Role of HDAC1 in the progression of gastric cancer and the correlation with lncRNAs

ZHIQIANG YU*, JUN ZENG*, HUI LIU, TIAN WANG, ZIQI YU and JIANYONG CHEN

Department of Gastroenterology, Jiangxi Provincial People's Hospital, Nanchang, Jiangxi 330006, P.R. China

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Abstract. Gastric cancer (GC) is a common life-threatening cancer type worldwide, with an increasing prevalence and a high rate of mortality. Due to limitations in clinical treatment, surgery has become the most efficient strategy for the treatment of GC. It is urgent to identify novel biomarkers, which are useful for the diagnosis of GC and for improving the survival rate of patients with GC. HDACs are multi-functional proteins and are involved in regulating gene expression, cell proliferation and the epigenetic regulation. However, the precise role of HDACs in the progression of GC remains unknown. The present study demonstrated that HDAC1 is involved in the promotion of GC cell proliferation, possibly by upregulating the expression of the lncRNAs, BC01600 and AF116637, in the tissues of patients with GC. Abnormal expression profiles of lncRNAs were observed in the tissues of patients with GC. lncRNAs were analyzed in the GSE64951 and GSE19826 databases, and it was revealed that BC01600 and AF116637 were two typically upregulated lncRNAs. Furthermore, it was revealed that BC01600 and AF116637 are regulated by HDAC1, as evidenced by decreased expression of these two lncRNAs in HDAC1-knockout SC-M1 cell lines, and by reduced expression of HDAC1 in these two lncRNA-knockout SC-M1 cell lines. Silencing of HDAC1 decreased the proliferation and increased the apoptosis of SC-M1 cell lines, but had no effect on the migration of the SC-M1 cell lines. The present study provided evidence of the importance of HDAC1 in the progression of SC-M1, and the association between HDAC1 and the expression of lncRNAs. The results of the present study indicated that HDAC1 may be a promising target for the clinical treatment of GC.

Introduction

Gastric cancer (GC) is a common life-threatening cancer type worldwide, with an increasing prevalence and high rate of mortality, particularly in China (1). Etiological factors, and genetic and epigenetic factors are all involved in the progression of GC (2). At present, surgery is the most effective treatment for GC, and the survival rate primarily depends on the stage of GC (non-invasive detection of GC) (3). Therefore, identifying specific and effective biomarkers in the early stage of GC is important for the efficient treatment of patients with GC in order to increase the survival rate. Previous studies have demonstrated that genetic abnormalities, including aberrant genes, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), participated in the initiation and progression of GC (competing endogenous RNA networks) (4).

Long non-coding RNAs are defined as transcripts >200 nucleotides, and are widely expressed in different tissues (5,6). lncRNAs are involved in a number of biological progresses, including the proliferation and differentiation of cells (7). lncRNA genes resemble protein-coding genes, and they have a variety of molecular functions across numerous cellular pathways and processes, including oncogenic signaling (8). Researchers have identified an increasing number of aberrantly expressed lncRNAs in GC, and have aimed to determine their role in the progression of GC (9-12).

Epigenetic modifications serve causative roles in the progression of GC (13). Histone deacetylases (HDACs), a family of 18 molecules divided into four sub-classes defined according to their structural similarities (14), serve functional roles, including modification of histone and non-histone proteins, regulation of chromatin structure, repression or activation of gene expression and regulation of cell metabolism (14). Previous studies have demonstrated that HDACs are crucial for the progression of several types of disease, including diabetes, melanoma, hepatocellular carcinoma and GC (15-18). However, the precise role of HDACs in the progression of GC remains unclear. HDACs have been demonstrated to serve roles in regulating the expression of microRNAs. However, little is known regarding whether HDACs may serve certain roles in regulating the expression of IncRNAs during the progression of GC.

The present study attempted to investigate the role of abnormal expression of HDACs in the progression of GC, and to determine the association between HDACs and lncRNAs.
The present study provided evidence of the importance of HDACs in the progression of GC and identified them as promising targets for the clinical treatment of GC.

**Patients and methods**

**Patient population.** Gastric cancer tissues from 63 patients (mean age 61 years, range 45-73 years; 45 males and 18 females) with gastric cancer and normal tissues from 31 healthy individuals (mean age 58 years, range 40-75 years; 20 male and 11 female) were used in the present study, which was obtained from Jiangxi Province People’s Hospital during the period from March 2015 to August 2016. Ethics approval for this study was approved by the Institutional Review Board and the Ethics Committee at Jiangxi Province People’s Hospital (ID no. 2015083). Written informed consent was obtained from each patient. Ten cancer tissues and normal tissues were randomly selected for examining the expression profile of lncRNAs in the GSE64951 database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64951). Additionally, 9 cancer tissues and normal tissues were randomly selected for examining the expression profile of lncRNAs in the GSE19826 database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi). The expression of lncRNAs in the tissues of patients with gastric cancer and healthy donors was analyzed by Affymetrix Human Genome U133 Plus 2.0 Array (platforms: GPL570, [HG-U133_Plus_2]; Affymetrix, Inc., California, USA).

**Sample preparation for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and gene chip analysis.** The differential genes were verified using RT-qPCR. The procedure was as follows: Total RNA was isolated from the cultured SC-M1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer’s protocol. Total RNA was used to synthesize cDNA using the PrimeScript™ RT reagent kit (cat. no. RR037A; Takara Bio, Inc.). The terminal concentration of siRNA was 50 nM according to the manufacturer’s protocol. RT-qPCR primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The 5'-3' primer sequences internal control. RT-qPCR primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The 5'-3' primer sequences for the si-HDAC1s were: siRNA1, 5'-gcaaagauguccgaggc-3'; siRNA2, 5'-ucaaaggacacgccaagug-3'; and siRNA3, 5'-gagaagaagaagucc-3'. For in vitro experiments, siRNAs were transfected into three gastric cancer cell lines using Lipofectamine iMAX (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. At 48 h following transfection, the efficiency of RNA interference was determined by RT-qPCR that the method has been described in the upper.

**Cell apoptosis assay.** Gastric cancer cells were stained using a fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Cells (1x10^6 cells/ml) were cultured in 12-well plates, and following transfection with siRNAs for 24 h, the cells were harvested and washed twice with cold phosphate-buffered saline with gentle agitation. SC-M1 cells were then re-suspended and added to binding buffer (1X), and cell density was adjusted to 2-5x10^5/ml. In the dark, 5 µl Annexin V-FITC was added to the cell suspension volume of 195 µl and incubated for 10 min at room temperature prior to the addition of 190 µl binding buffer and 10 µl PI. A total of 10,000 events per sample were acquired using a FACS-scan flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of apoptotic cells was analyzed by Acri (BD Biosciences). The percentage of cells undergoing apoptosis was analyzed using BD CellQuest™ Pro Software Analysis Tutorial (BD Biosciences; version 5.1).

**Stimulation of gastric cell line.** The human gastric cancer SC-M1 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. In certain experiments, cells (1x10^5 cells/ml) were cultured in 96-well plates for the examination of cell viability using an MTT assay (MTT Cell Proliferation kit; Sangon Biotech Co., Ltd., Shanghai, China). According to the manufacturer's protocol, formazan solubilization solution was used to dissolve the formazan crystals and measure the absorbance at 570 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

**RNA interference.** The siRNA sequence targeting HDAC1s (NCBI; GenBank ID: NM_0049646.2) and the scramble siRNA sequence were designed and synthesized by GenePharma (Shanghai, CHINA). The sequences for the si-HDAC1s were: siRNA1, 5’-gcaaagauguccgaggc-3’; siRNA2, 5’-ucaaaggacacgccaagug-3’; and siRNA3, 5’-gagaagaagaagucc-3’. For in vitro experiments, siRNAs were transfected into three gastric cancer cell lines using Lipofectamine iMAX (Invitrogen; Thermo Fisher Scientific, Inc.), the terminal concentration of siRNA was 50 nM according to the manufacturer’s protocol. At 48 h following transfection, the efficiency of RNA interference was determined by RT-qPCR that the method has been described in the upper.
cells according to the standard protocol. Cells were lysed in cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid and 1% Triton X-100) containing protease and phosphatase inhibitors. The protein determination method was BCA (BCA Protein Assay kit, Sangon Biotech Co., Ltd. Shanghai, China). Briefly, the loading of protein sample was 40 µg each hole, the proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and subsequently transferred to nitrocellulose membranes. The membrane was completely immersed in 5% skimmed milk powder for 60 min at room temperature. The following primary antibodies were used: Total p-21 (cat. no. PL0502562; dilution, 1:1,000; PLLABS, British Columbia, Canada; http://www.pllabs.com/total-p53 (cat. no. ab131442; dilution, 1:2,000; Epitomics; Abcam, Cambridge, MA, USA) and actin (cat. no. PA1-183; dilution, 1:2,000; Thermo Fisher Scientific, Inc.) antibodies. Incubate overnight at 4°C with primary antibodies, respectively. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (cat. no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA) at a dilution of 1:4,000 for 1 h at room temperature. Membranes were developed using the enhanced chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. Limma R package (1.10.1) was utilized to identify the DEGs in GSE64951 and GSE19826 database, with a padj ≤0.05. SPSS version 19.0 for Windows (IBM Corp., Armonk, NY, USA) was used for all analyses. All the data are expressed as the mean ± standard deviation. The correlation between HDAC1 and upregulated lncRNAs (BC016000 and AF116637 and uc004ebu.1) in gastric cancer were assessed using one-way analysis of variance followed by Tukey's
post-hoc test. P < 0.05 was considered to indicate a statistically significant difference.

Results

Abnormal expression of lncRNA is identified in gastric cancer. Accumulating evidence has demonstrated that the abnormal expression of lncRNAs is associated with the progression of certain diseases. In order to detect the expression profile of lncRNAs in gastric cancer tissues, the expression of lncRNAs in the tissues of patients with gastric cancer and healthy donors was analyzed by Affymetrix Human Genome U133 Plus 2.0 Array. Compared with normal controls, the expression profile of lncRNAs in gastric cancer tissues is completely different compared with normal tissues, indicating the abnormal expression profile of lncRNAs in gastric cancer.

The correlation between HDAC1 and upregulated lncRNAs in gastric cancer. HDACs are a family of multi-functional proteins, which are involved in tumor suppression. HDAC1 is a very important member of the HDAC family. Therefore, the present study investigated the association between HDAC1 and the upregulated lncRNAs in gastric cancer. Correlation analysis of HDAC1 and the upregulated lncRNAs, BC016000 and AF16637, was conducted. The Pearson’s correlation coefficient (PCC) between HDAC1 and upregulated lncRNAs were BC016000 and AF16637 was calculated using the expression value. The absolute values of Correlation Coefficient were shown in Fig. 2, for example: The absolute value of Correlation Coefficient between HDAC1 expression value and BC016000 expression value was 0.48426573 in the

Figure 2. Correlation analyses of lncRNAs with HDAC1. (A) The association between abnormally expressed lncRNA and HDAC1. Scatter plots were drawn to demonstrate the associations between BC016000 and AF16637 and HDAC1 in two databases, (B) GSE64951 and (C) GSE19826, respectively; lncRNA, long non-coding RNA. lncRNAs, long non-coding RNAs.
GSE64951 database. Correlation analysis was performed in the GSE64951 and GSE19826 databases. In the two aforementioned databases, lncRNAs BC016000 and AF116637 exhibited a moderate to strong positive correlation with HDAC1 with Correlation Coefficients of 0.545 and 0.421, respectively, in the GSE64951 database (Fig. 2A and B), and in the GSE19826 database, the Correlation Coefficients were 0.549 and 0.484, respectively (Fig. 2A and C).

Silencing of HDAC1 downregulates the expression of BC016000 and AF116637. To further investigate whether BC016000 and AF116637 were correlated with HDAC1, the expression levels of BC016000 and AF116637 were evaluated following HDAC1-silencing with its specific siRNA. Three pairs of siRNAs targeting HDAC1 were designed. It was revealed that all these three siRNA sequences could efficiently inhibit the expression of HDAC1 (Fig. 3A). HDAC1 siRNA transfection with these three siRNAs significantly inhibited the expression of BC016000 and AF116637 (Fig. 3B). Additionally, three pairs of siRNAs targeting lncRNA, BC016000 and AF116637 were designed. Next, the expression of BC016000 and AF116637 was efficiently decreased by their respective siRNA sequences (Fig. 4). Subsequently, it was demonstrated that BC016000 and AF116637 lncRNA siRNA transfection with their respective siRNAs significantly inhibited the expression of HDAC1 (Fig. 3B). Furthermore, the expression of BC016000, AF116637 and HDAC1 was analyzed in gastric cancer tissues. It was revealed that these two lncRNAs and HDAC1 have an increased expression trend in gastric cancer tissue (Fig. 5). Taken together, these results indicated that HDAC1 could promote the expression of BC016000 and AF116637 in gastric cancer.

**HDAC1 promotes the proliferation of gastric cancer cells.** To further examine the role of HDACs in the progression of gastric cancer, HDAC1 was silenced by transfecting gastric cancer cells with specific siRNAs. It was revealed that silencing of HDAC1 decreased cell viability, indicating that HDAC1 can inhibit the proliferation of gastric cancer cells (Fig. 6A). Furthermore, the present study also examined the effect of HDAC1 on apoptosis by flow cytometry. It was demonstrated that silencing of HDAC1 increased the percentage of apoptotic cells, indicating that HDAC1 can decrease the apoptosis of gastric cancer cells (Fig. 6B). Following transfection of the gastric cancer cells with specific siRNAs, the present study also examined the migration of gastric cancer cells. The results demonstrated that silencing of HDAC1 with specific siRNAs has no effect on the migration of gastric cancer cells (Fig. 6C). Furthermore, the role of HDAC1 in the activation of tissue-associated genes, p53 and p21, was examined. It was demonstrated that transfection with HDAC1 siRNAs significantly increased the expression of total-p53 (Fig. 6D). Taken together, the results of the present study demonstrated that HDACs could promote the proliferation of gastric cancer.
The present study demonstrated that HDAC1 could promote the proliferation of GC cells, possibly by upregulating the expression of lncRNAs, BC01600 and AF116637. High expression of HDAC1 and abnormal expression of lncRNA was observed in the tissues of patients with GC. It was demonstrated that the expression of BC01600 and AF116637, two typically upregulated lncRNAs, is correlated with the expression of HDAC1, as evidenced by the decreased expression of these two lncRNAs in HDAC1-knockout GC cell lines, and by reduced expression of HDAC1 in lncRNA, BC01600 and AF116637, knockout...
GC cell lines, respectively. Silencing of HDACs increased the p21-dependent apoptosis of the GC cell lines.

Investigating novel treatments for GC, one of the most common types of cancer, is necessary. Limitations of current treatment options include the following: The patients in the later phase of GC experienced a lot of painful and a high mortality rate, even after surgery (20). It is useful to identify efficient biomarkers for the diagnosis of GC in the early phase of GC. Epigenetics has become a popular field of molecular biology recently and accumulating evidence has demonstrated that epigenetic mechanisms contribute toward differentiation, aging and disease development, particularly during the progression of cancer (13,21,22).

lncRNAs serve important roles in the progression of cancer and numerous hallmarks of cancer are controlled by lncRNAs (8-10,23-25). Various lncRNAs have been implicated in the regulation of individual genes via epigenetic regulation (26). Previous studies have demonstrated that overexpression of lncRNAs is implicated in the metastasis of human tumors (27,28). An increasing volume of evidence

Figure 6. HDAC1 was involved in the proliferation of gastric cancer cells. (A) The viability of gastric cancer cells at different time points following HDAC1 siRNA treatment. "P<0.001 viability of GC cells siRNA (-1/2/3) group vs. siRNA-NC at different time points. (B) The apoptosis of gastric cancer cells was examined by flow cytometric analysis. "P<0.001 apoptosis of GC cells siRNA (-1/2/3) group vs. siRNA-NC at different time points. (C) The migrated gastric cancer cells were counted. (D) The repression of P53 and P21 in gastric cancer was examined by western blot analysis. siRNA, small interfering RNA.
has indicated that specific IncRNA mutations are associated with the progression of cancer (8). Therefore, IncRNAs could be a promising candidate for the diagnosis and therapy of cancer (28,29). The present study demonstrated that the expression profile of IncRNAs is completely different in gastric cancer, compared with normal controls by using the bioinformatics methods according to the GSE64951 and GSE19826 databases. The following three IncRNAs were selected: BC016600, AF116637 and uc004edu, which were overlapped in these two databases, as determined by constructing a Venn diagram using the upregulated IncRNAs in these two databases. Therefore, the abnormal expression profile of IncRNAs may be involved in or be a factor in promoting the progression of GC. However, the way in which the expression of these IncRNAs is regulated remains unknown.

HDACs are multi-functional proteins, which serve critical roles in transcription regulation (30). Aberrant expression of HDACs is frequently observed in various types of human cancer. In the majority of cases, upregulation of HDACs is associated with advanced disease and poor outcomes in patients (15). HDACs have also been revealed to be broadly dysregulated in multiple myeloma (31). HDACs are also involved in the progression of several types of cancer, including melanoma and hepatocellular carcinoma (15,18). Participating in a range of cellular and molecular pathways, HDACs can either repress tumor suppressor gene expression or modify key molecules in oncogenic cell-signaling pathways. However, the precise role of HDACs has not yet been revealed. A previous study demonstrated that HDACs could upregulate the expression of microRNAs, indicating that HDACs may serve certain roles in regulating the expression of IncRNAs (32). HDAC1 is an important member of the HDACs family. In order to determine the role of HDAC1 in the expression of IncRNAs, correlation analysis of HDAC1 and the upregulated IncRNAs, BC016600 and AF116637, was conducted, and BC016600 and AF116637 IncRNAs exhibited a positive and strong correlation with HDAC1, indicating that HDAC1 serves roles in the expression of IncRNAs. The results of the present study demonstrated that HDACs may be involved in the progression of GC by upregulating the expression of IncRNAs.

Cancer cells are abnormally proliferated and differentiated cells. Proliferation, apoptosis and migration are important for the progression of cancer. The role of HDAC1 in the proliferation, apoptosis and migration of GC cancer cells was also examined. It was demonstrated that silencing of HDAC1 may decrease the proliferation and increase the apoptosis of gastric cancer cells. However, HDAC1 has no effect on the migration of gastric cancer cells. Therefore, HDAC1 is specifically involved in the proliferation of gastric cancer cells.

In summary, HDAC1 is critical for the progression of GC, by promoting the proliferation of cancer cells, which may be regulated by the HDAC1-mediated decreased or increased expression of IncRNAs. The present study demonstrated the correlation of HDACs and IncRNAs, and provided a possible mechanism by which HDACs regulate the progression of gastric cancer. The results of the present study provided a possible target for the clinical treatment of gastric cancer.

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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
JC designed the research. ZhiY and JZ performed the research. TW and ZIY were responsible for clinical diagnosis and sample collection. HL contributed to data collection and statistical analysis. ZhiY, JZ and JC wrote the manuscript. All the authors read and approved the final manuscript.

Ethics statement and consent to participate
Human samples used in the present study were obtained from the patients with written informed consent. The present study was approved by the Ethics Committee of Jiangxi Province People’s Hospital and was conducted according to The Declaration of Helsinki.

Patient consent for publication
Consent for publication was obtained from all patients.

Competing interests
The authors declare that they have no competing interests.

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