YY1-modulated Long Non-coding RNA SNHG12 Predicts and Promotes Gastric Cancer Metastasis via Activating miR-218-5p/YWHAZ-dependent β-catenin: A Bed to Bench Approach

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Research

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Abstract

Background: Gastric Cancer (GC) is one of the leading causes of cancer-related deaths and mortality. Long non-coding RNAs (lncRNAs) such as SNHG12 play important roles in the pathogenesis and progression of cancers. However, the role and significanve of SNHG12 in the metastasis of GC has not yet been thoroughly investigated.

Methods: The SNHG12 expression pattern was detected in GC tissue samples from our faculty and cell lines using quantitative reverse transcription PCR. In vivo and in vitro gain and loss assays were conducted to observe the effects of SNHG12 regulation on GC cell metastasis potential. The underlying mechanisms of SNHG12 regulation on EMT and metastatic potential of GC cells were further determined by quantitative reverse transcription PCR, western blotting, dual luciferase reporter assays, co-immunoprecipitation, immunoprecipitation, RIP assays, TOPFlash/FOPFlash reporter assays and Ch-IP assays.

Results: SNHG12 was upregulated in GC tissues and cell lines. The expression levels of SNHG12 in GC samples was significantly related to tumor invasion depth, TNM staging and lymph node metastasis, and was associated with poorer DFS and OS in the GC patients. SNHG12 was significantly highly expressed in peritoneal metastatic tissues from GC patients and mice subjects, suggesting a possible role of SNHG12 in peritoneal carcinomatosis from GC. Further in vivo and in vitro gain and loss assays indicated that SNHG12 promoted GC metastasis and EMT. Based on hypothetical bioinformatic analysis findings, our mechanistic analyses revealed that miR-218-5p was a direct target of SNHG12 and suggested that both SNHG12 and miR-218-5p could collectively regulate YWHAZ, forming the SNHG12/ miR-218-5p/YWHAZ axis, hereby decreasing the ubiquitination of β-catenin, thus activating the β-catenin signaling pathway and facilitating metastasis and EMT. Further analysis also revealed that the transcription factor YY1 could negatively modulate SNHG12 transcription.

Conclusions: Our findings demonstrate that SNHG12 is be a potential prognostic marker and therapeutic target for GC. Negatively modulated by transcription factor YYI, SNHG12 promotes GC metastasis and EMT by regulating the miR-218-5p/YWHAZ axis and hence activating the β-catenin signaling pathway. Furthermore, we discovered high SNHG12 expression could be related to peritoneal carcinomatosis from GC but this requires further validation.

Background

Gastric Cancer (GC) is the 5th most common malignancy and 3rd leading cause of cancer deaths in the world(1). The prognosis of GC remains poor due to tumor metastasis and recurrence and the potential underlying factors, including the heterogeneity in the functions of key genes and interlink signaling molecules and regulatory networks involved in the initiation, progression and invasion of GC(2, 3). In this regard, the role of regulatory molecules in the tumor microenvironment such as long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and other non-coding RNAs (ncRNAs) has been scrutinized, and recent

findings have shown that lncRNAs play important roles in a wide range of physiological and pathophysiological processes, and act as oncogenes or tumor suppressor genes in tumorigenesis and cancer metastasis(4–6).

LncRNA small nucleolar RNA host gene 12 (SNHG12) is located on chromosome 1 in the 1p35.3 region, and was first reported to be significantly upregulated in endometrial cancer in human(7). Recent studies have shown that the altered expression of SNHG12 could be correlated with the viability, proliferation, metastasis, and invasion of tumor cells, impacting the prognosis and survival by diverse pathways(8). Although several studies suggest that SNHG12 could promote GC progression by sponging miR-320, miR-199a/b-5p, miR-16 or activating the PI3K/AKT pathway(9–12), the role of SNHG12 in the metastasis of GC has not yet been thoroughly.

Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein zeta, also named YWHAZ or 14-3-3ζ, is a key modulator on the β-catenin signaling pathway and is closely associated with tumorigenesis and cancer metastasis(13). YWHAZ has been shown to be overexpressed in multiple types of cancers and regulated by miRNAs or lncRNAs (14). Zeng Y et al reported that lncRNA LUCAT1/miR-134-5p/YWHAZ axis can promote proliferation and invasion of GC(15); Wang H et al found circ-SERPINE2/miR-375/YWHAZ axis promote proliferation(16) and Jin CX et al clarified how YWHAZ effects apoptosis and autophagy in GC(17). In this study, we demonstrate that SNHG12 shows promise as a possible biomarker and therapeutic target in GC and how SNHG12 regulates the β-catenin signaling pathway by modulating the YWHAZ protein.

Yin Yang 1 (YY1) belongs to the GLI-Kruppel class of Zinc-finger proteins and acts as transcription repressor or activator to regulate a series of biological processes, such as embryogenesis, cellular proliferation, differentiation, and tumorigenesis. Nevertheless, its role in the regulation of tumor progression remains controversial(18). In GC, miRNA-584-3p was shown to inhibit GC progression by repressing YY1-facilitated MMP-14 expression and Zou X et al reported that the expression of YY1 was negatively related to ATP6V1A, suggesting potential mechanistic and clinical implications in GC(19, 20).

Henceforth, in the present study, we profiled SNHG12, miR-218-5p, YWHAZ and YY1 expression in GC tissues and cells and investigated the role and underlying mechanisms of SNHG12 in GC metastasis. We demonstrate that SNHG12 is upregulated in GC cells, and its expression level is significantly related to GC malignant characters and short survival of GC patients. SNHG12 is critical in GC metastasis. Suppression of SNHG12 expression leads to impaired GC cell invasion and EMT in vitro, and inhibits tumor metastasis in vivo. SNHG12 directly sponges miR-218-5p to upregulate YWHAZ expression, which activates the β-catenin signaling pathway to facilitate GC cell invasion and EMT. Furthermore, SNHG12 expression is negatively regulated by transcription repressor YY1. These results indicate an important role for SNHG12 in regulating GC metastasis.

Materials And Methods

GC patients and tissue specimens

A total of 54 GC tissues and pair-matched normal gastric epithelial tissues were obtained from GC patients who underwent radical surgery at Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine from 2015 to 2019. No radiotherapy or chemotherapy was given to the patients before surgery. All cases were independently diagnosed histologically by two experienced pathologists and staged according to the TNM staging of the American Joint Committee on Cancer (AJCC 7th ed., 2010). All tissue samples were immediately frozen in liquid nitrogen after resection from GC patients and stored at − 80 °C for further analysis. The acquisition of the tissues was approved by the Ruijin Hospital Ethics Committee.

Cell lines and culture conditions

Six human GC cell lines (AGS, MGC-803, MKN-45, SGC-7901, HS-746T and HGC-27), HEK-293T and a non-malignant gastric mucosal epithelial cell line GES-1 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM (Gibco, BRL, San Francisco, USA) medium supplemented with 10% fetal bovine serum (Hyclone, Carlsbad, CA, USA) and 5 μg/ml penicillin and streptomycin maintained in a humidified atmosphere at 37 °C in 5% CO_2.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from patient tissues and cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, China) while micro RNA was reverse transcribed into cDNA using miRNA First Strand cDNA Synthesis (Sangon Biotech, Shanghai, China). cDNA was quantified by RT-PCR and the data were acquired with SYBR Green (Vazyme, Nanjing, China) using Applied Biosystems 7500 instrument. GAPDH, U6 and ACTB were used as internal controls. Primers are listed in Additional file 1.

Lentivirus production, siRNA, plasmids and cell transfection

The lentivirus-containing short hairpin RNA (shRNA) targeting SNHG12 was purchased from OBiO (Shanghai, China), and the pCDH-CMV-Human vector for SNHG12 overexpression was purchased from Allwin (Shanghai, China). The miR-218-5p mimics, miR-218-5p inhibitors, and negative control (NC) oligonucleotides were obtained from GenePharma (Shanghai, China). SiRNAs for YY1 and YWHAZ were obtained from Sangon Biotech (Shanghai, China) (Additional file 2). GC cells were transfected with the above-mentioned oligonucleotides and plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cell migration

Cell migration ability was measured using transwell chambers (8-μm pore size; Corning Costar, Cambridge, MA, USA). For the transwell assay, $6{\cdot}10^4$ cells suspended in serum-free DMEM medium were

seeded into the upper chamber. The lower chamber contained DMEM medium supplemented with 5% serum. After 10 h incubation, the filters were fixed in methanol and stained with 0.1% crystal violet. The upper faces of the filters were gently abraded, and the lower faces with cells migrated across the filters were imaged and counted under the microscope. These experiments were performed in triplicate and repeated three times.

Western blotting

Total proteins from cells were extracted using RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. The primary anti-bodies used in this assay included E-cadherin (ABclonal), vimentin (CST), N-cadherin (Proteintech), β-catenin (Proteintech), YWHAZ (ABclonal), GAPDH (Proteintech), Lamin B (Proteintech).

Fluorescence in situ hybridization (FISH)

The FISH assays of GC cells and tissues were conducted according to the method described as previously(21).

Luciferase reporter and TOPFlash/FOPFlash reporter assays

The luciferase reporter plasmid carrying the WT or mutated (MUT) 3′-UTR of SNHG12 and WT or mutated (MUT) 3′-UTR of YWHAZ were purchased from Public Protein/Plasmid Library (Nanjing, China). The above plasmids were transfected into GC cells along with the miR-218-5p mimics using lipofectamine 2000. After transfection (36–48 h), the cells were lysed, and luciferase activity was measured with the Dual-Luciferase Reporter Assay system (Promega). The TOPFlash/FOPFlash reporter assay were conducted according to the manufacturer's instructions of TCF Reporter Plasmid Kit (Millipore).

Co-immunoprecipitation and immunoprecipitation

Co-immunoprecipitation (Co-IP) and immunoprecipitation (IP) were conducted using the IP/Co-IP kit (ABsin) according to the manufacturer's instructions. The primary anti-bodies used in this assay included β-catenin (ABclonal), YWHAZ (Proteintech), Ubiquitin (CST), β-tubulin (ABclonal).

ChIP assay

ChIP assays were performed using the EZ-Magna ChIP Kit (Millipore 17-10086), as previously described(22).

RNA Binding Protein Immunoprecipitation

RNA Binding Protein Immunoprecipitation (RIP) were performed using the EZ-magna RIP Kit (Millipore 17–700), the antibody used in this assay include Ago2 (ABcam).

In vivo metastasis assays

Four-weeks-old immunodeficient BABL/c female nude mice were purchased and maintained under specific pathogen-free conditions. MGC-803 cells ($2{\cdot}10^6$) with stable sh-SNHG12 or empty vector were

separately injected into the abdomen of the mice and their body weight was measured and recorded every 3 days. After 1 month, the mice were sacrificed and the tumors in abdominal were dissected for immunohistochemistry and FISH assays.

Statistical analysis

All statistical analyses were conducted using SPSS 23.0 (SPSS, Chicago, IL, USA) or GraphPad PrismV8 (GraphPad Prism, Inc., La Jolla, CA, USA). Each experiment was performed at least in triplicate, and data are presented as the mean ± SD of three independent experiments. Student's t-test or one-way ANOVA was used to compare the means of two or three groups. P values less than 0.05 were considered statistically significant.

Results

Overexpression of SNHG12 is associated with advanced staging of GC and indicates poor prognosis in GC patients.

Previous studies have shown that SNHG12 plays a critical role in GC progression, however, the role of SNHG12 in regulating GC metastasis is yet unclear. To address this issue, we first determined the expression of SNHG12 in human GC samples and GC cell lines by qRT-PCR assays. As shown in Fig. 1a, SNHG12 was markedly highly expressed in tumor tissues than in the matched normal gastric epithelial tissues, suggesting that SNHG12 acted as an oncogene in GC. Further investigation in GC cell lines demonstrated that compared with the GES-1 normal gastric epithelial cell line, SNHG12 was significantly highly expressed in HS-746T, MGC-803, SGC-7901, AGS, and HGC-27 but poorly expressed in MKN-45 (Fig. 1b). Based on their SNHG12 expression pattern, MGC-803 and AGS were selected for further experiments. Based on their SNHG12 expression pattern, MGC-803 and AGS were selected for vitro experiments. FISH assays showed that the fluorescence intensity of SNHG12 in GC tissues was much higher than that in matched normal gastric epithelial tissues (Fig. 1c).

Since peritoneal metastasis still resides as one of the main forms of disease progression, we additionally investigated the expression of SNHG12 in peritoneal metastases obtained from our sample pool by FISH, as shown in Fig. 1c and the fluorescence intensity of SNHG12 in GC peritoneal metastasis tissues and GC tissues was much higher than that in primary tumor and matched normal gastric epithelial tissues, suggesting a possible underlying role of SNHG12 in the progression of peritoneal carcinomatosis from GC.

In order to verify the clinical significance of SNHG12, we analyzed the correlation between the expression levels of SNHG12 and the clinicopathological characteristics of the 54 GC patients. The results showed that higher SNHG12 expression was significantly related to depth of tumor invasion, extent of lymph node metastasis and the TNM staging of GC patients (Table 1). Moreover, Kaplan-Meier analysis indicated that patients with high SNHG12 expression had poorer survival outcome, and the respective 5-year disease free survival (DFS) of the high and low SHNG12 expression groups was 72.2% vs. 94.4%

respectively (p = 0.0112) (Fig. 1d) while the corresponding 5-year overall survival (OS) was 72.2% and 94.4% respectively (p = 0.0266) (Fig. 1e). Further analysis of the nature of disease progression revealed that most of the patients with poor survival showed disease progression involving the peritoneum.

Table1

Clinical parameter	SNHG12 expression		P value
	High expression cases(n=36)	Low expression cases(n=18)	
Gender			0.837
Male	25	12	
Female	11	6	
Age(years)	65.3±10.2	62.6±10.7	0.363
Tumor Size			0.126
≥5cm	22	7	
<5cm	14	11	
Borrmann Type			0.105
TypeI	4	1	
TypeII	12	12	
TypeIII	19	5	
TypeIV	1	0	
Histologic differentiation			0.344
Well and moderate	7	5	
Poor	29	13	
T Stage			0.000
T1/T2	5	9	
T3/T4	31	9	
N Stage			0.000
N0	5	11	
N1	6	2	
N2	7	4	
N3a	7	1	
N3b	11	0	
TNM Stage			0.000
I	1	5	
IIA	4	3	
IIB	4	8	
IIIA	4	1	
IIIB	7	0	
IIIC	16	1	
LNM Rate	25.6%±26.0%	6.7%±13.3%	0.007

SNHG12 promotes GC cell migration and EMT in vitro.

To verify the role of SNHG12 in GC metastasis, loss-and-gain assays were conducted in GC cells by shRNA and pCDH-CMV-Human vector. The knockdown and overexpression efficiencies of SNHG12 in GC cells were validated by RT-qPCR (Additional file3 figure S1). Since sh-SNHG12-2 manifested the optimal knockdown efficiency, it was used for the subsequent investigations. Transwell assays indicated that knockdown of SNHG12 significantly suppressed GC cell migration (Fig. 2a). Conversely, overexpression of SNHG12 promoted GC cell migration (Fig. 2b).

Epithelial-Mesenchymal Transition (EMT), a process in which epithelial cells transdifferentiated into motile mesenchymal cells, endows cancer cells with high invasion and mobility. We therefore investigated the effects of SNHG12 on EMT of GC cells. As shown in Fig. 2c, knockdown of SNHG12 induced morphological changes in GC cells, from a spindle-shaped mesenchymal appearance to a cobble-like and spherical epithelial phenotype. Western blotting (WB) results showed that knockdown of SNHG12 decreased the expression of the mesenchymal markers N-cadherin, vimentin and elevated the epithelial marker E-cadherin expression, while overexpression of SNHG12 demonstrated the opposite effects (Figs. 2d,e).

SNHG12 promotes GC metastasis in vivo.

To further investigate the metastatic potential of SNHG12 in vivo, a peritoneal metastasis mice model was constructed. Significant differences were noted in the body weight of the subjects: After 9 days, it was noted that the control mice (NC) weighed more than the sh-SNHG12 mice, most probably due to possible mass formation (Fig. 3a). After 1 month, the mice were sacrificed and the anatomical dissection findings were as follows: in the NC mice, there was notable inflammatory adhesion in the mesentery with nodular formations in the mesentery and intestinal surface (around 6 to 8 masses in each mouse) while in the sh-SNHG12 mice, the abdominal cavity was clear with rare nodular findings (3 masses obtained from 3 of the subjects) (Figs. 3b,c). FISH hybridization of the masses obtained from all the subjects were further investigated and the SNHG12 signals were higher in the NC group (Fig. 3d).

SNHG12 acts as a competitive endogenous RNA for miR-218-5p to regulate YWHAZ expression in GC cells.

FISH assays showed that SNHG12 is mainly located in cytoplasm (Fig. 4a). Further cytoplasmic and nuclear RNA purification assays further confirmed that the majority of SNHG12 transcripts were detected in cytoplasm instead of nucleus (Fig. 4b). This result suggested that SNHG12 mainly exerted its function at the post-transcriptional level and may sponge miRNAs to regulate downstream molecules. Bioinformatics databases (Starbase and miRcode) indicated that miR-218-5p exhibited the complementary binding sites with the 3’-UTR of SNHG12, suggesting the direct sponging of miR-218-5p by SNHG12 (Additional file3 figure S2). Moreover, YWHAZ was chosen as the putative downstream genes of SNHG12 and miR-218-5p by using the databases TargetScan Human 7.2 and Starbase (Additional file3 figure S3). The regulation of miR-218-5p was implemented in the MGC-803 and AGS cell lines by using miR-218-5p mimics for overexpression and inhibitors for suppression assays. Compared with miR-NC groups, the expression of SNHG12 and YWHAZ was suppressed in miR-218-5p mimics group while overexpressed in inhibitors group (Fig. 4c). We further investigated whether miR-218-5p could directly bind to the 3’-UTR of SNHG12 and YWHAZ and dual-luciferase reporter assays indicated a significant reduction in luciferase activities after the co-transfection of miR-218-5p-mimics and a wild-type SNHG12 reporter vector or a wild-type YWHAZ reporter vector, but this reduction was not observed upon transfection with mutant 3’-UTR of SNHG12 reporter vector or mutant 3’-UTR of YWHAZ reporter vector (Figs. 4d, e, f and g). To further elucidate the relationship between SNHG12, miR-218-5p and YWHAZ,

pCDH-CMV-SNHG12 or miR-218-5p mimics were transfected into MGC-803 and AGS cells, qRT-PCR assays indicated the expression of SNHG12 and YWHAZ was significantly increased or decreased respectively. On the other hand, when MGC-803 and AGS cells were co-transfected with pCDH-CMV-SNHG12 and miR-218-5p mimics, both of the above effects could be inverted (Fig. 4 h). In parallel, sh-SNHG12-2 or si-YWHAZ or miR-218-5p inhibitors were transfected into MGC-803 and AGS cells, the relative expression of SNHG12 and YWHAZ was decreased or increased respectively. Sh-SNHG12-2 or si-YWHAZ and miR-218-5p inhibitors were co-transfected into cells, both of the above trends could be inverted (Figs. 4i, j). RIP assays on Ago2, a component of the RNA-induced silencing complex (RISC), were conducted and the results revealed that SNHG12, miR-218-5p and YWHAZ could bind to Ago2, knockdown SNHG12 in MGC-803 and AGS cells led to the increase enrichment of YWHA (Fig. 4k). These results suggested that SNHG12 may compete with YWHAZ for miR-218-5p containing Ago2-based RISC.

SNHG12 activates β-catenin via YWHAZ encoded protein stabilizing β-catenin and reducing its ubiquitination degradation.

Wnt/β-catenin signaling pathway is well-established in cancer cell invasiveness and EMT. YWHAZ encoded protein, 14-3-3ζ, can interact β-catenin to increase its expression via decreasing its ubiquitination. According to previous studies, we determined the focused on the effects of SNHG12 on regulating β-catenin signaling activity via YWHAZ encoding protein, 14-3-3ζ. As shown in Figs. 5a, b, YWHAZ knockdown led to a decrease of β-catenin at protein level, while no obvious change at RNA level. In parallel, SNHG12 knockdown resulted in the decrease of β-catenin at both of RNA and protein levels (Figs. 5c, d). The nuclear expression of β-catenin dramatically decreased when SNHG12 knockdown, while, its nuclear expression increased when SNHG12 overexpressed (Fig. 5e). Moreover, Co-IP assays validated the interaction of YWHAZ protein and β-catenin in GC cells (Fig. 5f), and IP assays proved the ubiquitination level increased in YWHAZ or SNHG12 knockdown group, compared with mock control group (Figs. 5 g, h). Furthermore, TOPFLASH and FOPFLASH reporters were constructed to verify whether SNHG12 expression modulated the activation of β-catenin pathway, and as expected, the overexpression of SNHG12 in MGC-803 and AGS cells resulted in the remarkable increase in TOP/FOP reporter activity (Fig. 5i), suggesting activation of β-catenin-dependent transcription.

SNHG12/miR-218-5p/YWHAZ axis positively regulates GC cell metastatic potential via β-catenin pathway

To further understand the involvement of miR-218-5p/YWHAZ/β-catenin pathway in regulating metastatic potential of GC cells induced by SNHG12, transwell assays were performed in MGC-803 and AGS cells transfected with pCDH-CMV-SNHG12 or/and miR-218-5p mimics, respectively. Figures 6a, b showed that migrated cell count was significantly increased or decreased in MGC-803 and AGS cells transfected with pCDH-CMV-SNHG12 or miR-218-5p mimics only, however, when GC cells were co-transfected with pCDH-CMV-SNHG12 and miR-218-5p mimics, the increased migrated cells induced by SNHG12 overexpression could be inverted by overexpressing miR-218-5p and had no significant difference from control groups. In addition, we observed the abnormal expression of EMT-related proteins,

β-catenin and YWHAZ encoded protein induced by SNHG12 overexpression was reversed after introduction of miR-218-5p mimics (Fig. 6c). Similarly, transwell assays showed that migrated cell count was significantly decreased or increased in MGC-803 and AGS cells transfected with sh-SNHG12/si-YWHAZ or miR-218-5p inhibitors only. On the other hand, when GC cells were co-transfected with sh-SNHG12/si-YWHAZ and miR-218-5p inhibitors, the decreased migrated cells induced by SNHG12 knockdown could be inverted by inhibiting miR-218-5p and had no significant difference from control groups (Figs. 6d,e,g,h). Therefore, abnormal expression of EMT-related proteins, β-catenin and YWHAZ encoded protein induced by SNHG12 knockdown was reversed after introduction of miR-218-5p inhibitors (Fig. 6f).

Transcription factor YY1 modulates SNHG12 expression

To further elucidate the mechanism underlying SNHG12 overexpression in GC, we investigated the involvement of transcription factors in regulating the transcription of SNHG12. JASPAR and PROMO databases were used to analyze the potential TFs that may bind to the region of SNHG12 promotor, and the transcription factor YYI showed similar affinity to the binding site on the promotor of SNHG12. Ch-IP assays results showed that site1 (+ 232 to + 237) and site2 (+ 1357 to + 1362) regions in the SNHG12 promotor might mediate YY1 binding to the endogenous SNHG12 promotor (Fig. 7a,b). Initially, RT-qPCR was performed to determine YY1 expression in GC cell lines and tissues. As shown in Fig. 7c, compared with GES-1, YY1 was relatively poorly expressed in most of GC cell lines (MGC-803, AGS, MKN-28, HS-746T, and SGC-7901) except for MKN-45 and HGC-27. Moreover, YY1 expression was significantly lower in GC tissue samples than that in paired normal gastric epithelial tissues (Fig. 7d). Furthermore, the results from transwell assays indicated that YY1-silencing led to significant decrease in the migration of MGC-803 and AGS cells (Fig. 7e). Nevertheless, upon treatment with si-YY1, the expressions of SNHG12 and YWHAZ increased while that of miR-218-5p decreased (Fig. 7f), which was verified by RT-qPCR. Thus, these results suggested that low expressed YY1 in GC promotes the transcription of SNHG12.

Discussion

Previous reports have confirmed the role of SNHG12 in the tumorigenesis of several cancers but its significance in GC still needs further elucidation(8). In this study, we confirmed that SNHG12 is highly expressed in GC cell lines and tissues, and further analysis revealed that its high expression was clinically closely related to the invasion depth, TNM staging and extent of lymph node metastasis, findings which complement previous literature(12). Kaplan-Meier survival analysis confirmed that GC patients with high SNHG12 expression profile had poorer DFS and OS rates. While investigating the metastatic potential of SNHG12 in a mice model, we discovered that inhibiting the latter could decrease the metastatic activity of the GC cells. On the other hand, in peritoneal metastatic tissues obtained from GC patients, FISH assays revealed much stronger SNHG12-positive staining than the control, further confirming the role of SNHG12 in GC metastasis. Therefore, based on our results, of pivotal clinical significance, SNHG12 can be a promising prediction marker for peritoneal metastasis and poor survival in GC.

The elevated expression of SNHG12 has been reported in several types of cancers, and SNHG12 may affect proliferation, metastasis, apoptosis and cell cycle of cancer cells. Herein, we found SNHG12 promoted GC cell metastatic potential in vitro and in vivo, and these results indicated that SNHG12 acts as an important contributor to GC progression. There has been a lot of speculations about the underlying mechanisms behind the involvement of SNHG12 in regulating tumor progression. In this study, we clarified that SNHG12 mainly located in the cytoplasm, and sponged miR-218-5p. We first noted that the level of miR-218-5p expression in GC cell lines was low, suggesting that as a suppressor gene, it could negatively regulate metastasis. Hence, for the first time, it has been shown that SNHG12 and miR-218-5p have the negative correlation in GC where miR-218-5p can directly bind to the 3'UTR of SNHG12.

Further investigation using the TargetScan Human 7.2 and Starbase databases revealed YWHAZ as the downstream molecule of both SNHG12 and miR-218-5p. YWHAZ encoded protein, 14-3-3ζ is a member of the 14-3-3 family proteins affecting diverse vital biological processes, such as metabolism, signal transduction, apoptosis and cell cycle. YWHAZ expression is upregulated in multiple types of cancer including GC, and YWHAZ has been identified as potential biomarker for predicting prognosis of GC patients. Consistent with the previous studies, we here confirmed that YWHAZ was highly expressed in GC cell lines and tissues, and its expression level was significantly related with tumor invasion depth, TNM staging, lymph node metastasis and poor prognosis (Additional file 4). Dual-luciferase reported assays and Transwell assays in GC cell lines support that the SNHG12/miR-218-5p/YWHAZ axis forms a ceRNA net and positively regulates GC metastasis and EMT.

Wnt/β-catenin signaling pathway is well-established in regulating cancer cell EMT and invasiveness. lncRNAs can regulate tumor progression via activating β-catenin signaling pathway (23) and YWHAZ protein (14-3-3ζ) is capable of activating β-catenin pathway by inducing the accumulation of β-catenin in cytosol and nucleus(24). In this study, we discovered that SNHG12 could activate the β-catenin signaling pathway in GC cells by not only increasing the transcriptional level of β-catenin, but also regulating YWHAZ which binds to β-catenin to reduce the ubiquitination degradation of β-catenin, resulting in the over-expression of β-catenin, thus activating the downstream pathway and promoting metastasis and EMT, such as for instance via activating TCF/LEF transcription element.

Although SNHG12 is critical in regulating the metastasis of GC, the mechanisms underlying SNHG12 overexpression in GC cells have not been elucidated. In our study, we discovered that YY1, which can regulate many lncRNAs as a transcription factor, was poorly expressed in GC with the potential of inhibiting SNHG12 transcription. We cannot exclude other mechanisms behind the transcriptional suppression of SNHG12 by YY1, as YY1 can bind to HDAC which inhibits histone acetylation(25), we speculate that YY1 may suppress the transcription of SNHG12 via direct binding to SNHG12 promotor region and inducing epigenetic modification. However, this hypothesis requires more verification and the detailed mechanisms of the transcriptional suppression of SNHG12 by YY1 remain to be discovered in the future.

With the role of SNHG12 established in the GC, we assume that that based on the in vivo, in vitro and clinical analyses, the clinical significance of SNHG12 in the diagnosis, target therapy and combined therapy is promising, hence setting the foundation to more clinical studies.

Conclusion

Our findings demonstrate that SNHG12 is be a potential prognostic marker and therapeutic target for GC. Negatively modulated by transcription factor YYI, SNHG12 promotes GC metastasis and EMT by regulating the miR-218-5p/YWHAZ axis and hence activating the β-catenin signaling pathway (Fig. 7 g). Furthermore, we discovered high SNHG12 expression could be related to peritoneal carcinomatosis from GC but this requires further validation.

Abbreviations

YY1
Ying Yang 1
lncRNA
long non-coding Ribonucleic Acid
SNHG12
Small Nucleolar RNA Host Gene 12
GC
Gastric Cancer
YWHAZ
Tyrosine 3-monooxygenase/tryptophan5-monooxygenase activation protein; 14-3-3 protein zeta
EMT
Epithelial-Mesenchymal Transition
PCR
Polymerase Chain Reaction
qRT-PCR
Reverse Transcription- quantitative Polymerase Chain Reaction
Ch-IP
Chromatin Immunoprecipitation
DFS
Disease Free Survival
OS
Overall Survival
ncRNA
non-coding Ribonucleic Acid
miRNA
micro Ribonucleic Acid

shRNA
short hairpin Ribonucleic Acid
FISH
Fluorescence in situ Hybridization
RIP
RNA-Binding Immunoprecipitation
ceRNA
Competing Endogenous Ribonucleic Acid
TCF/LEF
T-Cell Factor/Lymphoid Enhancer Factor

Declarations

Ethics approval and consent to participate This study was approved by the Ethics Committee of Ruijin Hospital affiliated to Shanghai Jiao Tong University school of medicine. All participating patients provided informed consent.

Consent for publication All patients provided informed consent for publication of data.

Availability of data and materials Data and materials will be made available upon request.

Competing interest No competing interest to declare.

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Authors' contributions – Please use the author's initials (AI, ZE, SA) The concept of the study was conceived by TQZ, QQD, MKB, ZQW, LPS and CL. ZTQ and QQD performed the in vitro and in vivo experiments, MKB and ZQW performed the clinical data management and supported the in vitro and vivo experiments. LPS provided valuable guidance and professional comments during the design and implementation of the study. CL is the corresponding author and supervised the whole design and implementation of the study. The manuscript was drafted by TQZ and QQD, MKB revised the manuscript and conducted language editing, ZQW, LPS and CL approved the final version of the manuscript.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

2. Zong L, Abe M, Seto Y, Ji J. The challenge of screening for early gastric cancer in China. Lancet. 2016;388(10060):2606.
3. Wang Q, Chen C, Ding Q, Zhao Y, Wang Z, Chen J, et al. METTL3-mediated m(6)A modification of HDGF mRNA promotes gastric cancer progression and has prognostic significance. Gut. 2019.
4. Khandelwal A, Malhotra A, Jain M, Vasquez KM, Jain A. The emerging role of long non-coding RNA in gallbladder cancer pathogenesis. Biochimie. 2017;132:152–60.
5. Arun G, Diermeier SD, Spector DL. Therapeutic Targeting of Long Non-Coding RNAs in Cancer. Trends Mol Med. 2018;24(3):257–77.
6. Schmitt AM, Chang HY. Long Noncoding RNAs in Cancer Pathways. Cancer Cell. 2016;29(4):452–63.
7. Zhai W, Li X, Wu S, Zhang Y, Pang H, Chen W. Microarray expression profile of lncRNAs and the upregulated ASLNC04080 lncRNA in human endometrial carcinoma. Int J Oncol. 2015;46(5):2125–37.
8. Tamang S, Acharya V, Roy D, Sharma R, Aryaa A, Sharma U, et al. SNHG12: An LncRNA as a Potential Therapeutic Target and Biomarker for Human Cancer. Front Oncol. 2019;9:901.
9. Yang BF, Cai W, Chen B. LncRNA SNHG12 regulated the proliferation of gastric carcinoma cell BGC-823 by targeting microRNA-199a/b-5p. Eur Rev Med Pharmacol Sci. 2018;22(5):1297–306.
10. Zhang H, Lu W. LncRNA SNHG12 regulates gastric cancer progression by acting as a molecular sponge of miR320. Mol Med Rep. 2018;17(2):2743–9.
11. Zhao G, Wang S, Liang X, Wang C, Peng B. Oncogenic role of long non-coding RNA SNHG12 in gastric cancer cells by targeting miR-16. Exp Ther Med. 2019;18(1):199–208.
12. Zhang R, Liu Y, Liu H, Chen W, Fan HN, Zhang J, et al. The long non-coding RNA SNHG12 promotes gastric cancer by activating the phosphatidylinositol 3-kinase/AKT pathway. Aging. 2019;11(23):10902–22.
13. Zhao JF, Zhao Q, Hu H, Liao JZ, Lin JS, Xia C, et al. The ASH1-miR-375-YWHAZ Signaling Axis Regulates Tumor Properties in Hepatocellular Carcinoma. Mol Ther Nucleic Acids. 2018;11:538–53.
14. Gan Y, Ye F, He XX. The role of YWHAZ in cancer: A maze of opportunities and challenges. J Cancer. 2020;11(8):2252–64.
15. Chi J, Liu T, Shi C, Luo H, Wu Z, Xiong B, et al. Long non-coding RNA LUCAT1 promotes proliferation and invasion in gastric cancer by regulating miR-134-5p/YWHAZ axis. Biomed Pharmacother. 2019;118:109201.
16. Liu J, Song S, Lin S, Zhang M, Du Y, Zhang D, et al. Circ-SERPINE2 promotes the development of gastric carcinoma by sponging miR-375 and modulating YWHAZ. Cell Prolif. 2019;52(4):e12648.
17. Guo F, Jiao D, Sui GQ, Sun LN, Gao YJ, Fu QF, et al. Anticancer effect of YWHAZ silencing via inducing apoptosis and autophagy in gastric cancer cells. Neoplasma. 2018;65(5):693–700.
18. Khachigian LM. The Yin and Yang of YY1 in tumor growth and suppression. Int J Cancer. 2018;143(3):460–5.

19. Zheng L, Chen Y, Ye L, Jiao W, Song H, Mei H, et al. miRNA-584-3p inhibits gastric cancer progression by repressing Yin Yang 1- facilitated MMP-14 expression. Sci Rep. 2017;7(1):8967.
20. Wang P, Wang L, Sha J, Lou G, Lu N, Hang B, et al. Expression and Transcriptional Regulation of Human ATP6V1A Gene in Gastric Cancers. Sci Rep. 2017;7(1):3015.
21. Yang XZ, Cheng TT, He QJ, Lei ZY, Chi J, Tang Z, et al. LINC01133 as ceRNA inhibits gastric cancer progression by sponging miR-106a-3p to regulate APC expression and the Wnt/beta-catenin pathway. Mol Cancer. 2018;17(1):126.
22. Wang ZQ, Cai Q, Hu L, He CY, Li JF, Quan ZW, et al. Long noncoding RNA UCA1 induced by SP1 promotes cell proliferation via recruiting EZH2 and activating AKT pathway in gastric cancer. Cell Death Dis. 2017;8(6):e2839.
23. Yang G, Shen T, Yi X, Zhang Z, Tang C, Wang L, et al. Crosstalk between long non-coding RNAs and Wnt/beta-catenin signalling in cancer. J Cell Mol Med. 2018;22(4):2062–70.
24. Chen CH, Chuang SM, Yang MF, Liao JW, Yu SL, Chen JJ. A novel function of YWHAZ/beta-catenin axis in promoting epithelial-mesenchymal transition and lung cancer metastasis. Mol Cancer Res. 2012;10(10):1319–31.
25. Tang W, Zhou W, Xiang L, Wu X, Zhang P, Wang J, et al. The p300/YY1/miR-500a-5p/HDAC2 signalling axis regulates cell proliferation in human colorectal cancer. Nat Commun. 2019;10(1):663.

Figures

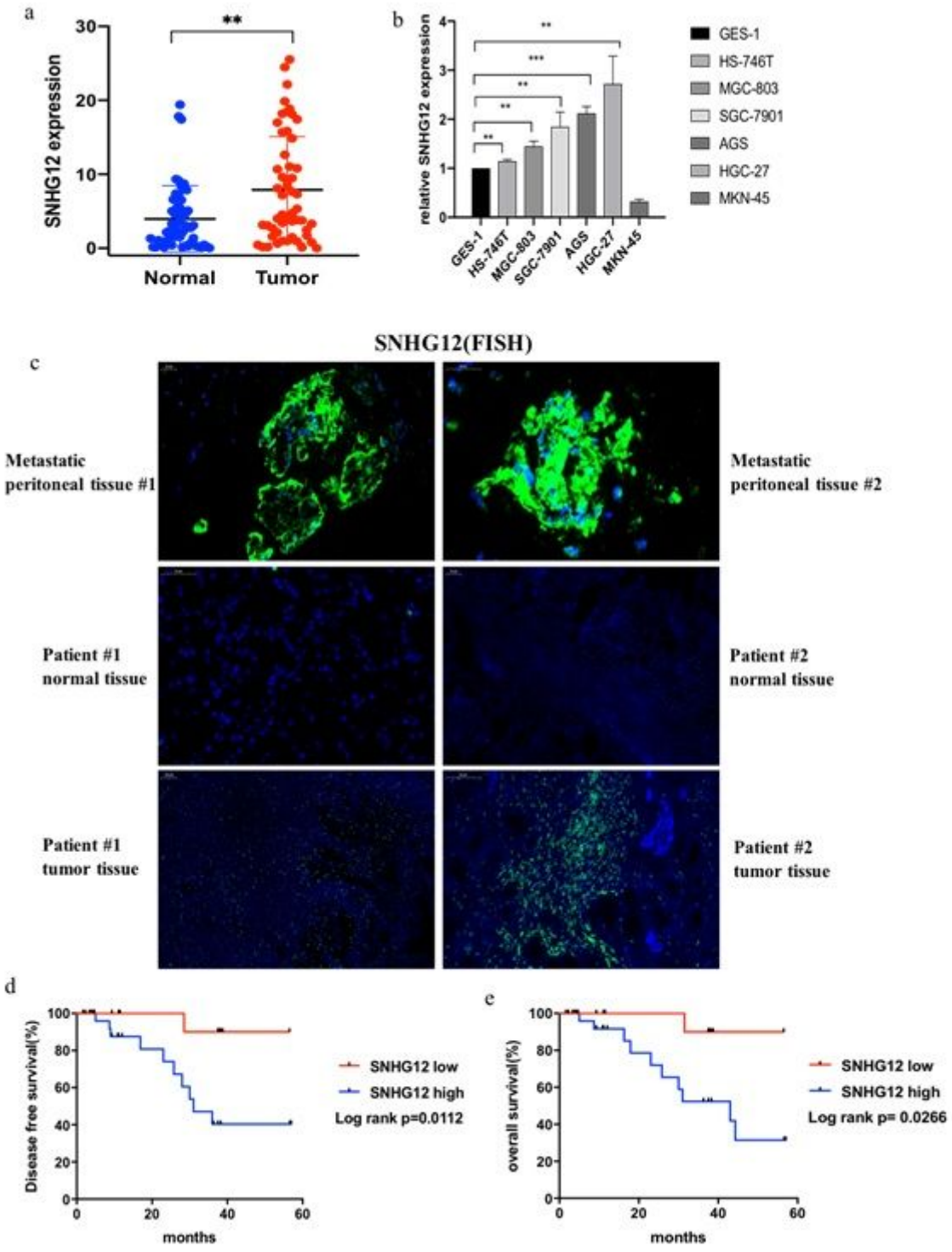

Figure 1

SNHG12 is overexpressed in GC tissues and cell lines and indicates poor prognosis in GC. a SNHG12 expression in 54 pairs GC tissues and matched normal gastric epithelial tissues. b qRT-PCR assays showing the relative SNHG12 expression in GC cell lines and GES-1. c FISH assays showing SNHG12 expression in GC metastatic peritoneal, non-tumor and tumor tissues, magnification ×200. d,e Kaplan-Meier analysis showing the 5-year DFS and OS of GC patients with high SNHG12 expression or low SNHG12 expression. Scale bar, 50μm. Significant results were presented as **P<0.01, ***P<0.001.

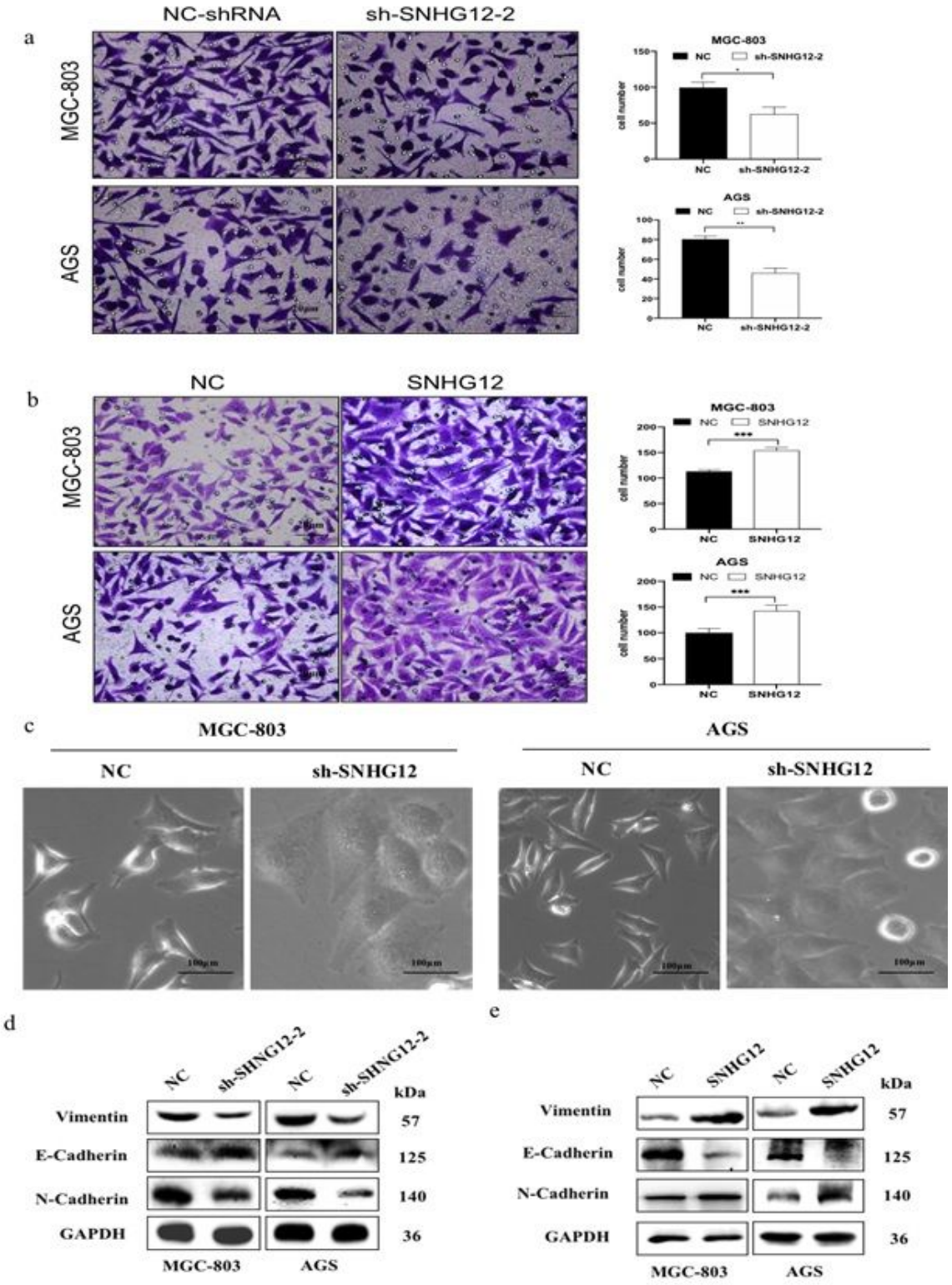

Figure 2

SNHG12 promotes GC cell migration and EMT in vitro. a,b Transwell assays showing the effects of the regulation of SNHG12 on GC cell migration. Magnification ×200, Scale bar 20μm. Significant results were presented as *P<0.05, **P<0.01, ***P<0.001. c Morphological change of the cells with stable SNHG12 knockdown (sh-SNHG12) compared with mock control cells (NC). Magnification ×200, Scale bar 100μm.

d WB assays exhibit change of EMT markers among stable SNHG12 knockdown cells (sh-SNHG12), stable SNHG12 overexpressed cells (SNHG12) and mock control cells (NC).

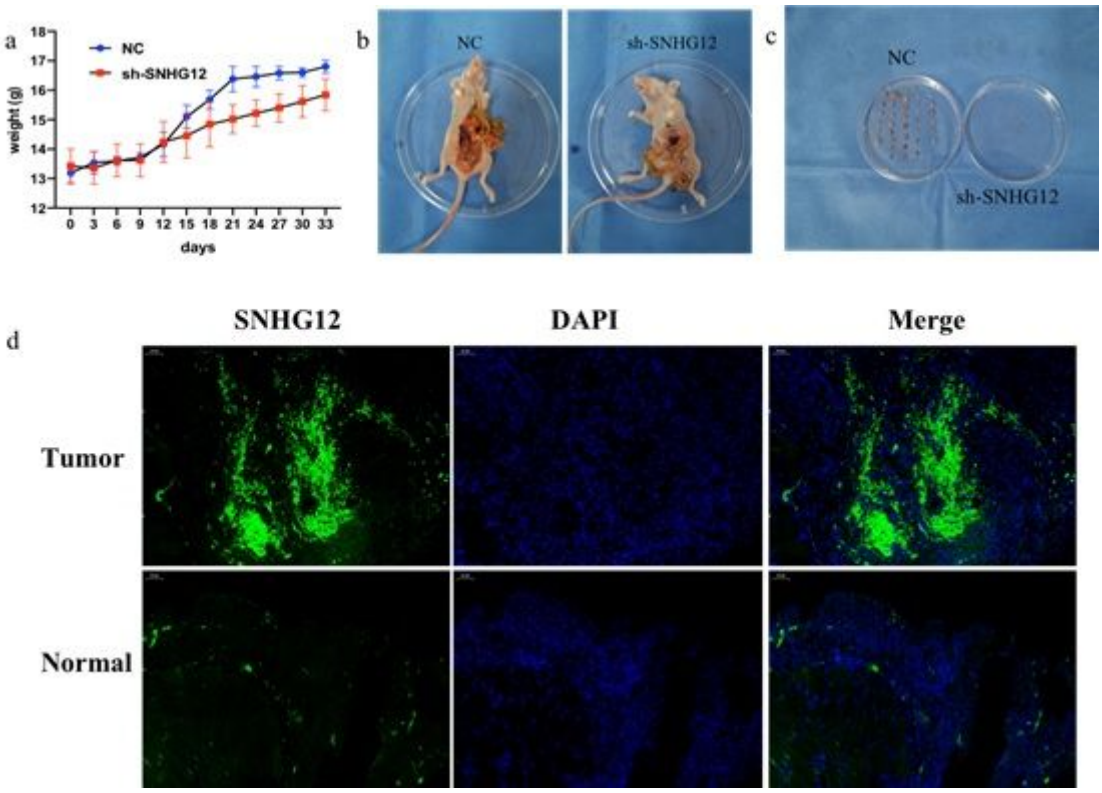

Figure 3

SNHG12 promotes GC metastasis in vivo. a Body weight of the animal subjects were recorded every 3 days for 1 month. b,c Obvious peritoneal metastatic formations in the control group (NC) as compared with the SNHG12 knockdown group (sh-SNHG12). d FISH assays showing SNHG12 fluorescence intensity between control groups (NC) and stable SNHG12 knockdown groups (sh-SNHG12). Magnification ×200, Scale bar 50μm.

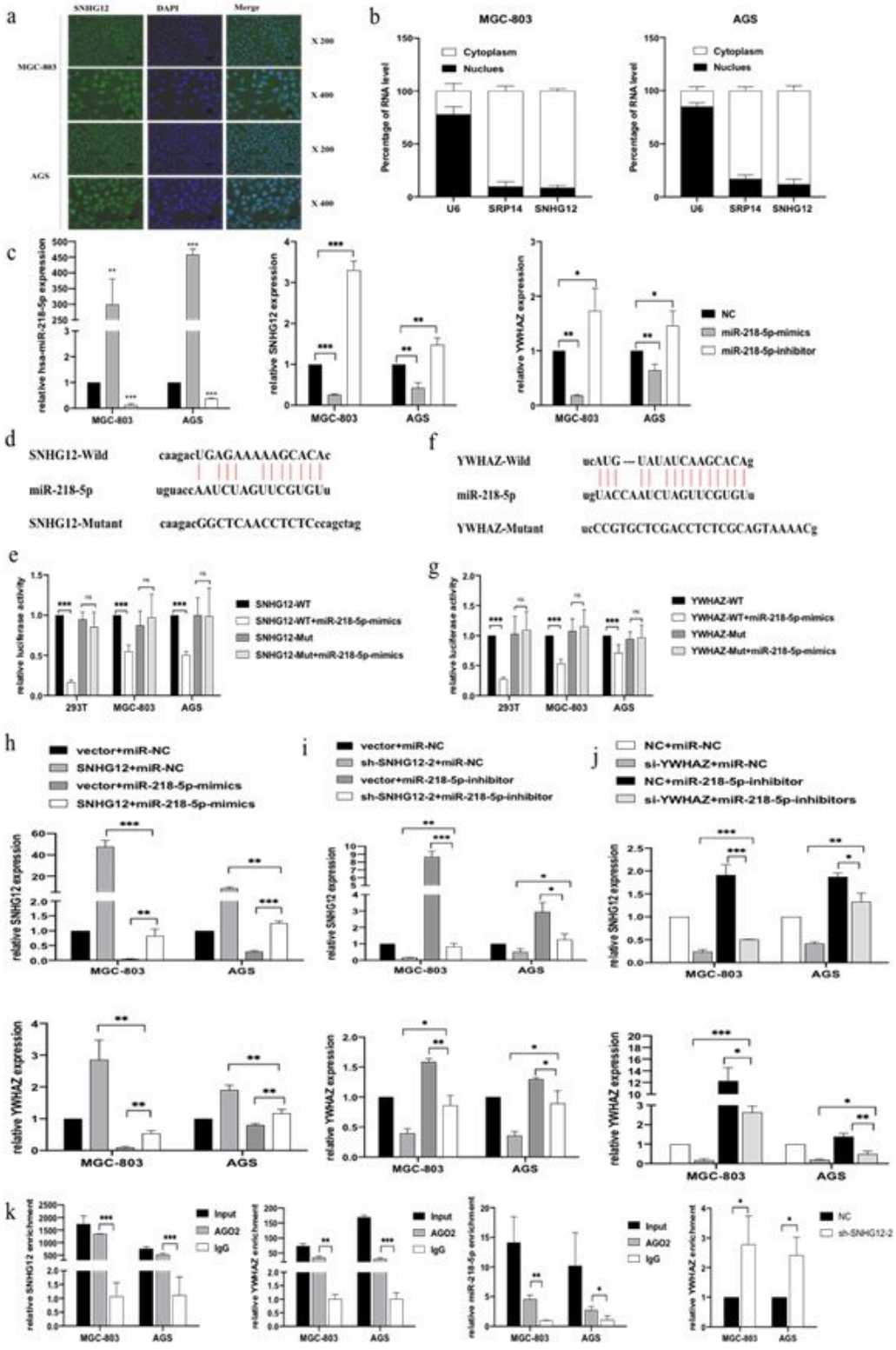

Figure 4

SNHG12 acts as a competitive endogenous RNA for miR-218-5p to regulate YWHAZ expression in GC cells. a, b FISH assays and cytoplasmic and nuclear RNA purification assays indicate that SNHG12 is located in GC cell cytoplasm. c qRT-PCR assays showing relative expression of miR-218-5p, SNHG12 and YWHAZ in GC cells transfected with miR-218-5p mimics or inhibitors. d,e Luciferase assays revealed the interaction between miR-218-5p and SNHG12. f,g Luciferase assays revealed the interaction between

miR-218-5p and YWHAZ. h, i, j qRT-PCR assays indicated the relative expression of SNHG12 and YWHAZ in GC cells transfected with miR-218-5p mimics or pCDH-CMV-SNHG12 and sh-SNHG12-2 or si-YWHAZ or miR-218-5p inhibitors. k RIP assays indicated the binding of SNHG12, miR-218-5p, or YWHAZ with Ago2. Significant results were presented as *P<0.05, **P<0.01, ***P<0.001.

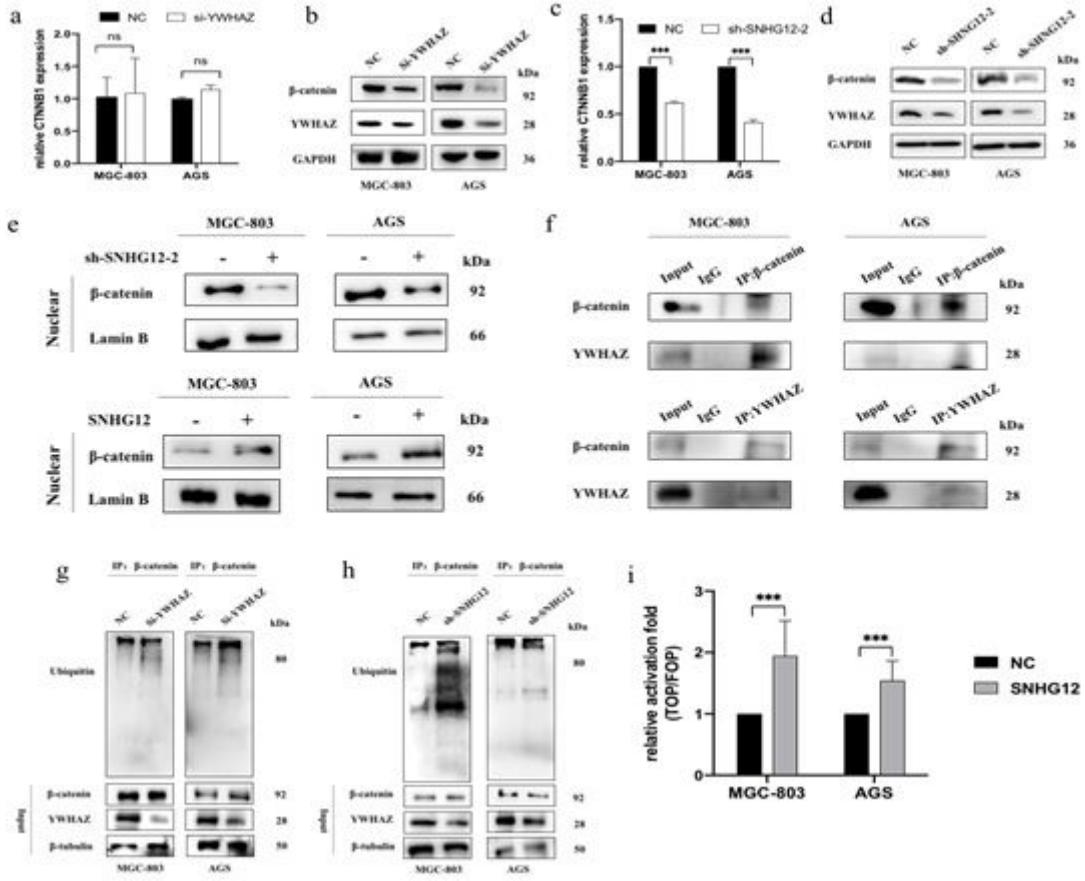

Figure 5

SNHG12 increases the expression of β-catenin via YWHAZ stabilizing β-catenin. a, b The RNA and protein expressions of β-catenin after YWHAZ knockdown were verified by qRT-PCR and WB. c, d The RNA and protein expressions of β-catenin after SNHG12 knockdown were verified by qRT-PCR and WB. e The nuclear expression of β-catenin after SNHG12 knockdown and overexpression was tested by western blotting. f Co-IP assays showing the interaction between YWHAZ and β-catenin. g,h Ubiquitination changes after knockdown YWHAZ and SNHG12 tested by IP in GC cells. i Luciferase assays showing the effects on TOP/FOP reporter activity in MGC-803 and AGS cells with SNHG12 overexpression. Significant results were presented as *P<0.05, **P<0.01, ***P<0.001.

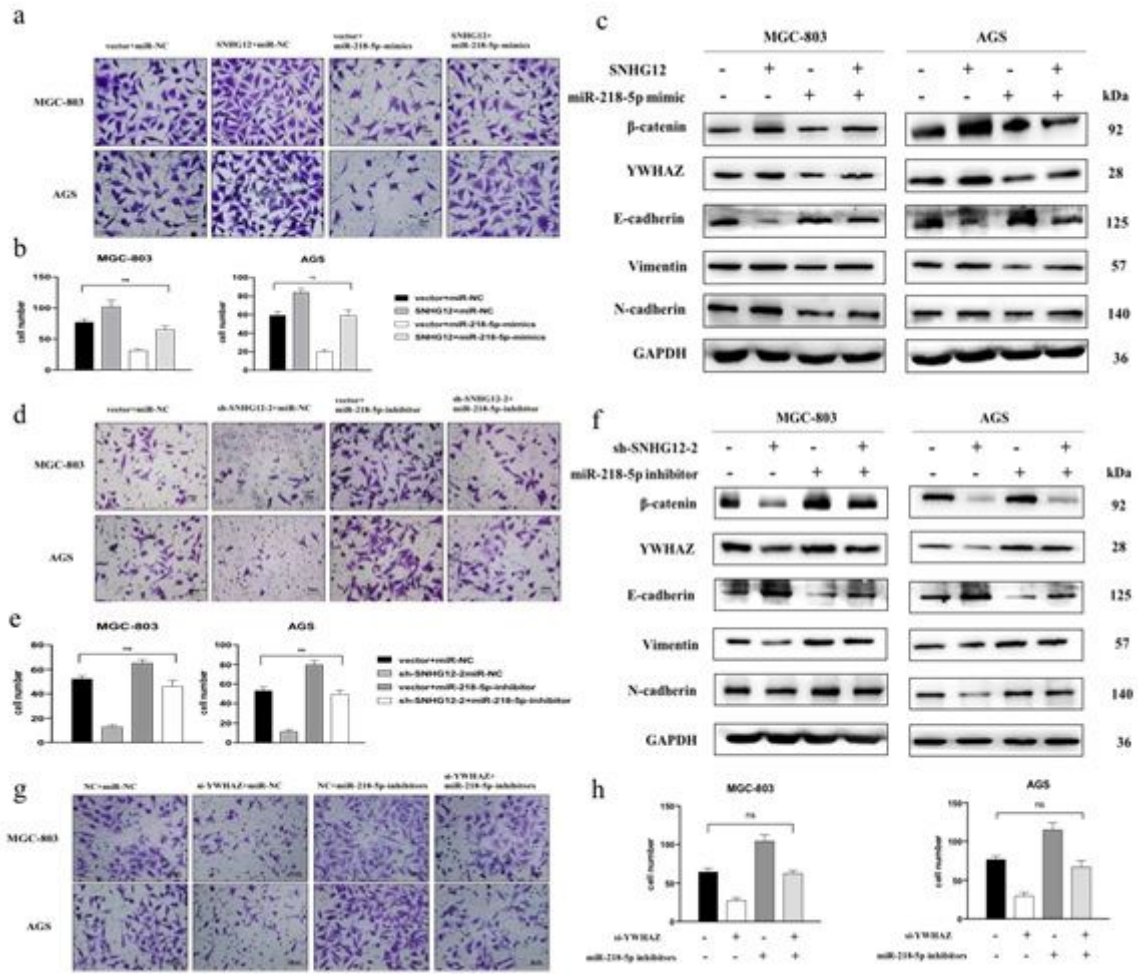

Figure 6

SNHG12/miR-218-5p/YWHAZ axis positively regulates GC cell metastatic potential via β-catenin pathway. a,b Transwell assays transfected with pCDH-SNHG12 or miR-218-5p mimics or both in MGC-803 and AGS. c WB assays showing the expression of EMT-related proteins, YWHAZ encoded protein and β-catenin transfected with pCDH-SNHG12 or miR-218-5p mimics or both in MGC-803 and AGS. d,e Transwell assays transfected with sh-SNHG12-2 or miR-218-5p inhibitors or both in MGC-803 and AGS. f WB assays showing the expression of EMT-related proteins, YWHAZ encoded protein and β-catenin transfected with sh-SNHG12-2 or miR-218-5p inhibitors or both in MGC-803 and AGS. g,h Transwell assays transfected with si-YWHAZ or miR-218-5p inhibitors or both in MGC-803 and AGS. Scale bar, 20μm. Significant results were presented as *P<0.05, **P<0.01, ***P<0.001.

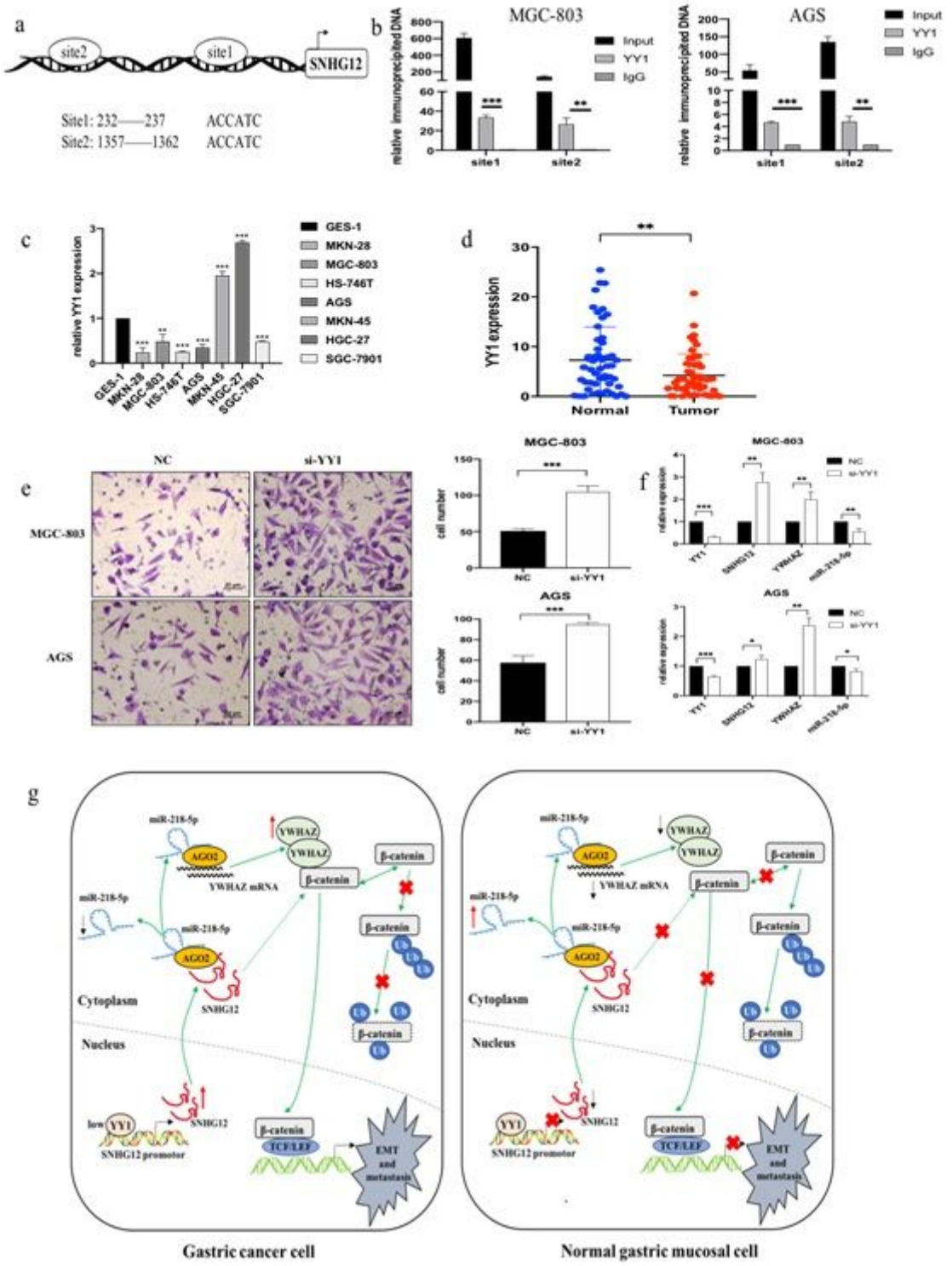

Figure 7

Transcription factor YY1 regulates the expression of SNHG12. a, b Bioinformatics analysis and CHIP assay showing YY1 binds to the promotor of SNHG12. c, d The expression of YY1 in GC tissues and cell lines. e Transwell assays showing the effects of the regulation of YY1 on GC metastasis. f RT-qPCR assays showing the expression of YY1, SNHG12, YWHAZ, miR-218-5p after YY1 knockdown. g Schematic illustration of the mechanism underlying SNHG12 regulation of GC metastasis and EMT. Scale bar, 20μm. Significant results were presented as *P<0.05, **P<0.01, ***P<0.001.

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