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ORIGINAL ARTICLE

LncRNA CASC9 Suppressed the Apoptosis of Gastric Cancer Cells through Regulating BMI1

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Abstract

Long noncoding RNAs (lncRNAs) play important roles in regulating the apoptosis of gastric cancer (GC) cells. This study aims to investigate the underlying mechanism of lncRNA CASC9 in regulating the apoptosis of GC cells. The expressions of lncRNA and protein in GC tissues and cell lines were detected by qRT-PCR and western blot. GC cell apoptosis was detected by flow cytometry analysis. RNA pull-down and RNA immunoprecipitation (RIP) assays were conducted to verify the interaction between CASC9 and BMI1. LncRNA CASC9 was upregulated in GC tissue and GC cells, and high CASC9 expression was positively correlated with TNM stage and lymph node metastasis. Silencing CASC9 promoted the apoptosis of GC cells. LncRNA CASC9 could interact with BMI1 and positively regulate BMI1 expression. Silencing CASC9 promoted the ubiquitination of BMI1. In addition, lncRNA CASC9 regulated the apoptosis of GC cells through BMI1. Furthermore, interfering CASC9 inhibited the tumor growth of GC. LncRNA CASC9 could interact with BMI1 to regulate the degradation of BMI1, thus to affect the apoptosis of GC cells and suppressed tumor growth.

Keywords lncRNA CASC9 · BMI1 · Gastric cancer · Ubiquitination · Degradation

Introduction

Gastric cancer (GC) is the third leading cause of death in cancers around the world which can be induced by high salt intake, smoking, virus infection and genetic mutation [1–3]. Although the mortality rate of GC has been decreased in the past few years with the development of methods for diagnosis and treatment [4], the five-year overall survival rate of patients with GC is still lower than 31% in China [5]. Therefore, finding effective treatment of GC is important to improve the survival rate of GC patients. GC cell apoptosis is an important basic process of GC cell life activity. Studies have shown that suppression of GC cell apoptosis promoted the development and progression of GC, whereas induction of GC cell apoptosis decreased tumor growth of GC [6, 7]. Hence, it is meaningful to investigate the molecular mechanism of GC cell apoptosis, which may provide directions for the treatment of GC.

B Lymphoma Mo-MLV Insertion Region 1 (BMI1), as a major component of the polycomb group complex 1, is expressed in many cancer cells which regulate the apoptosis, proliferation and invasion of cancer cells [8, 9]. Many studies have found that BMI1 was involved in the progression of GC [10, 11]. For example, Liu et al. reported that BMI1 was upregulated in GC cells, silencing BMI1 reduced the anti-apoptotic protein Bcl-2 expression and increased caspase 3 expression, thus to induce the apoptosis of GC cells [12]. Studies also reported that BMI1 protein can be regulated by ubiquitination and degradation pathway [13]. However, whether BMI1 can be regulated by ubiquitination and degradation pathway in GC is still not known.

Long noncoding RNAs (lncRNAs) are a kind of RNAs longer than 200 nucleotides that can regulate fundamental molecular and cellular processes [14]. Studies have shown that lncRNAs have potential abilities to regulate proteins with the aid of modifications in cancer [15]. Importantly,

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researchers have found that several lncRNAs induced the ubiquitination of proteins to modulate the progression of GC [16, 17]. Our preliminary experiment screened several lncRNAs from GC tissues and paired adjacent tissues by performing qRT-PCR, and identified lncRNA CASC9 was highly expressed in GC tissues. However, the underlying mechanism of lncRNA CASC9 in GC cell apoptosis is not identified. It has been reported that lncRNA CASC9 could interact with heterogeneous nuclear ribonucleoprotein L (HNRNPL) protein in liver cancer, which indicated that lncRNA CASC9 might interact with proteins to regulate cancer progression [18]. Therefore, we speculated that lncRNA CASC9 may be involved in BMI1-mediated apoptosis of GC cells through interacting with BMI1.

Materials and Methods

Clinical Samples

Twenty primary GC tissues and paired adjacent tissues were collected from patients in The First Affiliated Hospital of Anhui Medical University who did not receive chemotherapy or radiotherapy before surgery and confirmed by histopathological examination. The tissues were kept in liquid nitrogen and transferred to −80 °C refrigerator until use. All patients signed informed consents. And this study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University.

Cell Culture and Transfection

Human normal gastric epithelial cell line GES-1 and GC cell lines (SGC7901 and MKN-45) were purchased from American Type Culture Collection (ATCC, VA, USA). All cells were cultured in RPMI-1640 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA) and maintained in 37 °C, 5% CO2 incubator.

Small interference RNA targeting CASC9 (si-CASC9), pcDNA-BMI1, CASC9-short hairpin RNA (shRNA), and their negative controls si-control, pcDNA, NC-shRNA were all purchased from GENECHEM (Shanghai, China). SGC7901 cells and MKN-45 (5×10^5 cells/well in 6-well plates, 70–80% confluency) were transfected with si-CASC9 or pcDNA-BMI1 plasimd for 48 h to downregulate CASC9 expression or upregulate BMI1 expression. SGC7901 cells (5×10^5 cells/well in 6-well plates, 70–80% confluency) were stably transfected with CASC9-shRNA for 48 h to downregulate CASC9 expression using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from GC tissues, paired adjacent tissues, GES-1, SGC7901 and MKN-45 cells by a Total RNA Kit (Invitrogen, CA, USA). cDNA synthesis was conducted using High-Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA). Then, qRT-PCR was performed in duplicate using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, CA, USA) on QuantStudio 5 Real-Time PCR System (Applied Biosystems, CA, USA). The expression of GUSBP11, CASC9, CTDHUT, RAB6C-AS1 and DQ786243 was quantified by $2^{-\Delta\Delta Ct}$ method. The primer sequences were presented as follows: GUSBP11: F 5′-GGCGCGGACGACCTAAGTCGCGT-3′, R 5′-TTGCGCTGCCACGACGGGCT-3′; CASC9: F 5'-AGATGAAGCCGGTACCTCAGAT-3′, R 5'-TCACTTTAAAGAGGGAGAGGAG-3′; CTDHUT: F 5′-GGCCCAGGGCTCAGTGGGGTGTT -3′, R 5′-ACTCCAGCCTGGGCAACAAGAGCGA -3′; RAB6C-AS1: F 5'-CAT TCA GAAGTG GAG AGT GTA GG-3′, R 5′-GAG GAT TGC GAG TCA TCAGC-3′; DQ786243: F 5'-TGCCCTGTCACCCCCTGCCCAA-3′, R 5′- ACGCTGGG CCAACTGCAGGGCT-3′.

Western Blot

Total protein was extracted from GES-1, SGC7901 and MKN-45 cells using RIPA Lysis and Extraction Buffer (Thermo Scientific, CA, USA), and the concentration of proteins was measured by Pierce BCA Protein Assay Kit (Thermo Scientific, CA, USA). Then, the protein was separated by 10% SDS-PAGE, transferred into the PVDF Transfer Membrane (Thermo Scientific, CA, USA) and blocked with 5% skim milk, the menbrance was incubated with primary antibodies against BMI1 (1:10000; Abcam, Cambridge, UK), Bcl-2 (1:2000; Abcam, Cambridge, UK), cleaved-caspase 3 (1:500; Abcam, Cambridge, UK), caspase 3 (1:1000; Abcam, Cambridge, UK), and β-actin (1:5000; Abcam, Cambridge, UK). The membranes were incubated with anti-rabbit horseradish peroxidase-conjugate secondary antibody (1:2000; Cell Signaling Technology, USA) for 1.5 h at 25 °C. ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, CA, USA) was used to visualize the proteins.

Flow Cytometry Analysis

SGC7901 and MKN-45 cells in different groups (6×10^4 cells) were added with 195 μl Annexin V-FITC binding buffer. Then, cells were stained with 5 μl Annexin V-FITC and 10 μl propidium iodide using Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China). The apoptosis of cells was analyzed by flow cytometry (FACSCalibur; BD, New Jersey, USA).

RNA Pull-Down Assay

Biotin labeled lncRNA CASC9 was transcribed with the Biotin RNA labeling mix (Roche, Switzerland) and T7 RNA polymerase (Roche, Switzerland), then purified with RNeasy Mini Kit (Qiagen, Germany). Cells extract (2 μg) was mixed with 100 pmol biotin labeled lncRNA CASC9. Then, cell extract was added with 100 ml streptavidin agarose beads and incubated at 25 °C for 2 h. Western blot was used to detect BMI1 in CASC9 pull-down complex.

RNA Immunoprecipitation (RIP) Assay

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, New Jersey, USA) was used to conduct RIP assay. Cells were lysed using complete RIP lysis buffer. Cell lysates were incubated with RIP buffer containing magnetic beads conjugated to human anti-BMI1 antibody (Invitrogen, CA, USA). The negative control was IgG. The precipitated RNAs were isolated by Trizol reagent (Invitrogen, CA, USA) and lncRNA CASC9 level was detected by qRT-PCR.

Ubiquitination Assay

FLAG-BMI1, HA-TrCp, si-CASC9 or si-control were transfected into SGC7901 and MKN-45 cells. 36 h's later, 10 nm MG132 (Sigma, MO, USA) was added into RPMI-1640 medium, and cells were incubated for another 8 h. Then, cells were collected for western blot. The cell lysates were immunoprecipitated with the labeled antibody at 4 °C overnight. Western blot was used to detect the protein of BMI1.

Animal Model

Five-week-old female BALB/c-nu/nu nude mice were purchased from Laboratory Animal Center of Anhui Medical University. SGC-7901 cells were stably transfected with NC-shRNA or CASC9-shRNA. Then, 0.1 ml SGC-7901 cells (2×10^7 cells/ml) were subcutaneously injected into the right side of the abdomen of each nude mice. So, BALB/c-nu/nu mice were divided into NC-shRNA and CASC9-shRNA groups ($n = 6$). Tumor volume was measured every two days for 16 days using the equation: Tumor volume = length×width^2 × 0.5. Sixteen days later, mice were sacrificed and the tumor tissues were collected to detect the expression of CASC9 and BMI1.

Statistical Analysis

All experiments were repeated three times. The data were presented as mean ± standard deviation (SD), and analyzed using SPSS 18.0. The difference between groups was analyzed by Student's t test or one-way analysis of variance. p value <0.05 was considered as statistically significant.

Results

LncRNA CASC9 Was Upregulated in GC Tissue and GC Cells

We selected several abnormally expressed lncRNAs (lncRNA GUSBP11, lncRNA CASC9, lncRNA CTDHUT, lncRNA RAB6C-AS1 and lncRNA DQ786243) in GC tissues based on the literature, and detected the expressions of these lncRNAs. As shown in Fig. 1a, only lncRNA CASC9 expression was significantly upregulated in GC tissue than adjacent tissue. We further detected the expressions of lncRNA CASC9 and BMI1 in normal gastric epithelial cell line GES-1 and GC cell lines (SGC7901 and MKN-45). Compared with GES-1 cells, the expressions of lncRNA CASC9 and BMI1 were significantly upregulated in SGC7901 and MKN-45 cells (Fig. 1b). In addition, the correlation between CASC9 expression and clinicopathological features in GC was shown in Table 1, suggesting high CASC9 expression was positively correlated with TNM stage and lymph node metastasis.

LncRNA CASC9 Regulated the Apoptosis of GC Cells

To investigate the role of lncRNA CASC9 in regulating GC cell apoptosis, we transfected si-CASC9 into SGC7901 and MKN-45 cells. As shown in Fig. 2a, lncRNA CASC9 expression was significantly downregulated in SGC7901 and MKN-45 cells transfected with si-CASC9. BMI1 protein level was also downregulated in SGC7901 and MKN-45 cells transfected with si-CASC9 (Fig. 2a). Then, we found the apoptosis of SGC7901 and MKN-45 cells significantly increased in si-CASC9 group than si-control group (Fig. 2b). Apoptotic protein cleaved-caspase 3 and anti-apoptotic protein Bcl-2 expressions were upregulated and downregulated in SGC7901 and MKN-45 cells transfected with si-CASC9, respectively (Fig. 2b). These results indicated silencing CASC9 could promote the apoptosis of GC cells. pcDNA-CASC9 and pcDNA were also transfected into SGC7901 and MKN-45 cells. Results showed that CASC9 overexpression promoted the expression of BMI1 and Bcl-2 (Supplemental Fig. 1), indicating CASC9 overexpression could inhibit the apoptosis of GC cells.

LncRNA CASC9 Regulated the Expression of BMI1

To investigate the interaction between lncRNA CASC9 and BMI1, RNA pull-down RIP assays were conducted. As shown in Fig. 3a and b, BMI1 expression was detected in CASC9 pull-down complex, and lncRNA CASC9 was

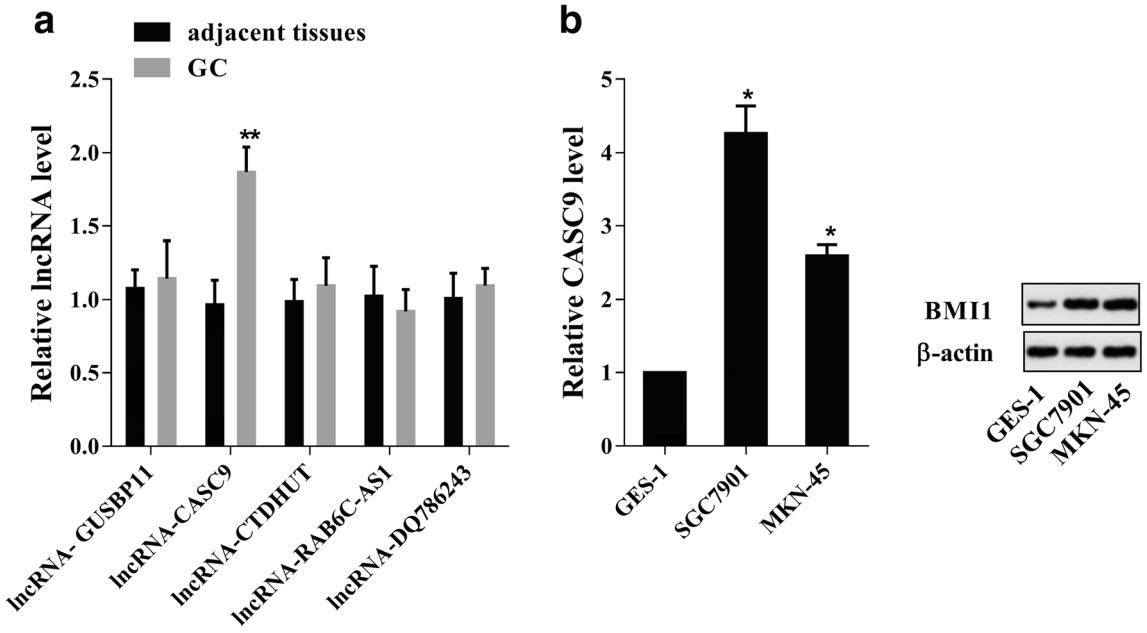

Fig. 1 LncRNA CASC9 was upregulated in GC tissue and GC cells. **a** LncRNA GUSBP11, lncRNA CASC9, lncRNA CTDHUT, lncRNA RAB6C-AS1 and lncRNA DQ786243 expressions were detected in GC tissue and adjacent tissues using qRT-PCR. **b** LncRNA CASC9 expression was detected in human GC cell lines (SGC7901 and MKN-45) and human normal gastric epithelial cell line GES-1 using qRT-PCR. BMI1 protein level was detected in GES-1, SGC7901 and MKN-45 cells using western blot. *$p < 0.05$, **$p < 0.01$ vs adjacent tissue or GES-1

accumulated in BMI1 precipitates, suggesting lncRNA CASC9 could interact with BMI1. To observe the role of CASC9 in regulating the expression of BMI1, pcDNA-CASC9 or si-CASC9 were transfected into SGC7901 and MKN-45 cells. BMI1 expression was upregulated in SGC7901 and MKN-45 cells after transfection of pcDNA-CASC9, whereas BMI1 expression was downregulated in SGC7901 and MKN-45 cells after transfection of si-CASC9 (Fig. 3c). Then, CHX was used to treat GC cells to detect the role of CASC9 on the half-life of BMI1 protein. We found si-CASC9 promoted the degradation of BMI1 protein with time (Fig. 3d). Ubiquitination assay showed a significant decrease in ubiquitinated BMI1 protein in SGC7901 and MKN-45 cells transfected with si-CASC9 (Fig. 3d). These findings indicated that lncRNA CASC9 could positively regulate BMI1, and silencing CASC9 promoted the ubiquitination of BMI1.

Table 1 Correlation between CASC9 expression and clinicopathological features in GC

Parameters	n	CASC9 expression		P
		Low	High	
Gender				0.291
Male	11	5	6	
Female	9	5	4	
Age (years)				0.288
<55	8	4	4	
≥55	12	5	7	
Stage				0.011*
I-II	10	7	3	
III-IV	10	2	8	
Histology				0.552
Squamous	7	4	3	
Adenoma	13	6	7	
Lymph node metastasis				0.002*
Negtive	6	5	1	
Positive	14	2	12	

*P < 0.05 was considered significant

LncRNA CASC9 Regulated the Apoptosis of GC Cells through BMI1

To detect whether lncRNA CASC9 regulated the apoptosis of GC cells through regulating BMI1, SGC7901 and MKN-45 cells were divided into si-control, si-CASC9, si-CASC9 + pcDNA, and si-CASC9 + pcDNA-BMI1 groups. Western blot was used to detect BMI1 expression in SGC7901 and MKN-45 cells. si-CASC9 downregulated BMI1 protein level in SGC7901 and MKN-45 cells, and pcDNA-BMI1 reversed the effect of si-CASC9 on BMI1 expression (Fig. 4a). We also found si-CASC9 significantly promoted the apoptosis of SGC7901 and MKN-45 cells, and pcDNA-BMI1 reversed the effect of si-CASC9 on the apoptosis of GC cells (Fig. 4b). The expressions of cleaved-caspase 3 and Bcl-2 further confirmed the effect of si-CASC9 and pcDNA-BMI1 on the apoptosis of SGC7901 and MKN-45 cells (Fig. 4c).

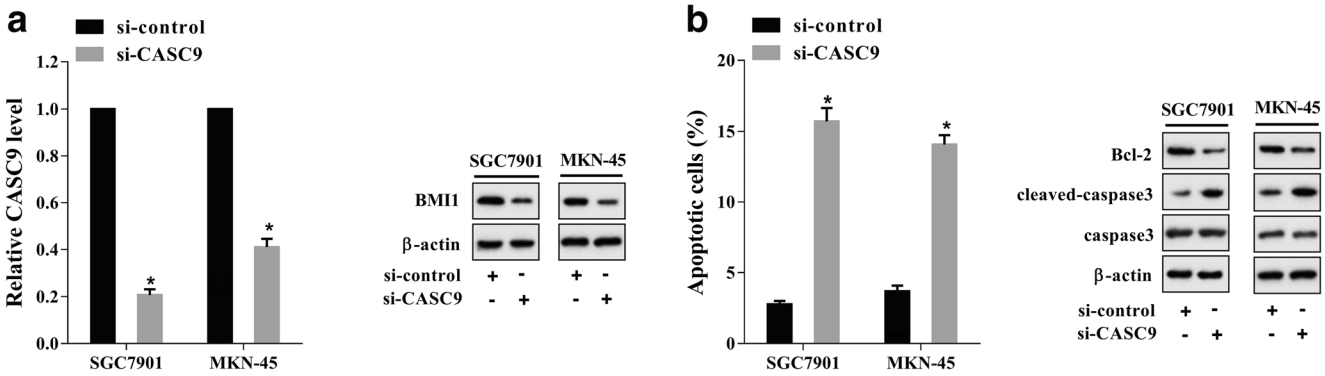

Fig. 2 LncRNA CASC9 regulated the apoptosis of GC cells. SGC7901 and MKN-45 cells were transfected with si-control or si-CASC9. **a** LncRNA CASC9 expression was detected in SGC7901 and MKN-45 cells using qRT-PCR. BMI1 protein level was detected in SGC7901 and MKN-45 cells using western blot. **b** The apoptosis of SGC7901 and MKN-45 cells was detected using Flow cytometry. Apoptotic protein cleaved-caspase 3 and anti-apoptotic protein Bcl-2 expressions were detected in SGC7901 and MKN-45 cells using western blot. *$p < 0.05$ vs si-control

Interfering CASC9 Inhibited Xenograft Tumor Growth in Nude Mice

To confirm whether CASC9 affects the growth of GC cells in nude mice, SGC-7901 cells transfected with NC-shRNA or CASC9-shRNA were subcutaneously injected into BALB/c-nu/nu mice to establish xenograft mouse model. Compared with NC-shRNA group, tumor volume was significantly reduced in CASC9-shRNA group (Fig. 5a). LncRNA CASC9 expression was significantly downregulated in tumors of CASC9-shRNA group than NC-shRNA group (Fig. 5b). BMI1 expression was decreased in tumors of CASC9-shRNA group than NC-shRNA group (Fig. 5b). These findings

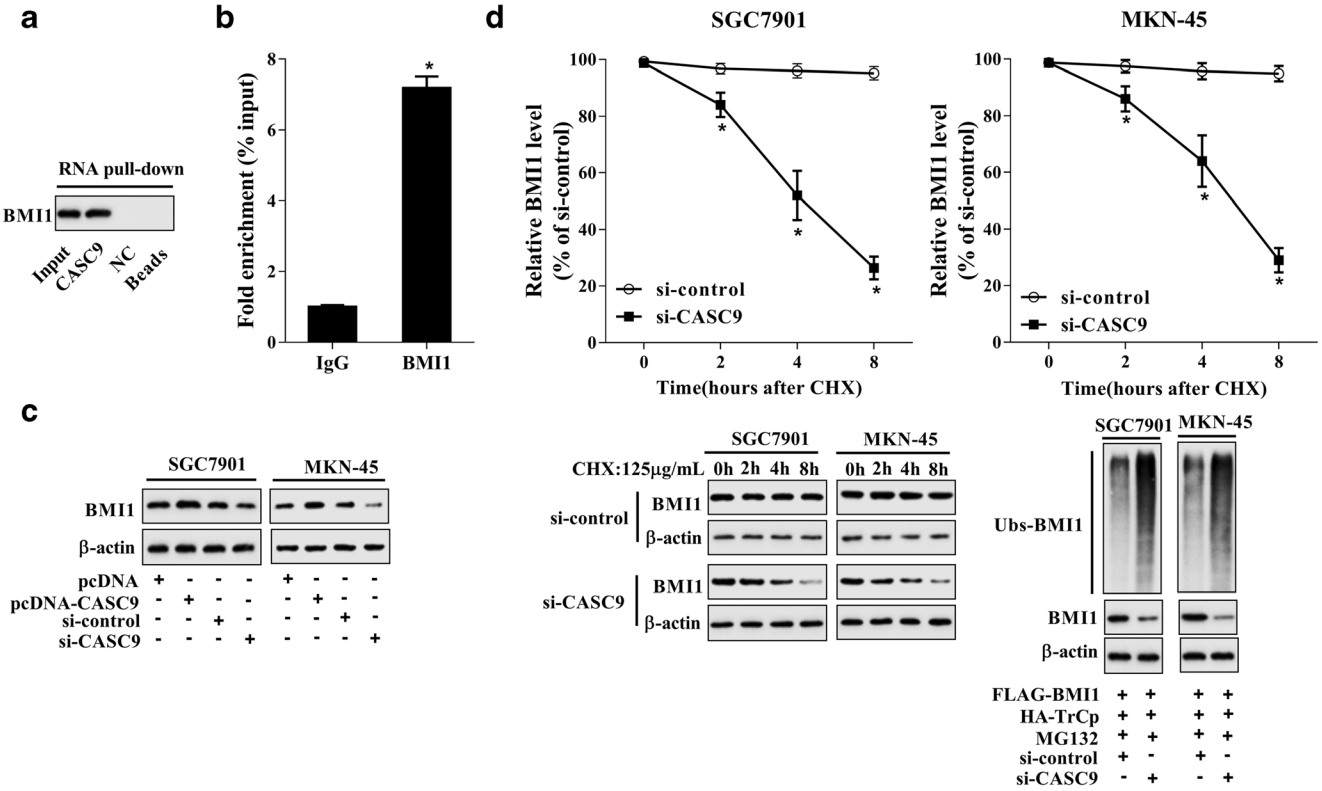

Fig. 3 LncRNA CASC9 regulated the expression of BMI1. **a** The efficiency of RNA pull-down assay was verified by western blot. BMI1 expression was detected in CASC9 pull-down complex. NC was the negative control of CASC9. **b** LncRNA-CASC9 was accumulated in BMI1 precipitates. *p < 0.05 vs NC or IgG. SGC7901 and MKN-45 cells were transfected with pcDNA-CASC9 or si-CASC9. **c** BMI1 expression was detected in SGC7901 and MKN-45 cells using western blot. **d** SGC7901 and MKN-45 cells were transfected with si-CASC9, then CHX was used to treat SGC7901 and MKN-45 cells for 0 h, 2 h, 4 h and 8 h. The expression of BMI was detected using western blot. Ubiquitination assay showed a decrease in BMI1 expression in GC cells transfected with si-CASC9

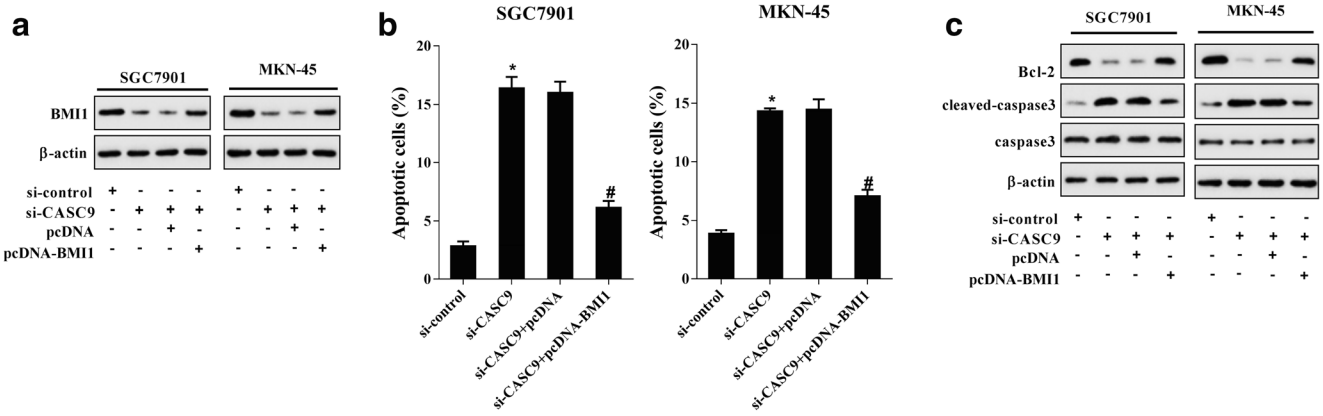

Fig. 4 LncRNA CASC9 regulated the apoptosis of GC cells through BMI1. SGC7901 and MKN-45 cells were divided into si-control, si-CASC9, si-CASC9 + pcDNA, and si-CASC9 + pcDNA-BMI1 groups. **a** BMI1 expression was detected in SGC7901 and MKN-45 cells using western blot. **b** The apoptosis of SGC7901 and MKN-45 cells was detected using Flow cytometry. **c** The expressions of cleaved-caspase 3 and Bcl-2 were detected in SGC7901 and MKN-45 cells using western blot. *p < 0.05 vs si-control, #p < 0.05 vs si-CASC9 + pcDNA

suggested that interfering CASC9 could inhibit the tumor growth of GC.

Discussion

In this study, we investigated the underlying mechanism of lncRNA CASC9 in the apoptosis of GC cells. Results indicated that lncRNA CASC9 was upregulated in GC tissues and cells, and lncRNA CASC9 could interact with BMI1. In vitro experiments demonstrated that lncRNA CASC9 regulated the apoptosis of GC cells through modulating BMI1, and in vivo experiments shown that silencing CASC9 suppressed tumor growth of GC.

Hundreds of lncRNAs have been reported to be tumor promoters or tumor suppressors in a variety of human cancers [19, 20]. According to previous reports, lncRNA CASC9 seems to be a tumor promoter that usually highly expressed in tumor tissues and cells, which promoted the progression of cancers [21–23]. For example, lncRNA CASC9 expression was upregulated in hemangiomas tissues and CASC9 overexpression promoted the proliferation, migration, and invasion of hemangiomas-derived endothelial cells through miR-125a-3p/Nrg1 pathway [23]. LncRNA CASC9 was elevated in oral squamous cell carcinoma tissues and cells, and enhanced the proliferation and suppressed cell apoptosis to promote tumor progression through AKT/mTOR pathway [22]. However, the role of lncRNA CASC9 in GC is not fully revealed, so more

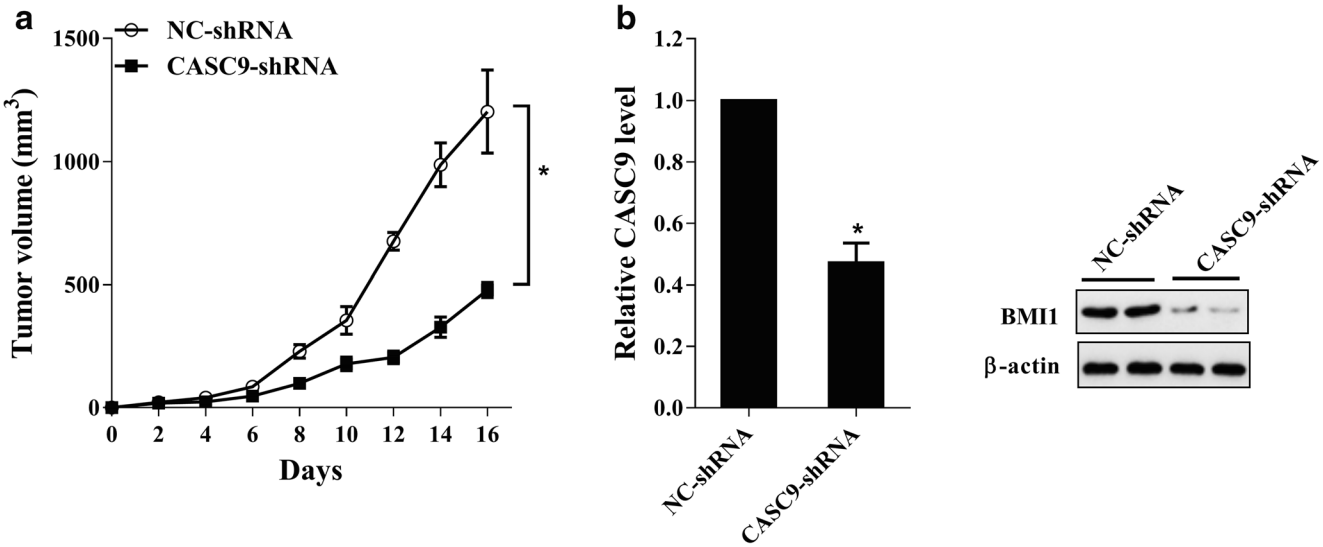

Fig. 5 Interfering CASC9 inhibited xenograft tumor growth in nude mice. SGC-7901 cells were stably transfected with NC-shRNA or CASC9-shRNA. Then, 0.1 ml SGC-7901 cells (2×10^7 cells/ml) were subcutaneously injected into the right side of the abdomen of the BALB/c-nu/nu mice. BALB/c-nu/nu mice were divided into NC-shRNA and CASC9-shRNA groups ($n = 6$). Tumor volume was measured every two days for 16 days. A. Tumor volume was measured in NC-shRNA and CASC9-shRNA groups. B. LncRNA CASC9 expression was detected in tumors of NC-shRNA and CASC9-shRNA groups using qRT-PCR. BMI1 expression was detected in tumors using western blot. *p < 0.05 vs NC-shRNA

studies focused on lncRNA CASC9 in the progression of GC are needed. In this study, lncRNA CASC9 was upregulated in GC tissues and cells, and silencing CASC9 promoted the apoptosis of GC cells. Hence, we further detected the underlying mechanism of lncRNA CASC9 in modulating the apoptosis of GC cells.

More and more evidences have shown that lncRNAs mediate gene expressions through multiple mechanisms, such as translation, transcription regulation, and post-transcription modification [15]. And interacting with the proteins to affect their expressions through ubiquitination is considered as one of these important mechanisms [24]. In GC, lncRNAs could induce the ubiquitination of proteins through interacting with proteins, thus to affect the progression of GC. For example, lncRNA HOTAIR can interact with Runx3 protein and HOTAIR can induce the degradation of Runx3 protein to regulate GC cell migration and invasion [17]. LncRNA HOXA11-AS can interact with WDR5, EZH2 and STAU1 in GC cells, and can induce the degradation of KLF2 [25]. In this study, our RNA pull-down and RIP assays showed that lncRNA CASC9 interacted with BMI1. In addition, BMI1 protein level was positively regulated by lncRNA CASC9. Ubiquitination assay confirmed that silencing CASC9 promoted the degradation of BMI1, suggesting lncRNA CASC9 can increase BMI1 expression through inhibiting the degradation of BMI1.

BMI1 is firstly identified as an oncoprotein that mediate protein-protein interaction. Numerous researches have shown that BMI1 is elevated in many cancers and is positively related with the progression of cancers [26, 27]. In GC, BMI1 is also highly expressed in tumor tissues and cells, and involved in the proliferation, invasion and metastasis of GC [28, 29]. Silencing BMI1 could decrease transformed phenotype of GC cells, inhibit the growth, migration and invasion of GC cells [29, 30]. In this study, we found BMI1 is upregulated in GC tissues and cells, which was consistent with previous reports [28, 29]. In addition, our data indicated that lncRNA CASC9 could interact with BMI1 to regulate the degradation of BMI1, which provided evidence for the ubiquitin modification of BMI1 by lncRNA in GC cell.

In conclusion, we identified that lncRNA CASC9 is highly expressed in GC tissues and cells. LncRNA CASC9 could interact with BMI1 to regulate the degradation of BMI1. In addition, silencing CASC9 decreased BMI1 expression to promote the apoptosis of GC cells in vivo and suppressed tumor growth in vivo.

Compliance with Ethical Standards

Conflict of Interests The authors have no actual or potential conflicts of interest to declare.

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