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Original Article

Long non-coding RNA CASC2 suppresses the proliferation of gastric cancer cells by regulating the MAPK signaling pathway

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Abstract: Long non-coding RNAs (lncRNAs) have been shown to play important roles in tumorigenesis. However, the biological functions and molecular mechanisms underlying aberrantly expressed lncRNAs in gastric cancer (GC) is still unclear. In this study, a novel lncRNA CASC2 was chosen to evaluate the effect on GC cell growth and the possible mechanism. Our results showed that CASC2 was significantly downregulated in human GC tissues and cell lines by quantitative RT-PCR. Overexpression of CASC2 in GC cells significantly inhibited the cell growth in vitro and in vivo. We further found that MAPK pathway especially the ERK1/2 and JNK component were involved in the CASC2 mediated GC cell proliferation. Moreover, combination treatment of CASC2 overexpression and suppression ERK1/2 or JNK produced synergistic inhibitory effects in vitro. Thus, these results indicated that CASC2 might serve as a tumor suppressor lncRNA that suppressed cell proliferation by inactivation of MAPK pathway. These findings suggested that CASC2 could be a potential therapeutic target for the treatment of GC patients.

Keywords: Long non-coding RNAs, CASC2, gastric cancer, proliferation, MAPK

Introduction

Gastric cancer (GC) is one of the most common tumors and the second leading death-related tumor worldwide [1]. GC is a complex genetic disease and several genes, including oncogenes or tumor suppressors related to the initiation and progression of GC have been identified [2]. Despite recent advances in therapeutic strategies such as radiotherapy, surgery, and adjuvant chemotherapy, the prognosis of GC remains poor [3]. Therefore, it is crucial to elucidate the regulatory network underlying gastric carcinogenesis to develop novel biomarkers for diagnosis and targeted therapy.

In recent years, genome-wide transcriptional studies found that only approximately 1% of the human genome serves as blueprints for proteins, whereas a much larger proportion of the genome is transcribed into non-coding RNAs [4, 5]. Among these non-coding RNAs are long non-coding RNAs (lncRNAs) which are more than 200 nucleotides in length with little protein-coding potential [6]. In recent years, several lncRNAs have been shown to be involved in carcinogenesis and cancer progression. For example, Zhang et al showed that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma [7]. Li et al showed that overexpression of lncRNA UCA1 could predicted a poor prognosis in patients with esophageal squamous cell carcinoma [8]. Shi et al found that lncRNA SPRY4-IT1 could increase the proliferation of human breast cancer cells by upregulating ZNF703 expression [9].

The long non-coding RNA cancer susceptibility candidate 2 (lncRNA CASC2) located at chromosome 10q26, was originally identified as a downregulated gene in endometrial cancer and acted as a tumor suppressor gene. Genomic and cDNA sequence comparisons revealed the presence of three alternatively spliced CASC2 transcripts (CASC2a, CASC2b and CASC2c) that share the first three exons but contain different downstream exons [10]. Exogenous expression of CASC2a in undifferentiated endometrial cancer cells significantly suppressed the

clonal growth [11]. Wang et al showed that lncRNA CASC2 suppressed malignancy in human gliomas via negative regulation of miR-21, which may be a novel therapeutic target for treating gliomas [12]. He et al indicated that low expression of lncRNA CASC2 indicated a poor prognosis and regulated cell proliferation in non-small cell lung cancer [13]. However, the expression and functional role of lncRNA CASC2 in GC is still unknown.

In the present study, we found that CASC2 expression was significantly down-regulated in GC tissues and cell lines. Moreover, we demonstrated that up-regulation of CASC2 expression suppressed GC cell growth in vitro and in vivo. Furthermore, we found that MAPK pathway especially the ERK1/2 and JNK component were involved in the CASC2 mediated proliferation of GC cells. Moreover, combination treatment of CASC2 overexpression and suppression ERK1/2 or JNK produced synergistic inhibitory effects in vitro. Thus, our results provided us a new insight into the role of lncRNAs CASC2 in the carcinogenesis of GC and indicated the potential application of CASC2 in the treatment of GC.

Materials and methods

Tissue specimens

A total of 67 fresh gastric cancer tissues and paired adjacent non-tumor tissues were obtained from patients who had undergone surgical resection of gastric cancer between 20011 and 2013 at the Department of Surgery, Affiliated Hospital of Nantong University. The gastric cancer diagnosis was confirmed by an experienced pathologist. All of the tissue samples were washed with sterile phosphate-buffered saline before being snap frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. No patients had been treated with radiotherapy or chemotherapy before surgery. This study was approved by the Ethics Committee of Nantong University and informed consent was obtained from each patient involved in the study.

Cell culture

The human gastric cancer cell lines SGC-7901, MGC-803, HGC-27, and BGC-823 and normal gastric mucosa cells line GES-1 were obtained from American Type Culture Collection (ATCC). All of the cell lines were grown and maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C in a 5% CO_2 atmosphere.

Plasmid construction and cell transfection

The CASC2 sequence was subcloned into the pcDNA3.1 vector (Invitrogen). CASC2 Ectopic expression was achieved through pcDNA3.1-CASC2 transfection using lipofectamine 2000 (Invitrogen), with an empty pCDNA3.1 vector used as a control. The expression levels of CASC2 were measured by qRT-PCR. Plasmid vectors (pcDNA3.1-CASC2 and pcDNA3.1) for transfection were extracted using Midiprep kits (Qiagen), and respectively transfected into SGC-7901 or BGC-823 cells.

Cell proliferation assays

Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8). Briefly, 100 μl of SGC-7901 or BGC-823 cells were seeded into a 96-well plate at a concentration of 1000 cells per well and were incubated at 37°C. At daily intervals (24 h, 48 h, and 72 h), the optical density was measured at 450 nm using a microtiter plate reader (Quant BioTek Instruments). The results represent the average of three replicates under the same conditions.

Cell cycle analysis

Both non-transfected and transfected SGC-7901 and BGC-823 cells were harvested by trypsinization, washed twice in cold PBS, and fixed in 70% ethanol at 4°C overnight. After fixation, the cells were washed and re-suspended in cold PBS and incubated in a solution of 10 mg/mL RNase and 1 mg/mL propidium iodide (PI, Sigma) at 37°C for 30 min in the dark. Finally, the DNA content was determined by flow cytometry (BD Biosciences). The percentages of the cells in G0/G1, S, and G2/M phases were counted and compared.

Cell apoptosis analysis

SSGC-7901 and BGC-823 cells were harvested 48 h after transfection for apoptosis analysis. Floating and adherent cells were collected using 0.1% trypsin, washed twice with cold PBS, and suspended in 1000 mL binding buffer. The cells were then treated with FITC-

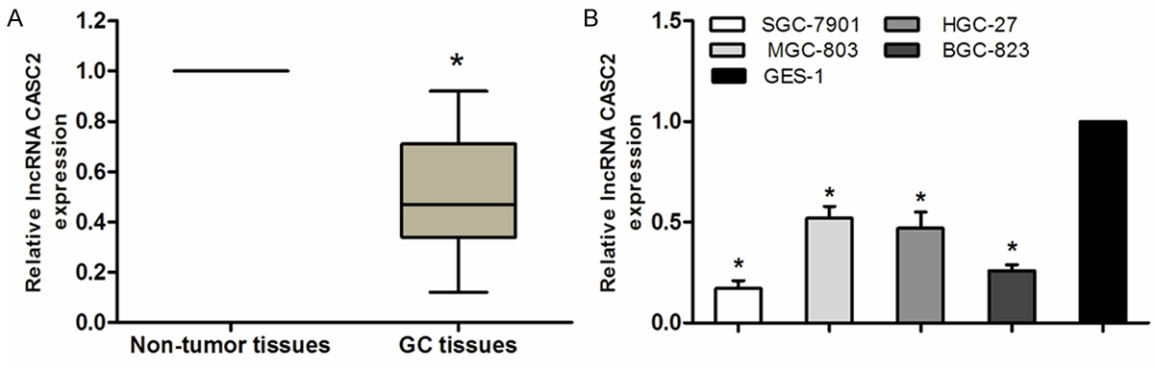

Figure 1. Expressions of lncRNA CASC2 was decreased in both GC tissues and cell lines. A. Relative CASC2 levels in GC tissues and adjacent non-tumor tissues were detected by qRT-PCR. GAPDH was used as an internal control. B. Relative CASC2 levels in GC cell lines (SGC-7901, MGC-803, HGC-27, and BGC-823) and normal gastric mucosa cells line GES-1 were detected by qRT-PCR. *P<0.05.

Annexin V and PI in the dark at room temperature, according to the manufacturer's protocol. The cells were then examined by flow cytometry (BD Biosciences) on in instrument equipped with CellQuest software (BD Biosciences). This assay was repeated three times.

Tumor xenografts in nude mice

Male athymic BALB/c nude mice (6 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). After 4 days of acclimatization, a total of 7×10^6 BGC-823 cells stably transfected with pcDNA3.1 vector or pcDNA3.1-CASC2 (Invitrogen) were injected subcutaneously into the right inside of the groin of each mouse. The mice were sacrificed 22 days after seeding the tumor cells. The tumor volumes were calculated using the following formula: Volume = length × width^2 × 0.5.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from GC tissues and cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The expression level of CASC2 in GC tissues and cell lines was measured by qRT-PCR using the SYBR-Green method (Takara) according to the manufacturer's protocol and normalized using GAPDH. The primers were as follows: CASC2 sense: 5'-GCACATTGGACGGTGTTTCC-3'; CASC2 antisense: 5'-CCCAGTCCTTCACAGGTCAC-3'; GAPDH sense: 5'-AGAAGGCTGGGGCTCATTTG-3'; GAPDH antisense: 5'-AGGGGCCATCCACAGTCTTC-3'. All experiments were performed using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

Western blot

Cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonylfluoride (Roche). Equal amount proteins were separated by 10% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad). Membranes were probed with primary antibodies at 4°C overnight followed by incubation with HRP conjugated secondary antibodies. The following primary antibodies were used in this study: anti-p-ERK1/2, anti-ERK1/2, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK (1:500, all from Abcam). β-actin (Abcam) was used as a loading control. Protein bands were visualized using the ECL-kit according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using SPSS version 18.0. Differences between groups were analyzed using Student's t test or one-way ANOVA analysis. All data are presented as the mean ± SD from at least three independent experiments. *P* values lower than 0.05 was considered statistically significant.

Results

CASC2 levels was down-regulated in gastric cancer tissues and cell lines

Expression of CASC2 in 67 GC patient tissue specimens and matched adjacent non-tumor

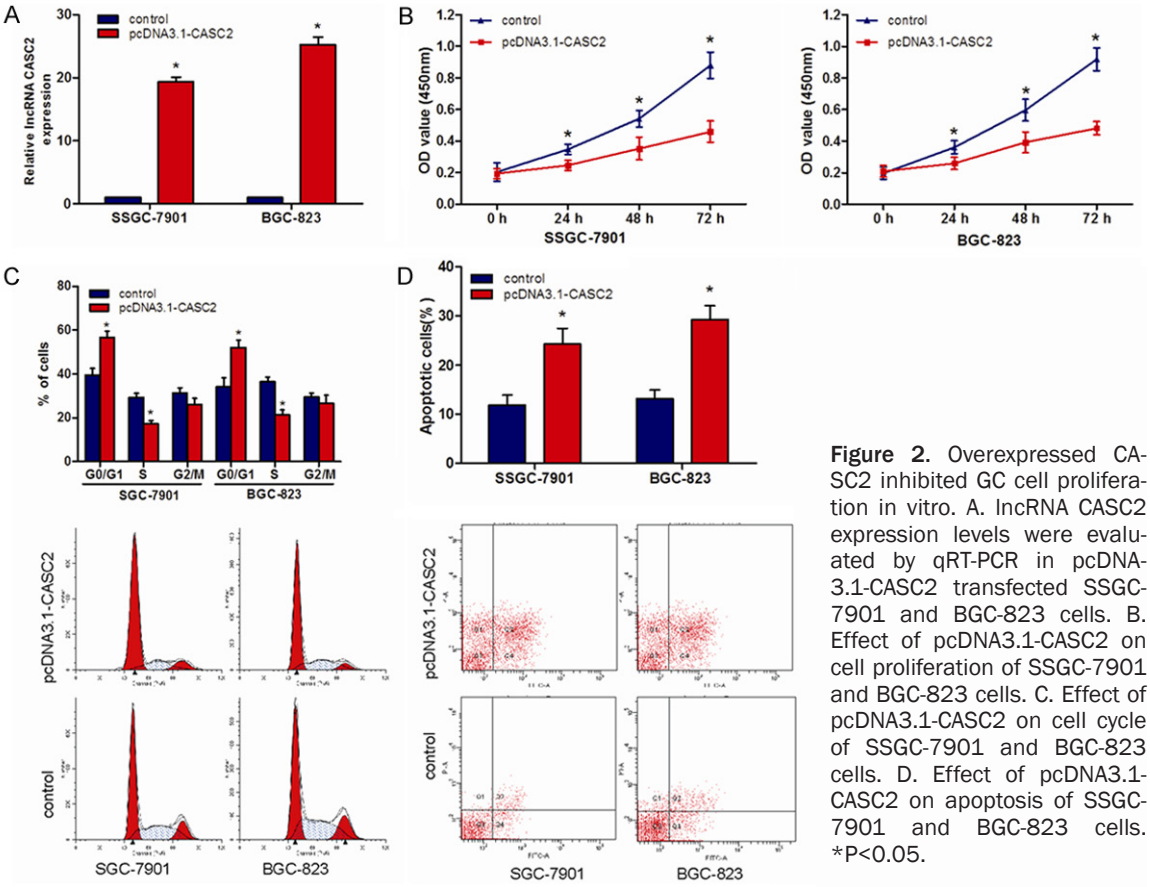

Figure 2. Overexpressed CASC2 inhibited GC cell proliferation in vitro. A. lncRNA CASC2 expression levels were evaluated by qRT-PCR in pcDNA3.1-CASC2 transfected SSGC-7901 and BGC-823 cells. B. Effect of pcDNA3.1-CASC2 on cell proliferation of SSGC-7901 and BGC-823 cells. C. Effect of pcDNA3.1-CASC2 on cell cycle of SSGC-7901 and BGC-823 cells. D. Effect of pcDNA3.1-CASC2 on apoptosis of SSGC-7901 and BGC-823 cells. *P<0.05.

tissues and GC cell lines was detected by qRT-PCR. Compared with the levels of the adjacent non-tumor tissues, a significant down-regulated of CASC2 was observed in GC patients (P<0.05, **Figure 1A**). Furthermore, GC cell lines were also tested, compared with human normal gastric mucosa cells line GES-1, CASC2 expression level was decreased in all four GC cell lines (SGC-7901, MGC-803, HGC-27, and BGC-823) (P<0.05, **Figure 1B**).

CASC2 inhibited GC cell proliferation in vitro

To identify the potential role of CASC2 in tumor progression, we investigated the impact of CASC2 on GC cell proliferation. The successful increased expression of CASC2 in GC cells was confirmed by qRT-PCR (P<0.05, **Figure 2A**). CCK8 assay demonstrated that evaluated expression of CASC2 significantly inhibited cell proliferation of SSGC-7901 and BGC-823 cells compared with the control group (P<0.05, **Figure 2B**). To explore the potential mechanisms underlying cell proliferation inhibition by CASC2 overexpression, flow cytometry was used. Cell cycle analysis showed that overexpression CASC2 expression increased the number of GC cells in G0/G1 phase and decreased the number of cells in S phase compared with the control group (P<0.05, **Figure 2C**). Apoptosis analysis showed that increased expression of CASC2 induced GC cell apoptosis in comparison with the control group (P<0.05, **Figure 2D**). Thus, our findings suggested that CASC2 effectively inhibited cell proliferation both by modulation of the G1-S checkpoint and induced cell apoptosis.

CASC2 inhibited GC cell proliferation in vivo

To further determine the role of CASC2 on tumorigenesis, MGC-803 cells transfected with either pcDNA3.1-CASC2 or pcDNA3.1-control were injected into nude mice. 22 days after injection, a palpable tumor could be observed, As shown in **Figure 3A**, pcDNA3.1-CASC2 significantly reduced tumor growth at the indicated time. In addition, tumors derived from the pcDNA3.1-CASC2 group grew at a slower rate than the pcDNA3.1-control group (P<0.05,

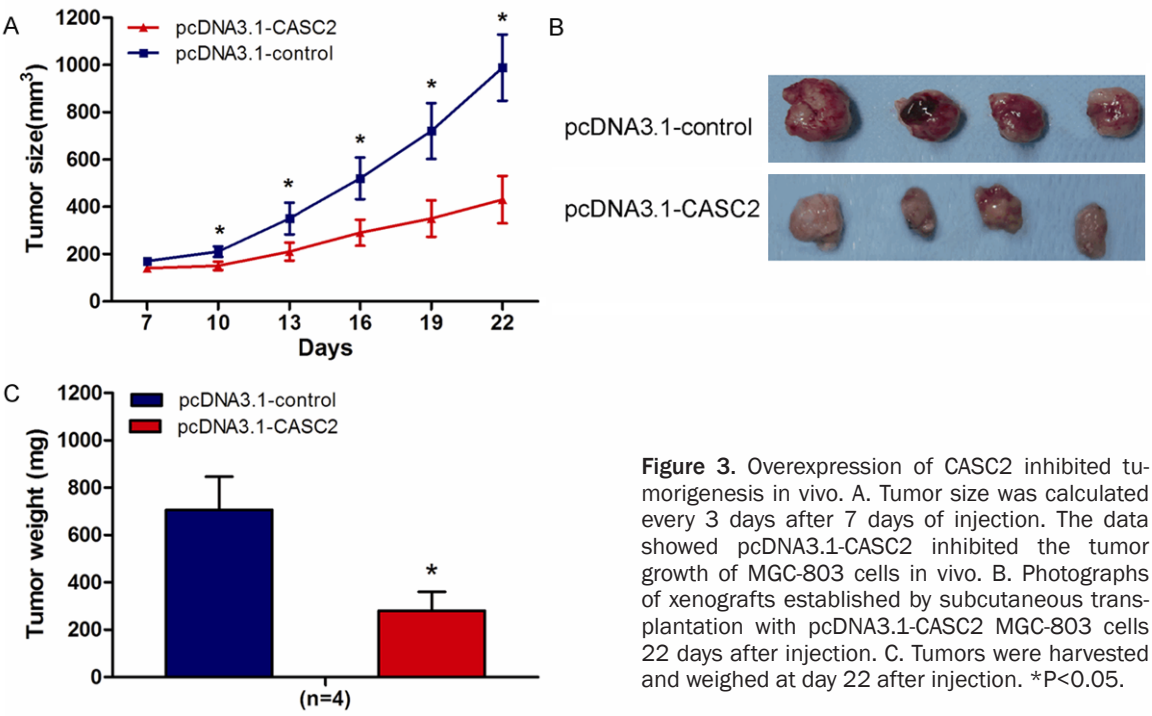

Figure 3. Overexpression of CASC2 inhibited tumorigenesis in vivo. A. Tumor size was calculated every 3 days after 7 days of injection. The data showed pcDNA3.1-CASC2 inhibited the tumor growth of MGC-803 cells in vivo. B. Photographs of xenografts established by subcutaneous transplantation with pcDNA3.1-CASC2 MGC-803 cells 22 days after injection. C. Tumors were harvested and weighed at day 22 after injection. *P<0.05.

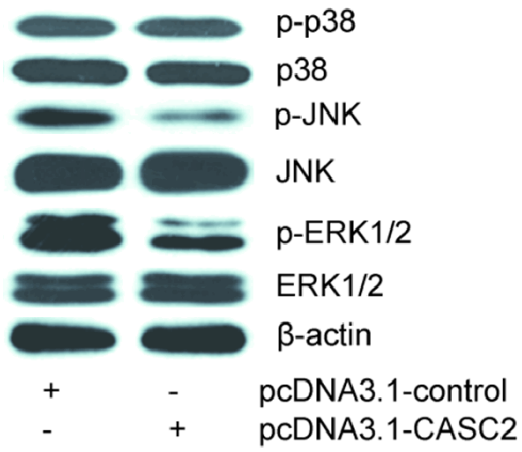

Figure 4. The activities of ERK1/2, and JNK in CASC2 overexpression BGC-823 cells were significantly repressed.

Figure 3B), and the tumor weight in the pcDNA3.1-CASC2 group was significantly less than the pcDNA3.1-control group (P<0.05, **Figure 3C**).

MAPK pathways were involved in the CASC2-mediated cell proliferation of GC

To better understand the detailed regulation mechanism of CASC2 in GC, we first examined whether overexpression CASC2 affect the MAPK signaling pathway, which is often aberrantly activated in human cancers and contributes to enhanced cell proliferation and metastasis. Western blot analysis showed that the levels of p-ERK1/2 and p-JNK were obviously decreased in the cells transfected with pcDNA3.1-CASC2 (P<0.05, **Figure 4**). However, no differences of p-p38 were observed in the cells transfected with pcDNA3.1-CASC2 (P> 0.05, **Figure 4**). Those data suggested that the MAPK pathway might participate in the CASC2 mediated proliferation of GC cells.

CASC2 regulated GC proliferation via ERK1/2 and JNK inactivation

We treated pcDNA3.1-CASC2 transfected cells with specific pharmacological inhibitors U0126 and SP600125 to investigate the effects of ERK1/2 and JNK on CASC2-related proliferation. Western blot was performed to detect total and phosphorylation changes of ERK1/2 and JNK after cells were treated with U0126 and SP600125. Our data showed that inhibitors induced significant inactivation of ERK1/2 (**Figure 5A**) and JNK (**Figure 5C**). Consistently, the cell proliferation decreased significantly (**Figure 5B** and **5D**) after 72 h. These results indicated that the ERK1/2 and JNK pathway inactivation might participate in the CASC2 regulated proliferation of GC cells.

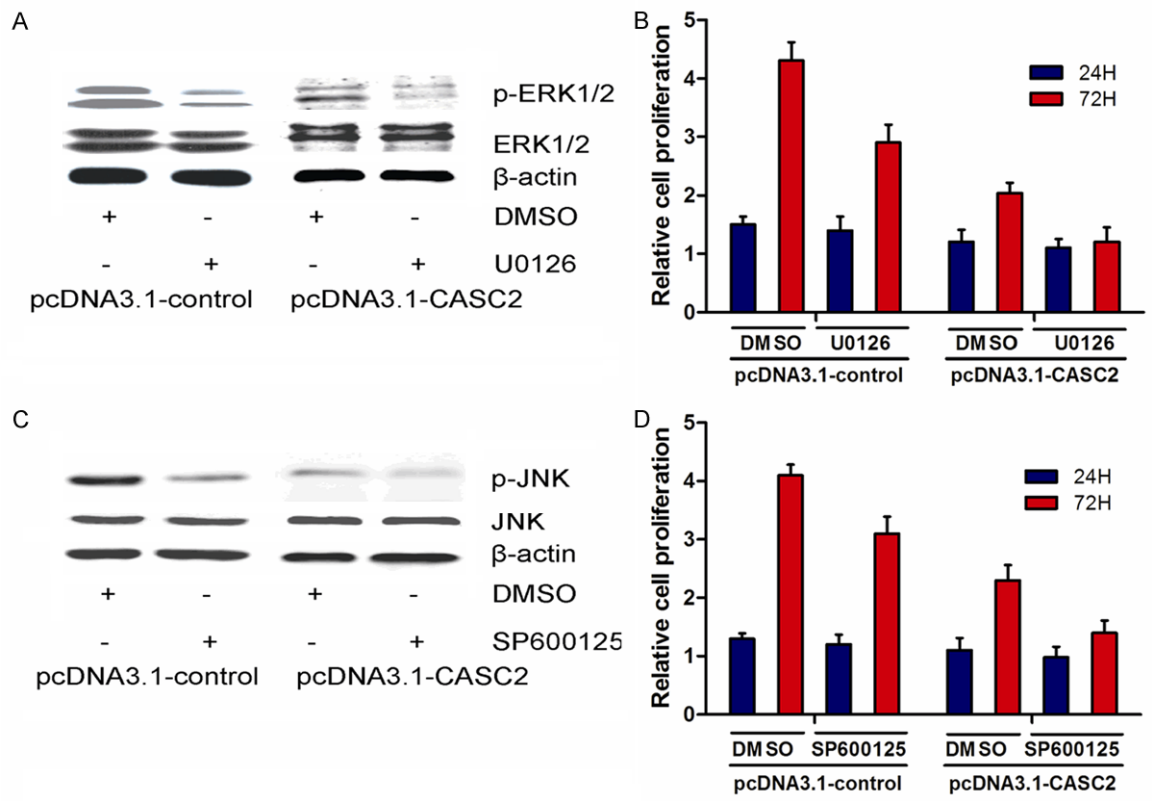

Figure 5. Inactivation of ERK1/2 and JNK participated in CASC2-regulated proliferation. Cells transfected with pcDNA3.1-CASC2 or pcDNA3.1-control plasmid were treated with U0126 or SP600125 respectively. Western blot was performed to detect total and phosphorylation changes of ERK1/2 and JNK. U0126 or SP600125 induced significant inactivation of ERK1/2 (A) and JNK (C). Cell proliferation decreased significantly after 72 h (B and D).

Discussion

Accumulating evidence shows that lncRNAs play more and more important roles in a wide range of biological processes [14]. Functional studies have indicated that some lncRNAs, which may act as oncogenes or tumor suppressors are involved in human cancer pathogenesis, including GC [15, 16]. For example, Ma et al reported that the expression of lncRNA NEAT1 was increased in GC, and NEAT1 might influence GC progression by promoting tumor growth [17]. Zhao et al evaluated that lncRAN LOWEG was low expressed in GC and acted as a tumor suppressor by inhibiting cell invasion [18]. Zhang et al found that increased expression of lncRNA TUG1 predicted a poor prognosis of GC and regulated cell proliferation by epigenetically silencing of p57 [19]. These studies indicated that lncRNAs play important roles during cancer progression.

CASC2, a recently found lncRNA, has been proved to have the ability to regulate non-small cell lung cancer cell proliferation. While, it is also associated with the prognosis of non-small cell lung cancer patients [13]. However, the role of CASC2 in GC is still unclear. In the present study, we found that lncRNA CASC2 was down-regulated in both GC tissues and cell lines. Moreover, we identified the function of CASC2 in GC cells by applying gain-of-function approaches. CCK8 assay showed that overexpression of CASC2 expression inhibited GC cell proliferation, FCM reported that increased expression of CASC2 could modulated the G1-S checkpoint and induced cell apoptosis in vitro. In addition, our in vivo studies showed that overexpression of CASC2 suppressed tumor growth in nude mice. Those data demonstrated that CASC2 may function as a tumor suppressor in GC progression.

MAPK intracellular signaling mediates various biological events in cells, such as gene expression, cell proliferation, differentiation, apoptosis, migration and invasion [20]. Recent studies

indicated that lncRNA could regulate tumor progression via MAPK pathway. For example, Huang et al suggested that HBx-related long non-coding RNA DBH-AS1 could promote cell proliferation and survival by activating MAPK signaling in hepatocellular carcinoma [21]. Wu et al suggested that lncRNA MALAT1 could promote the proliferation and metastasis of gallbladder cancer cells by activating the ERK/MAPK pathway [22]. Jiang et al demonstrated that lncRNA BANCR promoted proliferation and migration of lung carcinoma via MAPK pathways [23]. To elucidate the precise mechanism involved in CASC2-suppressed GC cell proliferation, the effects of CASC2 on MAPK activation or inactivation were examined. We found that MAPK pathways especially the ERK1/2 and JNK component were involved in the CASC2-mediated proliferation of GC cells. Moreover, combination treatment of CASC2 overexpression and suppression ERK1/2 or JNK produced synergistic inhibitory effects in vitro, indicating that overexpression of lncRNA CASC2 inhibit GC cell proliferation by regulating MAPK pathway inactivation.

In conclusion, our data showed that CASC2 was significantly downregulated in GC tissues. Overexpression of CACS2 could inhibit GC cell proliferation both in vitro and in vivo. Moreover, Overexpression of CACS2 led to the inactivation of the ERK1/2 and JNK/MAPK pathway. Therefore, CACS2 might serve as a tumor suppressor lncRNA that inhibits proliferation of GC and inactivates the MAPK pathway.

Disclosure of conflict of interest

None.

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