Linc-pint overexpression inhibits the growth of gastric tumors by downregulating HIF-1 α

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Abstract. Long intergenic non-protein coding RNA, p53 induced transcript (Linc-pint) has been reported to be down-regulated in various cancer cell lines; however, its expression profile and role in gastric cancer remains unknown. The present study aimed to investigate the involvement of Linc-pint in gastric cancer. Through quantitative polymerase chain reaction, western blotting and viability assays, it was observed that Linc-pint expression was significantly down-regulated in gastric biopsies from patients with gastric cancer, compared with healthy controls. Conversely, the expression of hypoxia-inducible factor-1 α (HIF-1 α) mRNA was significantly upregulated in patients with gastric cancer compared with in healthy controls. Using a variety of statistical inference tests, including receiver operating characteristic curve and correlation analyses, it was determined that the expression levels of Linc-pint and HIF-1 α exhibited a significantly negative correlation in patients with gastric cancer but not in healthy controls. Linc-pint expression was significantly and inversely associated with tumor size but not tumor metastasis. Linc-pint overexpression inhibited the proliferation of gastric cancer cells, whereas treatment with exogenous HIF-1 α promoted proliferation. Linc-pint overexpression downregulated the expression of HIF-1 α , whereas exogenous HIF-1 α did not significantly alter Linc-pint expression. Furthermore, treatment with exogenous HIF-1 α suppressed the inhibitory effects of Linc-pint overexpression on the proliferation of gastric cancer cells. In conclusion, overexpression of Linc-pint may inhibit the growth of gastric tumors via downregulation of HIF-1 α .

Introduction

The abnormally accelerated growth of cancer cells is an important characteristic of the development and progression of various types of cancer (1). Inhibition of the growth of cancer cells is considered to be a promising therapeutic approach in the treatment of cancer (2). Gastric cancer is one of the most frequently diagnosed malignancies (3); in China alone, gastric cancer is the third most common tumor-associated malignancy, following lung and breast cancer (4). Numerous drugs have been developed to inhibit the progression of gastric cancer (5,6); however, as the mechanisms underlying tumor growth remain unclear, treatment outcomes remain unsatisfactory. Therefore, the identification of novel molecular targets that efficiently inhibit the growth of gastric tumors is required.

Hypoxia-inducible factor-1 α (HIF-1 α) signaling serves important roles in the onset, development and progression of several types of cancer, and is considered to be a potential target in the treatment of these types of pathology (7). Hypoxia is an important regulator of tumor growth, and the role of HIF-1 α in the growth of gastric cancer cells has been verified (8,9). Long intergenic non-protein coding RNA, p53 induced transcript (Linc-pint) is a long noncoding RNA (lncRNA) with potential tumor suppressor function in various types of cancer (10,11). It was previously reported that HIF-1 α may interact with lncRNAs to affect physiological or pathological processes (12). In the present study, it was revealed that Linc-pint may have inhibited the growth of gastric cancer cells, potentially via the downregulation of HIF-1 α .

Materials and methods

Subject enrolment and gastric biopsies. A total of 167 patients with gastric cancer were diagnosed and treated at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between January 2015 and March 2017. Of these patients, 52 were included in the present study according to inclusion and exclusion criteria. The inclusion criteria were as follows: Patients were i) diagnosed via pathological gastric biopsies; ii) diagnosed and treated for the first time; and iii) willing to participate in the study. The exclusion criteria were as

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follows: Patients i) had received treatment prior to admission; ii) presented other malignancies; iii) were diagnosed with chronic diseases; or iv) were diagnosed with other gastric diseases. The cohort comprised 30 males and 22 females, aged 28-68 year old with a mean age of 46.5 ± 8.1 years. In addition, 221 patients received gastric biopsies at the Fourth Hospital of Hebei Medical University during the same period to confirm suspected gastric lesions. Gastric lesions were not diagnosed in 62 cases. From these 62 cases, gastric biopsies from 26 males and 18 females (to match the gender distribution of the patient group, 30-67 years, mean age 47.2 ± 8.5 years) were randomly selected to serve as the control group. Patients with any clinical disorders were excluded. No significant differences were observed between the general clinicopathological data of the two groups. The study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. All patients provided written informed consent.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol[®] reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total RNA from gastric biopsies, according to the manufacturer's protocols. cDNA was synthesized using a SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.) under the following conditions: 25°C for 5 min, 50°C for 25 min and 80°C for 10 min. qPCR reaction systems were prepared using SYBR[®] Green Quantitative RT-qPCR kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and qPCR was conducted under the following thermocycling conditions: 95°C for 1 min, followed by 40 cycles of 95°C for 20 sec and 58°C for 45 sec. The primers used for qPCR reactions were as follows: Linc-pint, forward 5'-CGTGGGAGCCCTTTAAGTT-3', reverse, 5'-GGGAGG TGGCGTAGTTTCTC-3'; HIF-1 α , forward 5'-AGCCCT AGATGGCTTTGTGA-3', reverse, 5'-TATCGAGGCTGT GTCGACTG-3'; and β -actin, forward 5'-GACCTCTATGCC AACACAGT-3' and reverse, 5'-AGTACTTGCGCTCAGG AGGA-3'. Expression levels were normalized to the endogenous control β -actin using the $2^{-\Delta\Delta C_q}$ method (13).

Cell lines, cell culture and cell transfection. The human gastric cancer cell lines AGS (gastric adenocarcinoma) and SNU-1 (gastric carcinoma) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured with ATCC-formulated RPMI-1640 medium (ATCC 30-2001; ATCC) containing 10% fetal bovine serum (ATCC 30-2020; ATCC) in an incubator (37°C, 5% CO₂). For hypoxic condition treatments, cells were cultivated at 37°C with 1% O₂, 94% N₂, and 5% CO₂. In cases of exogenous human recombinant HIF-1 α treatment, cells were treated with HIF-1 α (1, 10, 50 and 100 ng/ml; Sigma-Aldrich; Merck KGaA) for 24 h before use. Linc-pint cDNA flanked by NheI restriction sites was inserted into an NheI-linearized pEGFPC3 (Clontech Laboratories, Inc., Mountainview, CA, USA) vector to generate a Linc-pint expression vector. Cells were cultured overnight to reach 80-90% confluence and Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect 5×10^5 cells with 10 nM vector. Non-transfected cells were used as control cells. Cells transfected with empty vectors were used as negative control (NC) cells. RT-qPCR analysis demonstrated that, compared with control and NC cells, the

overexpression rate of Linc-pint was >200% at 24 h following transfection. All subsequent experiments were performed using cells with a Linc-pint overexpression rate of >200%, and the cells were harvested at 24 h after transfections (data not shown).

Cell proliferation assay. The proliferation of cells was evaluated via a Cell Counting Kit-8 (CCK-8) assay. AGS and SNU-1 cells were harvested and suspensions were produced with a density of 5×10^4 cells/ml using RPMI-1640 medium containing 10% fetal bovine serum. A total of 5×10^3 cells (0.1 ml) were inoculated in the wells of a 96-well plate. Cells were cultured in an incubator (37°C, 5% CO₂), and CCK-8 solution (10 μ l; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added 24, 48, 72 and 96 h later, respectively. Following culture for a further 4 h at 37°C, optical density values were obtained by detecting the absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. Radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) was used to extract total protein from AGS and SNU-1 cells according to the manufacturer's protocols. The protein concentration was measured using a bicinchoninic acid assay. Following denaturation, protein samples (20 μ g/lane) were separated via 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk for 1 h at room temperature and subsequently incubated with rabbit anti-human primary antibodies against HIF-1 α (1:1,500; cat. no. ab82832, Abcam, Cambridge, UK) and GAPDH (1:1,000; cat. no. ab9485, Abcam) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G secondary antibodies (1:1,000; cat. no. MBS435036, MyBioSource, Inc., San Diego, CA, USA) for 2.5 h at room temperature. Enhanced chemiluminescence reagent (Sigma-Aldrich; Merck KGaA) was then added to develop the signal. Protein expression was quantified using ImageJ v1.6 (National Institutes of Health, Bethesda, MD, USA); HIF-1 α expression was normalized to the endogenous control GAPDH.

Statistical analysis. All experiments were performed three times. Data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Gene expression and cell proliferation data were presented as the mean $\pm$ standard deviation. Comparisons between two groups were performed using t-tests. Comparisons across multiple groups were performed using one-way analyses of variance followed by the Least Significant Difference test. The 52 patients with gastric cancer were divided into high- (n=26) and low-expression (n=26) groups according to the median expression level of Linc-pint. Associations between Linc-pint expression and the various clinicopathological factors of patients with gastric cancer were analyzed using χ^2 tests. Receiver operating characteristic (ROC) curve analysis was performed using the default parameters of GraphPad Prism 6. Correlations between variables were determined using Pearson's correlation coefficient. $P < 0.05$ was considered to indicate a statistically significant difference.

Figure 1. Linc-pint is downregulated and HIF-1 α is upregulated in patients with gastric cancer. Expression of (A) Linc-pint and (B) HIF-1 α mRNA in gastric biopsies from patients with gastric cancer and healthy controls. Data are presented as the mean $\pm$ standard deviation. * $P<0.05$. HIF-1 α , hypoxia-inducible factor-1 α ; Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript.

Results

Linc-pint is downregulated and HIF-1 α is upregulated in patients with gastric cancer. The differential expression of a gene in a cohort of patients with a certain disease suggests that the gene may be involved in pathological processes associated with the disease. The expression of Linc-pint and HIF-1 α mRNA in gastric biopsies from patients with gastric cancer and healthy controls was determined via RT-qPCR. As presented in Fig. 1, it was demonstrated that Linc-pint was significantly downregulated ($P<0.05$) and HIF-1 α was significantly upregulated ($P<0.05$) in patients with gastric cancer compared with in healthy controls.

Downregulation of Linc-pint differentiates patients with gastric cancer from healthy controls. ROC curve analysis was performed to evaluate the diagnostic value of Linc-pint expression for gastric cancer. As presented in Fig. 2, the area under the curve (AUC) was 0.8910, with a standard error value of 0.03153 and 95% confidence interval of 0.8291-0.9528. It is generally believed that $AUC>0.65$ indicate good diagnostic potentials. Therefore, Linc-pint may serve as a potential diagnostic marker for gastric cancer.

Expression of Linc-pint and HIF-1 α mRNA is negatively correlated in patients with gastric cancer but not in healthy controls. The Pearson correlation coefficient was calculated to determine the association between the expression of Linc-pint and HIF-1 α mRNA. As presented in Fig. 3A, a significant negative correlation was observed between the expression of Linc-pint and HIF-1 α mRNA in patients with gastric cancer ($P<0.0001$); however, no significant correlation was reported between Linc-pint and HIF-1 α expression in healthy controls ($P=0.5484$; Fig. 3B).

Linc-pint expression in gastric biopsies is associated with the size of primary tumors. The 52 patients with gastric cancer were divided into high- ($n=26$) and low-expression ($n=26$) groups according to the median expression level of Linc-pint. Associations between Linc-pint expression and the

Figure 2. Receiver operator characteristic curve analysis of the diagnostic value of long intergenic non-protein coding RNA, p53 induced transcript for gastric cancer.

various clinicopathological factors of patients with gastric cancer were analyzed by a χ^2 test. As presented in Table I, Linc-pint expression was significantly associated with tumor size ($P<0.05$), but not with distant tumor metastasis, age or gender.

Linc-pint is a potential upstream inhibitor of HIF-1 α in gastric cancer cells. In the present study, one gastric adenocarcinoma cell line (AGS) and one gastric carcinoma cell line (SNU-1) were used to investigate the association between Linc-pint and HIF-1 α in gastric cancer. SNU-1 and AGS cells were transfected with a Linc-pint expression vector, and the expression of HIF-1 α was determined via western blot analysis. It was demonstrated that HIF-1 α was significantly downregulated in SNU-1 and AGS cells overexpressing Linc-pint under normal ($P<0.05$; Fig. 4A) and hypoxic conditions ($P<0.05$; Fig. 4B), compared with control and NC cells. Conversely, treatment with various concentrations of exogenous human recombinant HIF-1 α (1, 10, 50 and 100 ng/ml; Sigma-Aldrich; Merck KGaA) did not significantly alter Linc-pint expression

Table I. Correlation between Linc-pint expression and basic clinicopathological data of gastric cancer patients.

Variables	Groups	Cases	High expression	Low expression	χ^2	P-value
Age (years)	>50	23	10	13	0.70	0.40
	<50	29	16	13		
Sex	Male	30	13	17	1.26	0.26
	Female	22	13	9		
Primary tumor diameter (cm)	>5	24	8	16	4.95	0.03 ^a
	<5	28	18	10		
Tumor distant metastasis	Yes	27	11	16	1.93	0.17
	No	25	15	10		

^aP<0.05. Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript. Figure 3. Expression of Linc-pint and HIF-1 α mRNA is negatively correlated in gastric cancer patients but not in healthy controls. Association between Linc-pint and HIF-1 α mRNA expression in (A) patients with gastric cancer and (B) healthy controls. HIF-1 α , hypoxia-inducible factor-1 α ; Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript.

in gastric cancer cells under normal or hypoxic conditions, compared with cells without HIF-1 α treatment.

Linc-pint overexpression inhibits the proliferation of gastric cancer cells. The significant association between Linc-pint expression and tumor size suggested that Linc-pint is involved in tumor growth. The proliferation of SNU-1 and AGS cells following Linc-pint overexpression was determined using a CCK-8 assay. Gastric cancer cells overexpressing Linc-pint exhibited significantly reduced proliferation compared with the controls ($P<0.05$; Fig. 5). Conversely, treatment with exogenous HIF-1 α significantly increased the proliferation of gastric cancer cells compared with the controls ($P<0.05$). Furthermore, Linc-pint overexpression combined with HIF-1 α treatment significantly increased the proliferation of gastric cancer cells compared with Linc-pint overexpression alone ($P<0.05$).

Discussion

Linc-pint is an lncRNA with a downregulated expression profile and has potential roles as a tumor suppressor in various types of cancer (10,11); however, the involvement of Linc-pint in gastric cancer is yet to be determined. In the present study,

it was revealed that Linc-pint may be a tumor-suppressive lncRNA with diagnostic value in gastric cancer. The effects of Linc-pint may be potentially mediated via the downregulation of HIF-1 α .

The development of gastric cancer requires the involvement of numerous internal and external factors; however, genetic factors serve important roles (14). Overexpression of HIF-1 α has been reported in patients with gastric cancer (15). HIF-1 α overexpression promotes the proliferation, migration and invasion of cancer cells; it also induces drug resistance in gastric cancer cells, thereby leading to treatment failure (16). In the present study, it was observed that the expression of HIF-1 α mRNA was significantly upregulated in patients with gastric cancer compared with in healthy controls.

It has been reported that the development of gastric cancer is accompanied with alterations in the expression profiles of various lncRNAs (17), indicating the involvement of lncRNAs in the pathogenesis of this disease. Downregulation of Linc-pint has been observed in patients with pancreatic cancer; furthermore, the expression of Linc-pint is an indicator for the diagnosis and prognosis of pancreatic cancer (10). Additionally, downregulation of Linc-pint expression has been reported *in vitro* in various human malignant cell cultures (11).

Figure 4. Linc-pint overexpression downregulates the expression of HIF-1α in gastric cancer cells. Linc-pint overexpression significantly downregulated the expression of HIF-1α in SNU-1 gastric carcinoma and AGS gastric adenocarcinoma cells, under (A) normal and (B) hypoxic conditions. Conversely, treatment with exogenous human recombinant HIF-1α (1, 10, 50 and 100 ng/ml) did not significantly affect Linc-pint expression under the two conditions. Data are presented as the mean ± standard deviation. *P<0.05. C, non-transfected control; HIF-1α, hypoxia-inducible factor-1α; Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; NC, negative control transfected with empty vector.

Figure 5. Linc-pint overexpression inhibits the proliferation of gastric cancer cells. The proliferation of SNU-1 gastric carcinoma and AGS gastric adenocarcinoma cells following transfection with a Linc-pint overexpression vector and/or treatment with exogenous HIF-1α, as determined by a Cell Counting Kit-8 assay. Data are presented as the mean ± standard deviation. *P<0.05. C, non-transfected control; HIF-1α, hypoxia-inducible factor-1α; Linc-pint, long intergenic non-protein coding RNA, p53 induced transcript; NC, negative control transfected with empty vector.

In the present study, it was demonstrated that the expression of Linc-pint was decreased in patients with gastric cancer compared with in healthy controls. Furthermore, ROC curve analysis revealed that Linc-pint expression effectively differentiated patients with gastric cancer from healthy controls. Therefore, Linc-pint may serve as a potential diagnostic biomarker for gastric cancer.

Interactions between HIF-1 α and lncRNAs have been reported in the development of numerous human diseases, including gastric cancer (18-20). A significant negative correlation between HIF-1 α and Linc-pint expression was observed in patients with gastric cancer. Additionally, Linc-pint overexpression downregulated the expression of HIF-1 α in gastric adenocarcinoma and gastric carcinoma cell lines. Conversely, exogenous HIF-1 α treatment did not significantly alter Linc-pint expression. These findings indicated that Linc-pint may be an upstream inhibitor of HIF-1 α expression in the two types of gastric cancer. Of note, no significant correlation between Linc-pint and HIF-1 α was reported in healthy controls, suggesting that the inhibitory effects of Linc-pint on HIF-1 α expression occur in an indirect manner, and involve intermediate gastric cancer- or disease-specific stages. In the present study, HIF-1 α protein expression was not detected in certain patients due to low expression levels.

It has been reported that HIF-1 α interacts with certain glycolytic isoforms to promote the metabolism of cancer cells, increasing cell growth (21). In the present study, it was demonstrated that exogenous HIF-1 α promoted the proliferation of gastric cancer cells; however, Linc-pint overexpression reduced cell proliferation. Furthermore, treatment with exogenous HIF-1 α partially suppressed the effects of Linc-pint overexpression on gastric cancer cell growth. These findings suggested that Linc-pint inhibits the proliferation of gastric cancer cells via the downregulation of HIF-1 α . Additional experiments, including 5-ethynyl-2'-deoxyuridine and colony formation assays and *in vivo* experiments, are required to further support the conclusions of the present study.

In summary, Linc-pint was revealed to be downregulated in patients with gastric cancer. Furthermore, overexpression of Linc-pint inhibited the proliferation of gastric cancer cells, potentially by downregulating HIF-1 α .

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LH and WL designed experiments. LH, JW, HW, SW and FZ performed experiments. JH, YL, MM, CL and YX analyzed

data. WL drafted the manuscript and all authors approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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