Long non-coding RNA Inc-GNAT1-1 inhibits gastric cancer cell proliferation and invasion through the Wnt/β-catenin pathway in Helicobacter pylori infection

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Abstract. The aim of the present study was to investigate the effect of long non-coding (Inc)‑G protein subunit α transducin 1 (GNAT1)-1 in gastric cancer cells infected with Helicobacter (H.) pylori. Reverse transcription‑quantitative polymerase chain reaction was used to assess Inc‑GNAT1‑1 expression in normal gastric and gastric cancer cells infected with H. pylori. The overexpression of Inc‑GNAT1‑1 in SGC‑7901 and MNK45 cells was induced by transfection. Migration and invasion assays were performed in transfected gastric cancer cells to evaluate the effect of Inc‑GNAT1‑1. Histological and western blot analyses were used to determine the alterations in the Wnt/β-catenin signaling pathway protein expression. Cells transfected with Inc‑GNAT1‑1 were used to initiate gastric cancer tumor xenografts to compare tumor growth in mice inoculated with untransfected cells. The results revealed that H. pylori infection significantly downregulated Inc‑GNAT1‑1 expression. Lnc‑GNAT1‑1 overexpression inhibited gastric cancer cell migration and invasion. Wnt/β-catenin pathway protein expression was decreased by Inc‑GNAT1‑1 overexpression. In addition, Inc‑GNAT1‑1 overexpression reduced tumor growth. Thus, Inc‑GNAT1‑1 is downregulated in gastric cancer infected with H. pylori and Inc‑GNAT1‑1 overexpression inhibited gastric cancer growth through the Wnt/β-catenin signaling pathway.

Introduction

Gastric cancer is one of the most common cancers with a high prevalence worldwide (1). In total, >80% of diagnoses occur when gastric cancer has already progressed to later stages of the disease, which significantly impedes the effectiveness of therapy in this highly aggressive disease. Thus, the prognosis of patients with gastric cancer remains poor (2). Early diagnosis of gastric cancer and effective treatment to prevent gastric cancer progression is critical in improving the clinical management of gastric cancer. Hence, the underlying molecular mechanisms of gastric cancer require further investigation in order to develop successful therapeutic strategies to improve patient survival.

Helicobacter pylori (H. pylori) infection is an important oncogenic driver of gastric cancer. It was reported in 2008 that an estimated 660,000 cases of cancer were attributable to H. pylori infection, which is ~5.2% of all cancer cases (3). Upon H. pylori infection, a cascade of molecular alterations that may ultimately lead to tumorigenesis is induced in gastric cells (4). Long non-coding RNAs (lncRNAs), which constitute a large portion of the human genome, are increasingly identified as novel cancer regulators (5). They are typically >200 nucleotides in length and modulate gene expression post-transcriptionally (6). In gastric cancer, a number of IncRNAs have been confirmed to be involved in cancer promotion or suppression (5,7-9). The IncRNAs HOX Transcript Antisense RNA and H9 have been identified as potent gastric cancer inducers by increasing tumor cell invasiveness and metastasis (10). In addition, downregulation of the IncRNA Maternally Expressed 3 is associated with poor survivability and metastasis (10). In colorectal cancer, downregulation of Inc-GNAT1-1 is characteristic of high risk patients with poor prognosis. Lnc-G protein subunit α transducin 1 (GNAT1)-1 is a novel cancer suppressor (12). In colorectal cancer, downregulation of Inc-GNAT1-1 is characteristic of high risk patients with poor prognosis. Lnc-GNAT1-1 suppresses colorectal cancer by modulating the Raf kinase inhibitor protein (RKIP)-nuclear factor (NF)-κB-protein snail homolog 1 (Snail) circuit (12). However, to the best of our knowledge, the regulatory role of Inc-GNAT1-1 in gastric cancer has not yet been reported. Understanding the role and mechanism of Inc-GNAT1-1 in gastric cancer may contribute to the discovery of novel diagnostic and therapeutic tools to improve the clinical management of gastric cancer.
The present study focused on the role of Inc-GNAT1-1 in gastric cancer induced by *H. pylori* infection and aimed to suggest potential strategies for gastric cancer treatment based on Inc-GNAT1-1 regulation. The results of the present study may aid in uncovering the molecular mechanism of *H. pylori* in gastric cancer tumorigenesis and provide a gene therapy tool for the treatment of this highly aggressive cancer.

**Materials and methods**

**Cell culture.** Normal gastric epithelial cells were obtained from Wuhan Feiyi Group (Wuhan, China). Human gastric cancer cell lines SGC-7901 and MKN45 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS and 5% CO2 atmosphere of 95% air and 5% CO2.

**Infection of cells with *H. pylori* and cell transfection.** Cell transfection was performed as previously described (12). Human gastric cancer cell lines SGC-7901 and MKN45, obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China), were grown in Minimum Essential Medium with Earle's salts (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% FBS in a 5% CO2 atmosphere at 37°C, and collected during the logarithmic growth phase, and cells were seeded into 6-well plates at a density of 5x10^4 cells per well. Standard *H. pylori* (ATCC), which contained the entire cytotoxin-associated gene pathogenicity island (*cag*PAI), including *cagA*, grown in Brucella broth (Shanghai Biomart Co., Ltd., Shanghai, China) were inoculated with SGC-7901 and MKN45 cells to infect cells at a ratio of bacteria to SGC-7901 and MKN45 cells of 100:1 and with a multiplicity of infection of 10 (5x10^4 plaque forming units). Isogenic mutants lacking *cag*PAI or *cagA* (ATCC) were also inoculated into the gastric epithelial cells as control measures. Following 24 h, total RNA was extracted from the gastric epithelial cells using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) under the following temperature protocol: 55°C for 30 min and 85°C for 20 min. Transfection was performed using 2nd generational system using 293 cells. PCR was performed to amplify an EcoRI-EcoRI fragment containing full-length Inc-GNAT1-1 cDNA using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA) following thermocycling conditions: 98°C for 20 sec; followed by 30 cycles of 98°C for 5 sec, 55°C for 10 sec and 72°C for 30 sec; and then a final extension step at 72°C for 5 min. Primers were directly provided by GenePharma Co., Ltd. (Shanghai, China; sequences not available). This fragment was inserted into EcoRI linearized pIRSE2-EGFP vectors (Clontech Laboratories, Inc., Mountainview, CA, USA) to establish Inc-GNAT1-1 expressing vectors. Lipofectamine® 2000 reagent (cat. no. 11668-019; Invitrogen, Thermo Fisher Scientific, Inc.) was then used to transfect 10 nM vectors into 5x10^4 SGC-7901 and MKN45 cells. Empty pIRSE2-EGFP vectors were used as negative control.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from cells using TRizol® reagent (Thermo Fisher Scientific, Inc.). First-strand cDNA was generated using a Reverse Transcription system kit (Takara Biotechnology Co., Ltd., Dalian, China). RNA was heated to 70°C for 5 min, chilled on ice for 5 min and then incubated at 25°C for 5 min. Reagents within the Reverse Transcription kit were used to polyadenylate all lcnRNAs prior to cDNA conversion, as some of lcnRNAs did not have poly A tails. qPCR was performed using a SYBR-Green PCR kit (ABgene UK Ltd.; Thermo Fisher Scientific, Inc.) in a StepOne System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. PCR thermocycling conditions used were as follows: 50°C for 60 sec; followed by 40 cycles of 95°C for 15 sec and 56°C for 35 sec. The primer sequences used were as follows: Inc-GNAT1-1 forward 5'-ATG TGT CGC AGA GTA GC-3'; and GAPDH forward 5'-GCA AGA GCA CAA AGA GTA GC-3' and GAPDH reverse 5'-ACTGTGAGGAGGGAGATTGC-3'. GAPDH was used as an endogenous control. RT-qPCR results were quantified with ABI Prism 7900HT (Applied Biosystems; Thermo Fisher Scientific, Inc.) and were analyzed using the 2^-ΔΔCt method (13).

**Invasion assay.** Invasion assays were performed using Transwell invasion chambers coated with Matrigel (50 μl/filter; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. A total of 2x10^4 cells were transfected with pLV-Control (Ctrl) or pLV- Inc-GNAT1-1 and cultured for 48 h prior to transfer to the upper Matrigel-coated invasion chamber in a 1% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc.) and Dulbecco's modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.). DMEM/F12 containing 10% FCS (Sigma-Aldrich; Merck KGaA) was added to the lower chamber. Following incubation for 24 h at 37°C with 5% CO2, invasive cells in the lower chamber were stained with 1% crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at 25°C. Cells were subsequently counted under a light microscope (magnification, x100). Assays were repeated six times.

**Migration assay.** Migration assays were performed in a 24-well Transwell chamber system. Following transfection for 24 h, cells were seeded in the upper chamber at 2x10^4 cells/ml in 0.1 ml serum-free DMEM/F12 media. Media supplemented with 10% FBS was placed in the bottom well at a volume of 0.8 ml. Cells were incubated for 24 h at 37°C with 5% CO2. Following this, migrated cells in the lower chamber were stained with 1% crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at 25°C. Cells were subsequently counted under a light microscope (magnification, x100). All experiments were repeated six times over multiple days.

**In vivo assay of tumor growth.** A total of 8 of BALB/c nude mice (age, 6-8 weeks; weight, 20-22 g; 4 male and 4 female) were purchased from Charles River Laboratories, Inc. (Beijing, China) and housed at a temperature of 25°C with a humidity of 50%, a 12/12 h light/dark cycle and free access to food and water. The protocol of the present study was approved by the Ethics Review Committee of The First
Hospital of Lanzhou University (Lanzhou, China). SGC-7901 or MKN45 cells (1x10^3) that had been stably transfected with lnc-GNAT1-1-expressing vectors were suspended in 100 µl PBS and subcutaneously inoculated into the bilateral armpit, a thin subcutaneous fat and muscle layer between the fore limb and trunk of 4 BALB/c nude mice. An equivalent amount of SGC-7901 or MKN45 cells were injected into a further 4 BALB/c nude mice to generate a control group, and the mice did not demonstrate any clinical signs of tumor burden. The tumors were measured every 7 days following implantation, and the volume of each tumor was calculated by length x width^2 x 0.5. At 4 weeks following inoculation, mice were sacrificed and tumor tissues were collected and weighed. Humane end-points were strictly observed. Mice exhibiting signs of moderate to severe discomfort were euthanized. This was accomplished by anesthetizing the animals with 2,2,2-tribromoethanol (0.25 ml/kg) (14) followed by cervical dislocation, in accordance with the American Veterinary Medical Association guidelines on euthanasia (15). Analgesics were not used as they had the potential to affect the experimental outcomes of the study.

Western blot analysis. Total protein from cell lysates of both tumor samples and tissue samples from healthy mice were extracted using 1% SDS buffer (cat. no. P0013K; Beyotime Institute of Biotechnology, Haimen, China), and protein concentration was then determined using a BCA protein assay. Protein samples (40 µg/lane) were separated by 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. Following blocking for 1 h at 4°C in 5% non-fat milk and then washing with Tris-buffered saline with 10% Tween 20, membranes were incubation with anti-β-catenin (1:1,000; cat. no. 9582; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Cyclin D (1:1,000; cat. no. sc-8396; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-c-Myc (1:900; cat. no. sc-789; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:2,000; cat. no. ab8227; Abcam, Cambridge, UK) primary antibodies overnight at 4°C. Following this, blots were incubated with anti-rabbit IgG-horseradish peroxidase secondary antibodies (1:3,000; cat. no. TA130024; OriGene Technologies, Inc., Rockville, MD, USA) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescent reagent (PerkinElmer, Inc., Waltham, MA, USA) and quantified with ImageJ 1.37 software (version 1.37; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance followed by Tukey's post-hoc test was used to determine significant differences between ≥2 groups. Comparisons between two groups were performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

*H. pylori* infection downregulates Inc-GNAT1-1 expression in normal gastric and gastric cancer cell lines. The effect of *H. pylori* infection on the expression of Inc-GNAT1-1 in normal and gastric cancer cells was investigated. The RNA expression of Inc-GNAT1-1 was detected by RT-qPCR analysis. As shown in Fig. 1A, normal gastric GES cells had significantly lower Inc-GNAT1-1 levels following *H. pylori* infection at a MOI of 1:1 and 10:1 (1:1, P=0.02 and 10:1, P=0.007; Fig. 1A). A higher MOI induced a greater reduction in Inc-GNAT1-1 expression, indicating a dose-dependent manner in this effect. The reduction in Inc-GNAT1-1 expression was also observed in SGC-7901 cells (1:1, P=0.01 and 10:1, P=0.002; Fig. 1B) and MKN45 cells (1:1, P=0.02 and 10:1, P=0.004; Fig. 1C). The RNA expression of Inc-GNAT1-1 was significantly decreased in SGC-7901 and MKN45 cells (P=0.009 and P=0.012, respectively; Fig. 1D) compared with normal gastric GES cell at the same MOI (10:1). Therefore, the results revealed that normal gastric cells and gastric cancer cells were subject to Inc-GNAT1-1 reduction upon *H. pylori* infection; however, the Inc-GNAT1-1 reduction was more marked in gastric cancer cells.

**Ectopic Inc-GNAT1 overexpression inhibits gastric cancer cell invasion and migration.** To further confirm the role of Inc-GNAT1-1 in gastric cells under *H. pylori* infection, Inc-GNAT1-1 was overexpressed through transfection of a Inc-GNAT1-1 expression vector. Lentiviral transfection was used to transfect Inc-GNAT1-1 expression vector to induce Inc-GNAT1-1 overexpression. RT-qPCR was used to analyze Inc-GNAT1-1 expression in transfected SGC-7901 and MKN45 cells, compared with that of untransfected cells. As presented in Fig. 2, SGC-7901 and MKN45 cells transfected with Inc-GNAT1-1 had significantly higher levels of Inc-GNAT1-1 (P=0.004).

In addition, the effects of Inc-GNAT1-1 overexpression on gastric cancer cell migration and invasion was explored. The results revealed that Inc-GNAT1-1 overexpression significantly reduced the migration and invasion ability of SGC-7901 (Fig. 3A-C) and MKN45 cells (Fig. 3D-F).

**Lnc-GNAT1 downregulates Wnt/β-Catenin pathway protein expression in gastric cancer cells infected with *H. pylori*.** To investigate the association between Wnt/β-catenin signaling and tumor growth, the expression of key proteins in the Wnt/β-catenin signaling pathway was evaluated. β-catenin, cyclin D and c-Myc expression was significantly decreased in tumors overexpressing Inc-GNAT1-1 (Fig. 4).

To elucidate the underlying mechanism of Inc-GNAT1-1 in inhibiting gastric cancer migration and invasion, the association between Inc-GNAT1-1 overexpression and the Wnt/β-catenin pathway was also investigated in gastric cancer cell lines. Consistently, Inc-GNAT1-1 overexpression significantly decreased Wnt/β-catenin pathway protein expression in SGC-7901 cells (Fig. 5A and B) and MKN45 cells (Fig. 5C and D). These results indicated that Inc-GNAT1-1 may have the potential to be developed as a cancer therapeutic that may impede cancer cell migration through Wnt/β-catenin pathway protein expression inhibition. Thus, the efficacy of Inc-GNAT1 overexpression in attenuating tumor growth in vivo was further investigated.

**Overexpression of Inc-GNAT1-1 inhibits tumor tissue growth in nude mice.** MKN45 cells transfected with or without
lnc-GNAT1-1 were used to construct gastric cancer tumor xenografts and tumor growth was subsequently monitored. As shown in Fig. 6, tumor volume and tumor weight were monitored 4 weeks following inoculation. A much slower rate of volume increase was observed in gastric tumors derived from MNK45 cells overexpressing lnc-GNAT1-1 (Fig. 6A). As presented in Fig. 6B and C, tumor size and tumor weight of lnc-GNAT1-1 overexpressing tumors was also decreased when compared with the control group.

Taken together, these results indicated that lnc-GNAT1-1 overexpression may impede gastric cancer progression.

Discussion

It is well established that H. pylori infection is an important risk factor in gastric cancer. H. pylori is categorized as a group I carcinogen by the International Agency for Research on Cancer (16). Therefore, substantial effort has been devoted to reduce H. pylori infection in order to decrease the incidence of gastric cancer; this has been successful in developing countries (17). Despite significant progress, chemotherapy and radiation therapy are combined with surgery to improve therapeutic outcomes, and advanced strategies and effective treatments are required for patients with gastric cancer. At present, the highly invasive gastrectomy remains the principle treatment for gastric cancer (18) and more efficient approaches in gastric cancer treatment must be developed.

Encouraged by progress in gene therapeutics for various types of cancer (19), the present study aimed to evaluate the role of an emerging cancer regulating lncRNA, lnc-GNAT1-1, in gastric cancer. The results revealed that...
lnc-GNAT1-1 expression was significantly downregulated by H. pylori infection. A previous report has demonstrated that lnc-GNAT1-1 may act as a cancer suppressor by inhibiting cancer cell invasion and migration (12), suggesting that decreased levels of lnc-GNAT1-1 may be an indicator for high-risk gastric cancer.

In the present study, lnc-GNAT1-1 overexpression induced by lentiviral transfection resulted in decreased cancer cell invasion and migration in vitro. In vivo, gastric tumor growth was also attenuated by lnc-GNAT1-1 overexpression, which demonstrated the function of lnc-GNAT1-1 in gastric cancer. Compared with surgery, gene therapy strategies are less invasive. In addition, as normal tissues express high levels of lnc-GNAT1-1 (12), non-specific transfection of lnc-GNAT1-1 expressing vector presumably would not induce toxic effects on normal tissues, making it a potential tumor-specific treatment strategy.

The Wnt/β-catenin signaling pathway promotes the growth of aggressive cancer cells (20). The present study identified the Wnt/β-catenin signaling pathway as an underlying mechanism of lnc-GNAT1-1 in gastric cancer cell regulation. Activation of the Wnt/β-catenin pathway is a putative mechanism in the promotion of cancer cell invasion, migration and dissemination (21). H. pylori infection has also been associated with increased Wnt/β-catenin signaling to generate gastric cancer cells with stem cell-like properties (22). However, to the best of our knowledge, the association between lnc-GNAT1-1 and the Wnt/β-catenin signaling pathway has not been previously reported. Previous findings have suggested that lnc-GNAT1-1 is associated with RKIP-NF-κB-Snail pathway modulation (12). As Wnt/β-catenin and the RKIP-NF-κB-Snail signaling
pathways may contribute to malignant cancer development, this finding may account for the therapeutic potential of lnc-GNAT1-1 in inhibiting gastric cancer progression (12). In the present study, it was demonstrated that H. pylori infection significantly downregulated the expression of lnc-GNAT-1. Furthermore, lnc-GNAT1-1 overexpression was revealed to decrease the protein expression associated with the Wnt/β-catenin pathway, suppress tumor growth and suppress gastric cancer cell migration and invasion abilities. However, the results of the present study suggested that lnc-GNAT1-1 did not fully suppress Wnt/β-catenin signaling, and thus there may be a requirement for other potent Wnt/β-catenin signaling suppressors combined with potential lnc-GNAT1-1 targeting therapies for the treatment of gastric cancer. It was not investigated if lnc-GNAT1-1 overexpression may be useful in the treatment of chemoresistant gastric cancer in the present study. However, it is probable that chemoresistance may be alleviated, as Wnt/β-catenin signaling has been reported to be involved in the development of chemoresistance (23).

In conclusion, the present study demonstrated that lnc-GNAT1-1 may have an important role in gastric cancer induced by H. pylori infection. Upon infection, lnc-GNAT1-1 expression was significantly downregulated, and lnc-GNAT1-1 overexpression inhibited gastric cancer cell migration and invasion. In addition, Wnt/β-catenin signaling pathway protein expression was reduced by lnc-GNAT1-1 overexpression. Furthermore, tumor growth was slower in mice inoculated with cells overexpressing lnc-GNAT1-1. Therefore, gene therapy targeting lnc-GNAT1-1 may be a potential strategy for gastric cancer suppression; however, further studies are required to validate lnc-GNAT1-1 as a useful biomarker for gastric cancer diagnosis.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
LL, TS, BL, LZ and XL were responsible for the conception and design of the study. LL and XL performed the experiments. LL, TS and BL analyzed and interpreted the data. LL and BL drafted the article. LZ and XL were responsible for the revision of the manuscript.
Ethics approval and consent to participate

The protocol of the present study was approved by the Ethics Review Committee of the First Hospital of Lanzhou University (Lanzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References
