNA3.1 was transfected according to the manufacturer's instructions into QGY-7703 cells, the apoptosis of which was then analyzed by flow cytometry. The rate of apoptosis was drastically higher among QGY-7703 cells transfected with pcDNA3.1–CASC2c than those transfected with the empty vector ($P < 0.001$, Fig. 4a). In addition, we found that overexpression of CASC2c can also promote apoptosis of GC and CRC cells ($P < 0.001$, Fig. 4b, c).

## CASC2c overexpression inhibits activation of ERK1/2 signaling in HCC

Since CASC2c was found to be able to influence cell proliferation and migration, we next investigated the mechanism by which it regulates such cell behaviors. Many signaling pathways have been shown to be involved in cell growth and migration. In the present study, western blotting was used to

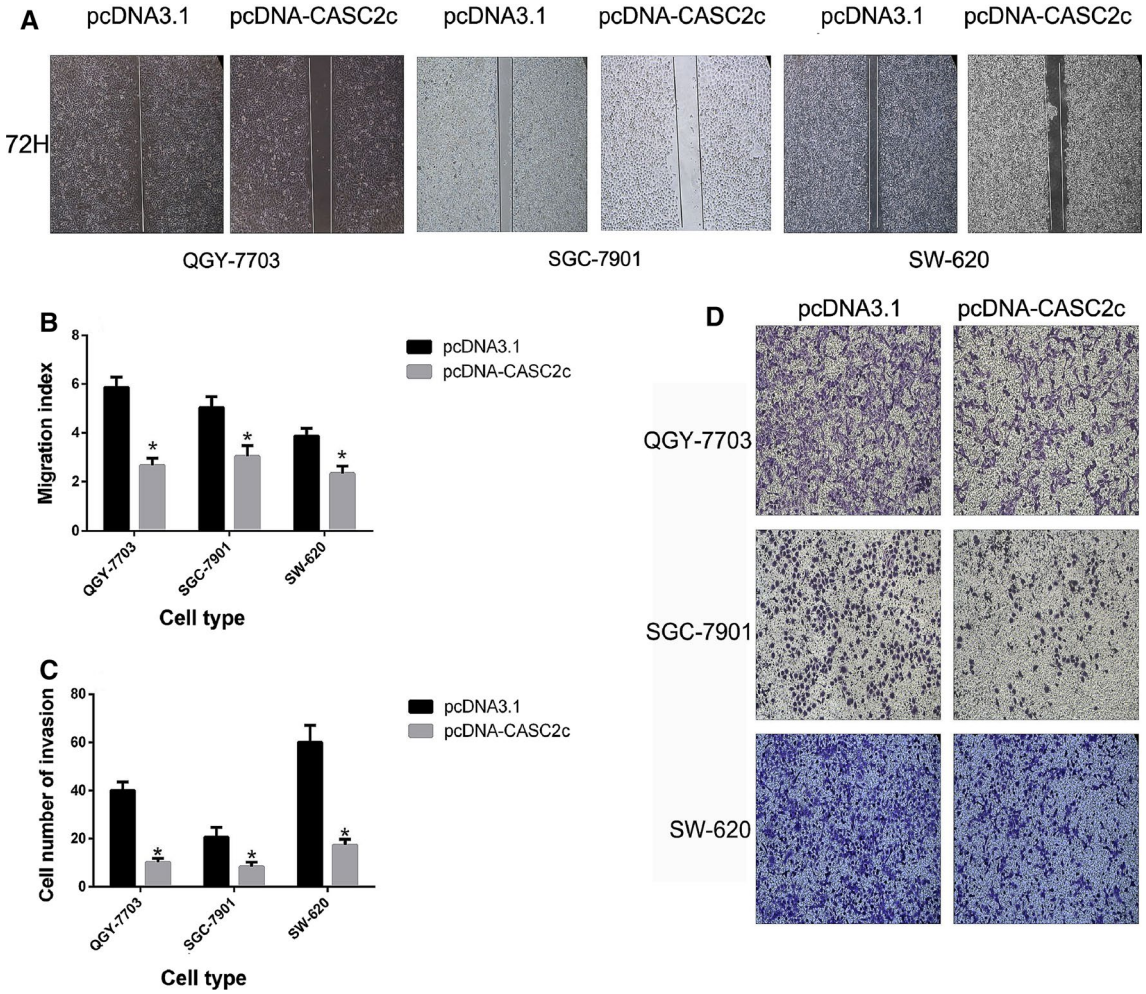


**Fig. 3** CASC2c overexpression inhibited cell migration and invasion. **a**, **b** A wound healing assay showed that overexpression of CASC2c inhibited migration by QGY-7703 (HCC), SGC-7901 (GC), and SW-620 (CRC) cells. For **a**, × 40 magnification was used. **c**, **d** A transwell assay indicated that CASC2c overexpression restrained invasion by QGY-7703 cells, SGC-7901, and SW-620 cells. For **d**, ×100 magnification was used (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

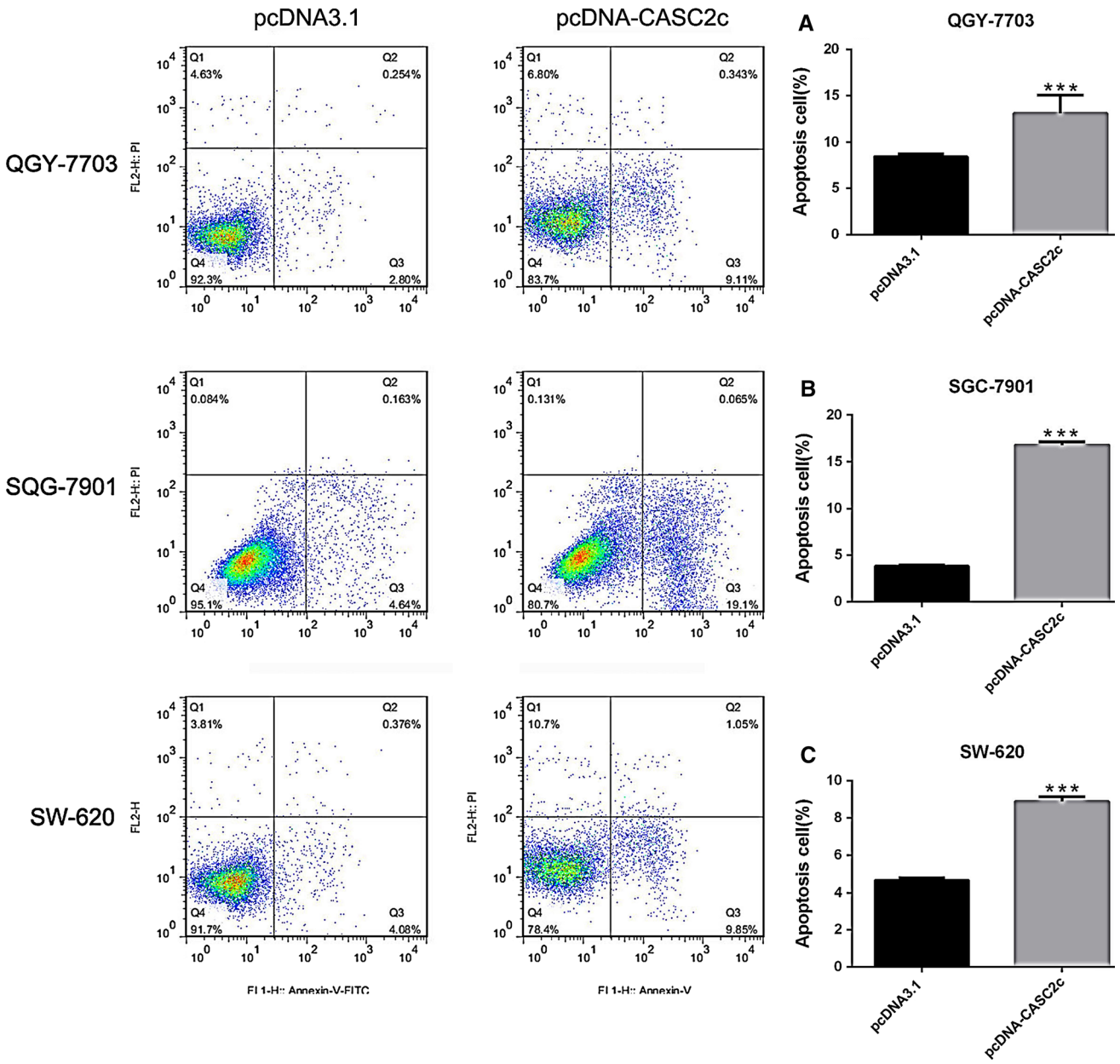


**Fig. 4** Flow cytometry revealed increased apoptosis due to CASC2c overexpression. Overexpression of CASC2c promoted apoptosis of QGY-7703 (**a**), SGC-7901 (**b**), and SW-620 cells (**c**) (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

identify correlations between altered expression of CASC2c and that of signaling pathway components. By transfecting pcDNA3.1–CASC2c or empty pcDNA3.1 into QGY-7703 cells, we established that CASC2c overexpression in HCC cells down-regulated ERK1/2 and Wnt/β-catenin signaling, with levels of p-ERK1/2 and β-catenin being significantly decreased ($P<0.01$, Fig. 5a, b), but no effect on ERK1/2 ($P>0.05$, Fig. 5e). And we found overexpression of CASC2c had no effect in AKT, p-AKT, STAT3 and p-STAT3 signaling pathways ($P>0.05$, Fig. 5c, d, f, g). To test whether CASC2c also affects p-ERK1/2 and β-catenin levels in GC and CRC cells, the expression construct and empty vector were transfected into SGC-7901 and SW-620 cells. Western blotting showed that CASC2c overexpression reduced levels of p-ERK1/2 in cells of both lines ($P<0.01$, Fig. 5a); however, while it inhibited expression of β-catenin in GC cells ($P<0.05$, Fig. 5b), levels of this protein were increased in CRC cells transfected with pcDNA3.1–CASC2c ($P<0.05$, Fig. 5b).

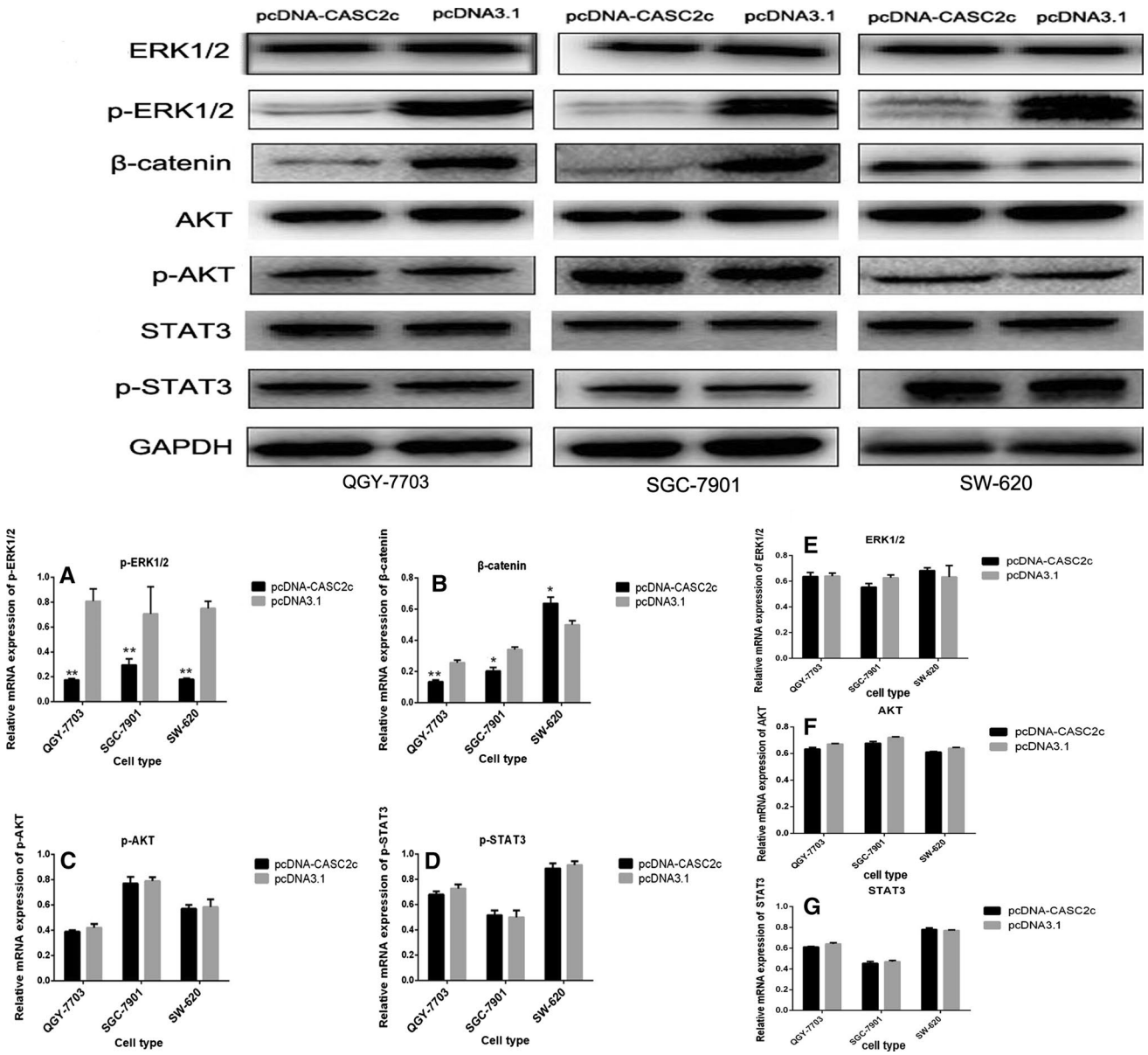


**Fig. 5** Effect of CASC2c on ERK1/2 and Wnt/β-catenin signaling pathways. **a** Overexpression of CASC2c reduced p-ERK1/2 levels in QGY-7703, SGC-7901, and SW-620 cells. **b** Western blotting showed that CASC2c overexpression reduced β-catenin expression in QGY-7703 (HCC) and SGC-7901 (GC) cells, but increased that in SW-620 (CRC) cells. **c**–**g** CASC2c overexpression had no effect on p-AKT, p-STAT3, ERK1/2, AKT and STAT3 levels in QGY-7703, SGC-7901, and SW-620 cells (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

## Discussion

Accumulating evidence suggests that lncRNAs are crucial to a wide range of biological processes [22]. Moreover, functional studies have indicated that some lncRNAs are involved in cancer pathogenesis and progression in humans, acting as oncogenes or tumor suppressors. For instance, Mo et al. reported that the lncRNA XIST is up-regulated in HCC tissues and cell lines and can promote cell growth in this cancer [23], and Yuan et al. established that HCC cell stemness features are increased by DANCR, another lncRNA, via derepression of CTNNB1 [24]. However, the functional and clinical significance of many lncRNAs and the molecular mechanisms responsible remain incompletely understood.

Three different lncRNAs are generated by alternative splicing of five exons of the novel gene *CASC2*: CASC2a, CASC2b, and CASC2c. By comparing sequences obtained from the NCBI database, we observed that CASC2a and CASC2b are very similar, but that CASC2c clearly differs

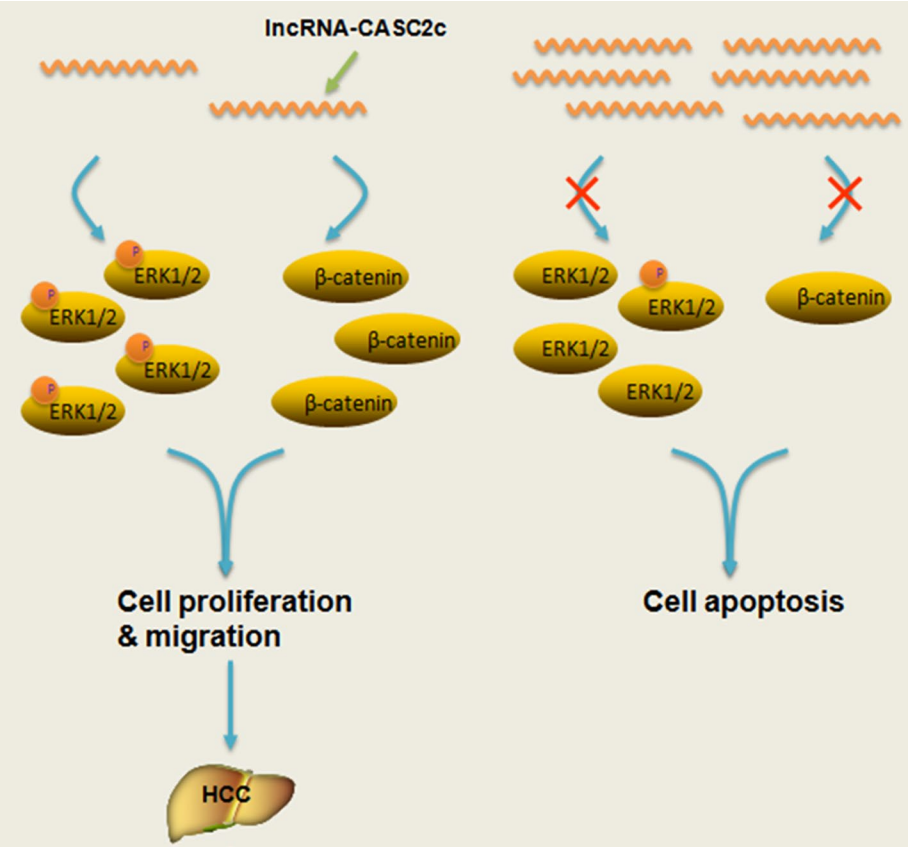


**Fig. 6** The schema of the effect of CASC2c on tumorigenesis of hepatocellular carcinoma

from these isoforms. Previous studies have demonstrated that CASC2a/b are involved in several malignancies, including GC and CRC, among others, but few reports concerning the influence of CASC2c in cancers have been published. In the current study, we measured the expression of CASC2c in HCC tissues and QGY-7703, SMMC-7721, HepG2, and HuH-6 cells and compared it to that in normal tissues and L-02 cells by qRT-PCR. Furthermore, we also tested levels of these lncRNAs in SW-620 CRC cells and SGC-7901 GC cells. CASC2c was found to be down-regulated in HCC tissues and HCC, CRC, and GC cells. A CCK-8 assay was used to test cell proliferation, and transwell and wound healing assays were employed to measure cell migration and invasion. Apoptosis was assessed with flow cytometry. Overexpression of CASC2c was found to inhibit the proliferation, migration, and invasion of HCC cells and promote their apoptosis. The same effect was observed using CRC and GC cells. Many signaling pathways serve critical functions in various cellular events, including regulation of gene expression, growth, metabolism, apoptosis, and metastasis. In this study, we attempted to identify the signaling pathways associated with CASC2c. Western blotting revealed that CASC2c is able to regulate ERK1/2 and Wnt/β-catenin signaling, the first time that this relationship has been described. We found that overexpression of CASC2c resulted in significantly lower levels of p-ERK1/2 and β-catenin in HCC cells (Fig. 6). Based on these results, we then aimed to establish whether CASC2c also affects the presence of these proteins in CRC and GC cells. Overexpression of this lncRNA reduced p-ERK1/2 levels in both cell types. However, to our confusion, while it decreased β-catenin expression in GC cells, it increased that in CRC cells. The specific mechanism underlying these contrasting effects is unknown. CASC2c may regulate other target genes or signaling pathways to modify β-catenin expression, and this is an area that merits further investigation.

In summary, we found CASC2c to be down-regulated in HCC tissues, and observed that its overexpression inhibited the proliferation, migration, and invasion and promoted the apoptosis of HCC, CRC, and GC cells. Furthermore, although CASC2c overexpression down-regulated ERK1/2 signaling in all of these cell types, it inhibited the β-catenin expression in HCC and GC cells and activated Wnt/β-catenin signaling in CRC cells. To conclude, CASC2c may constitute a cancer prognostic marker and therapeutic target.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights statement** This article does not contain any studies with animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.



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