Analysis of Long Non-Coding RNA Expression Profile and Functional Study of LOC389332 in Early Gastric Cancer

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Background: Long non-coding RNAs (LncRNAs) could potentially function as diagnostic markers for gastric carcinoma. Nevertheless, the expression profile and biological feature of LncRNAs in early gastric cancer (EGC) remains to be explored.

Material/Methods: LncRNA expression microarray analysis was performed on 6 paired EGC tissues. One deregulated LncRNA, LOC389332, was validated using a quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assay using independent tissue samples and cell lines. The Cell Counting Kit-8 (CCK-8) assay and wound healing assay were conducted to evaluate its influences on the proliferation and migration of gastric cancer cells. LncRNA expression microarray and gene ontology (GO) analysis were also performed on the LOC389332 knockdown cell line model to explore the molecular feature of LOC389332 in gastric carcinoma.

Results: The LncRNA expression profiling showed that 72 LncRNAs were significantly differentially expressed in EGC tissues. The results in the validation phase revealed that LOC389332 was remarkably overexpressed in gastric carcinoma tissues, precancerous lesions, and gastric cancer cells. Functional study showed that knockdown of LOC389332 expression could inhibit cell proliferation and migration. LncRNA expression microarray on the LOC389332 knockdown cell line model revealed that 393 mRNAs were differentially expressed. The GO enrichment analysis indicated that the downregulated genes were mainly associated with cell membrane function, signal transmission process, and cell adhesion process.

Conclusions: The LncRNA expression profile between EGC and gastritis tissues was significantly different. LOC389332 was potential non-coding oncogenes in gastric cancer, and it may perform its function through altering cell membrane function, signal transmission, and cell adhesion.

MeSH Keywords: Gene Expression Profiling • RNA Interference • RNA, Long Noncoding • Stomach Neoplasms

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Background

Gastric carcinoma is a common malignant disease in the digestive system, and the mortality rate is extremely high. In China, gastric cancer causes approximately 300,000 deaths each year [1]. Early gastric cancer (EGC) is defined as a tumor which is limited to the mucosa and submucosa layer of stomach, regardless of lymph node metastasis. The prognosis for patients with metastatic gastric carcinoma is extremely poor and few therapeutic methods are available. However, the 5-year survival rate could be greatly improved for patients who are diagnosed at an early stage and treated properly [2–5]. As a result, it is of great significance to explore neoteric diagnostic biomarkers for EGC or even precancerous lesions.

Long non-coding RNA (LncRNA) is an important subtype of non-coding RNA with a length greater than 200 nucleotides. Accumulating evidence shows that deregulation of LncRNAs may play vital roles in the pathogenesis and progression of various carcinomas, including gastric cancer [6]. It has been demonstrated that LncRNAs could potentially function as diagnostic biomarkers for gastric cancer [7]. However, the expression profile and biological features of LncRNAs in EGC and precancerous lesions require further investigation.

In the present research, we analyzed the expression profile of LncRNAs in EGC patients to explore key LncRNAs that may participate in the generation of EGC and confirm their expression pattern in gastric cancer tissues and cell lines. Furthermore, we conducted a functional study to investigate the biological feature of the differentially expressed LncRNAs.

Material and Methods

Specimens and relative clinical data

All subjects we recruited were patients who underwent endoscopic therapy in Peking Union Medical College Hospital and Qinghai Provincial People’s Hospital. The lesion tissue together with adjacent chronic non-atrophic gastritis tissue samples of 6 patients diagnosed with EGC were selected for LncRNA expression profile analysis. Other independent tissue samples, including 28 low-grade intraepithelial neoplasia (LGIN), 30 high-grade intraepithelial neoplasia (HGIN), 18 EGC, 19 advanced gastric cancer (AGC), and 30 gastritis without H. pylori infection, were used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR). All patients underwent magnifying endoscopy from March 2010 to March 2015. All specimens were immersed in RNAlater Solution for 24 h at 4°C and then were stored at –80°C until RNA extraction.

The pathological diagnoses of all specimens were based on WHO classification and diagnosis criteria of gastroenterological malignancies and were checked by 2 independent pathologists [8]. The invasion depth of EGC tissues was confirmed by subsequent surgery or endoscopic submucosal dissection (ESD).

The Ethics Committee of Peking Union Medical College Hospital audited and approved the present study. Consent papers are available for all included subjects.

Cell lines and culture conditions

The cell lines used in this study, including human gastric epithelial mucosa cell line (GES-1) and 2 gastric cancer cell lines (HGC-27, AGS), were acquired from the Cell Center of the Institution of Basic Medical Science, Chinese Academy of Medical Science. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in an atmosphere at 37°C with 5% CO₂.

RNA preparation

The RNeasy Mini Kit (Qiagen, MD, United States) was used for tissue total RNA extraction, while TRIzol reagent (Invitrogen, Carlsbad, CA) was used for RNA extraction of cell lines. The ND-1000 UV-VIS spectrophotometry (NanoDrop Technologies, DE, United States) was applied for RNA concentration measurement.

LncRNA expression microarray analysis of tissues

The expression microarray analysis was conducted according to one-color microarray-based gene expression analysis protocol, which introduced the detailed procedures of sample labeling, hybridization, rinsing, and scanning. GeneSpring software GX 12.6 (Agilent Technologies) was used for data processing.

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses

The expression of differentially expressed LncRNA LOC389332 in tissues and cell lines were further analyzed on the MX 3005P QPCR System. The probes ID number of LOC389332 and POLR2A were Hs01013790_s1 and Hs00172187_m1, respectively. Expression levels of LOC389332, represented by –ΔCt value, were quantified and normalized relative to those of POLR2A.

SiRNA transfection

To further explore the function of LOC389332, the expressing level of LOC389332 in malignant gastric cells was modified by gene knockdown using siRNA. The siRNA transfection of siRNA-LOC389332 and siRNA-NC (negative control) was performed
using Lipofectamine RNAiMAX transfection agent (Invitrogen, United States) according to the protocol. Cells were harvested after 48h for expression analyses by qRT-PCR.

**Cell proliferation assays**

The cell proliferation assays were conducted using Cell Counting Kit-8 (Dojingdo, Japanese) based on the manufacturer’s protocol. We seeded the cells into 96-well plates and then cultured them for 0, 24, 48, and 72 h after siRNA transfection. Afterwards, we added CCK-8 into each well and incubated the plates for 2 h. The absorbance of each well at 450 nm was read on a Bio Rad 680 reader (Bio Rad, United States). The experiment was conducted at least 3 times independently.

**Wound healing assay**

We first seeded cells into 6-well plates at a density of 4×10^5 per well. After siRNA transfection, when the cells grew to 100% confluency, we made a wound in the well center with a sterile pipette tip by removing the adherent cells. The cells were then cultured for 24 h. Thereafter, cells migrating into the wound were observed and imaged by a microscope.

**LncRNA expression microarray analysis of cells**

LncRNA expression microarray analysis using Agilent 8×60K Whole Human Genome microarray was performed on the LOC389332 knockdown cells and negative control cells to investigate their gene expression profile. The procedures were as described above. We then conducted a gene ontology (GO) enrichment analysis to identify gene clusters which were functionally relevant.

**Statistical analysis**

The SPSS software package (version 22.0) was applied for statistical analysis. Numerical significance was measured by a t test, ANOVA, or a chi-squared test, as appropriate. Differences were deemed statistically significant with P values less than 0.05. The GO enrichment analysis was performed on the DAVID Bioinformatics Resources 6.7 open platform (https://david.ncifcrf.gov). The unsupervised cluster analysis was conducted on open platform R (http://www.r-project.org).

**Results**

**LncRNAs were differentially expressed between EGC and gastritis**

In the screening phase, the LncRNA expression microarray data of 6 paired EGC tissues were analyzed. Differentially expressed genes were picked out on the basis of the threshold of absolute fold changes>2 and P value <0.05. A total of 72 LncRNAs were differentially expressed between EGC and gastritis tissues, 34 of which were upregulated and 38 of which downregulated. The top-20 deregulated LncRNAs are listed in Table 1. Furthermore, the 72 differentially expressed LncRNAs easily distinguished EGC from gastritis tissues, which are shown in the heat map in Figure 1, indicating that the expression pattern of these LncRNAs were significantly different between EGC tissues and their paired gastritis tissues.

**LOC389332 was overexpressed in gastric cancer and precancerous lesions**

Among the 72 differentially expressed LncRNAs, we selected LOC389332 for further validation and investigation. To validate the results of LncRNA expression microarray and quantify the expression level of LOC389332, qRT-PCR was performed in independent tissue samples, which contained 19 AGC, 18 EGC, 30 HGIN, 28 LGIN, and 30 gastritis tissues. Results showed that expression of LOC389332 was significantly elevated in LGIN, HGIN, EGC and AGC tissues compared with the gastritis tissues.

| LncRNA      | Gene ID      | Fold change |
|-------------|--------------|-------------|
| HOXA11-AS   | 221883       | 34.36       |
| FLJ22763    | 401081       | 14.95       |
| LOC389332   | 389332       | 7.42        |
| LINC00659   | 100652730    | 6.62        |
| LOC100507639| 100507639    | 5.71        |
| HOTAIR      | 100124700    | 4.93        |
| CASC9       | 101805492    | 4.87        |
| SH3PXD2A-AS1| 100505839    | 4.69        |
| LOC100131626| 100131626    | 3.60        |
| C21orf90    | 114043       | 3.49        |
| LOC100507464| 100507464    | 0.07        |
| LINCO0871   | 100506412    | 0.10        |
| LOC283731   | 283731       | 0.13        |
| SNHG17      | 388796       | 0.13        |
| LINCO0982   | 440556       | 0.14        |
| GATA6-AS1   | 100128893    | 0.17        |
| LOC400043   | 400043       | 0.20        |
| LOC100507520| 100507520    | 0.27        |
| LOC101927372| 101927372    | 0.28        |
| LOC100240734| 100240734    | 0.30        |

Table 1. Top 20 deregulated LncRNAs in EGC tissues.
tissues (P values less than 0.05), which was consistent with the microarray results (Figure 2A). However, LOC389332 was not significantly differentially expressed among LGIN, HGIN, EGC, and AGC tissues (P values varied from 0.147 to 0.999).

**LOC389332 was overexpressed in gastric cancer cell lines**

To further explore the influence of LOC389332 on cancer progression, we then measured the expressing level of LOC389332 in gastric cancer cells and normal gastric epithelium cells.
We confirmed that the LOC389332 expression level was remarkably elevated in gastric cancer cells comparing with that of normal gastric epithelium cells (Figure 2B).

**Knockdown of LOC389332 inhibited gastric cancer cell proliferation**

To examine the biological effect of LOC389332 on cell growth, the LOC389332 knockdown cell line model was constructed by siRNA transfection. Knockdown of LOC389332 expression in HGC-27 cells by transfecting siRNA-LOC389332 was confirmed by qRT-PCR. Cell Counting Kit-8 (CCK-8) assays showed that after 24 h, the cell growth rate of HGC-27 transfected with siRNA-LOC389332 was significantly decreased compared with those transfected with siRNA-NC, indicating that inhibition of LOC389332 expression could suppress the proliferation of HGC-27 (Figure 3). These findings indicate that LOC389332 exerted significant effects on the regulation of cell proliferation.

**Knockdown of LOC389332 suppressed migration of gastric cancer cells**

The migration of HGC-27 cells after transfection with siRNA-LOC389332 was then investigated. We determined the influence of LOC389332 on HGC-27 cell migration using wound healing assay. Results revealed that LOC389332 silencing cells showed a significantly slower scratch closure rate than negative control cells, which suggests knockdown of LOC389332 could inhibit the migration of HGC-27 cells (Figure 4).

**LOC389332 knockdown cells showed different gene expression profile compared with negative control cells**

To further investigate the influences LncRNA LOC389332 exerted on the gene expression profile of gastric cancer cells, we conducted LncRNA expression microarrays on LOC389332 knockdown HGC-27 cells and negative control cells. Differentially expressed genes were picked out on the basis of the threshold of absolute fold change>3 and P value <0.05. Results showed
that, compared with negative controls, there were 393 differentially expressed mRNAs, 210 of which were upregulated and 183 of which downregulated. The top 20 abnormally expressed mRNAs are listed in Table 2.

Then, we conducted GO enrichment analysis on 393 differentially expressed mRNAs to verify the gene clusters that were functionally relevant. No enriched GO terms were identified among the 210 upregulated mRNAs. The enriched GO terms in 183 downregulated mRNAs were associated with cell membrane function process, signal transmission process, and cell adhesion process (Table 3).

**Discussion**

LncRNAs, initially argued to be “junk RNAs” of the transcriptome, have been shown to play major biological roles in a variety of human diseases [9–12]. Recently, the functional significance of LncRNAs in tumors has been attracting more and more attention [13]. In the past few decades, a variety of novel LncRNAs have been identified with gene expression microarray analysis and demonstrated to be involved in generation and development of cancers [14,15]. Deregulation of LncRNAs may provide cellular growth advantages epigenetically, leading to uncontrolled and progressive tumor growth [16–19]. Thus, elucidating the biological roles of LncRNAs may help reveal the underlying events in different carcinomas.

In terms of gastric cancer, research has focused mainly on the abnormal expression of LncRNAs in advanced tumors. The expression profile and biological feature of LncRNAs in early gastric cancer and even precancerous lesions is less well characterized. As a result, in this study, we performed LncRNA gene expression profiling on paired EGC tissues to elucidate the key LncRNAs in the early process of gastric carcinogenesis and further explored their biological functions. A total of 72 deregulated LncRNAs were identified with a threshold of P value <0.05 and...
Hierarchical analysis revealed that the 72 differentially expressed LncRNAs easily distinguished EGC from non-malignant gastric tissues, showing that the expression pattern of LncRNAs in EGC were significantly different. Research has shown that the 5-year survival rate of patients with EGC was remarkably higher than that of those with advanced cancer [20–22]. Therefore, the LncRNAs differentially expressed in EGC mentioned above may function as potential biomarkers for EGC and further improve the prognosis of gastric cancer, but this needs further investigation and clinical verification.

Among the differentially expressed LncRNAs, we selected LOC389332 for further validation and investigation. LOC389332, located in chromosome 5, is an intronic LncRNA with a length of 723 nucleotides [23]. A previous study demonstrated that LOC389332 was downregulated in clear cell renal cell carcinoma and was related to progressive disease with poor prognosis [24]. However, no research has been performed to elucidate its function in gastric cancer. The present research validated by qRT-PCR that LOC389332 was remarkably overexpressed in the gastric cancer tissue samples, precancerous lesions, and gastric cancer cells compared with normal gastric tissues and epithelium mucosa cells. To further explore the biological function of LOC389332 in the generation and progression of gastric cancer, we then performed CCK-8 and wound healing assays to evaluate its role in cell proliferation and migration. The results showed that knockdown of LOC389332 remarkably repressed the growth and migration rate of HGC-27 cells, indicating that aberrant upregulation of LOC389332 could promote the generation and invasion of gastric carcinoma. All in all, these results suggest that LOC389332 could function as a tumor oncogene through monitoring growth and migration of cells in gastric cancer. Nevertheless, the specific mechanisms by which LOC389332 promotes the growth and migration of gastric cancer cells remain unclear and need to be explored in our future studies.

To further illustrate the molecular feature of LOC389332 in gastric cancer cells, we performed LncRNA expression microarray on the LOC389332 knockdown cell line model. Results showed that a total of 393 mRNAs were differentially expressed in LOC389332 knockdown gastric cancer cells compared with negative control cells. From the microarray, we found that 210 mRNAs were upregulated and 183 were downregulated in the LOC389332 knockdown gastric cancer cells compared with the negative control cells. These differentially expressed mRNAs were further analyzed using the GO enrichment analysis tool (http://jasonlab.org/goral). The results showed that a total of 51 functional categories were significantly enriched (FDR < 0.05), among which the top 5 enriched GO terms were associated with the biological process of cell adhesion, membrane, signal, receptor, and transmembrane region. The details are shown in Table 2.

### Table 2. Top 20 abnormally expressed mRNAs in LOC389332 knockdown gastric cells.

| mRNA   | Gene ID | Fold change |
|--------|---------|-------------|
| C1orf137 | 388667 | 25.14       |
| GABRG1  | 2565   | 11.00       |
| R51     | 6247   | 8.44        |
| FGF8P1  | 9982   | 7.39        |
| HNF4A   | 3172   | 6.37        |
| GFIB    | 8328   | 6.31        |
| CDYL2   | 124359 | 6.25        |
| WNT4    | 54361  | 5.78        |
| SGLCE8  | 27181  | 5.71        |
| CACNA2D3| 55799  | 5.64        |
| MYO16   | 23026  | 0.02        |
| ANKRD53 | 79998  | 0.03        |
| SFTPA2  | 729238 | 0.04        |
| POU2A1  | 5450   | 0.05        |
| TEK5    | 146279 | 0.05        |
| C14orf119| 55017  | 0.05        |
| SPAG11B | 10407  | 0.05        |
| KIR3D3  | 115653 | 0.05        |
| TMEM39B | 161291 | 0.07        |
| TSPY10  | 100289087 | 0.08 |

### Enrichment GO term | Genes involved | P value |
|----------------------|----------------|---------|
| Glycoprotein         | 72             | 1.3E-8  |
| Transmembrane        | 73             | 2.0E-6  |
| Receptor             | 34             | 2.9E-6  |
| Transmembrane region | 71             | 7.0E-6  |
| Cell membrane        | 40             | 1.4E-5  |
| Plasma membrane      | 61             | 2.4E-5  |
| Integral to membrane | 77             | 3.0E-5  |
| Intrinsic to membrane| 78             | 5.9E-5  |
| Membrane             | 79             | 1.8E-4  |
| Signal               | 52             | 1.6E-5  |
| Signal peptide       | 52             | 1.9E-5  |
| Disulfide bond        | 47             | 2.1E-5  |
| Cell adhesion         | 14             | 6.0E-3  |
| Biological adhesion   | 14             | 6.1E-3  |

**GO – Gene Ontology.**

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that LOC389332 may regulate gastric cancer cell generation and invasion through its effects on these deregulated mRNAs. However, it is possible that the target mRNAs of LncRNAs varies in different tissues and cell lines because the specific target mRNAs modulated by LOC389332 in gastric cancer remain unclear. Furthermore, a GO enrichment analysis of these 393 differentially expressed mRNAs was performed to distinguish the functionally relevant gene clusters. We found no enriched GO terms among the 210 upregulated mRNAs. Nevertheless, in the 183 downregulated mRNAs, the enriched GO terms were associated with cell membrane function process, signal transmission process, and cell adhesion process, meaning that LOC389332 may exert its function by regulating these processes. Cancer cells are characterized by infinite growth and metastasis, which means that the cell recognition, cell contact inhibition, and cell adhesion of malignancies are impaired. It has also been reported that with aberrant cell signal transmission, deregulated expression of signal transduction molecule and cause disturbance of apoptosis, proliferation, and invasion. Therefore, it could be speculated that dysfunction of these processes mentioned above are essential in the initiation and metastasis of various carcinomas [25]. Subsequent study on these processes is needed to explore the specific mechanisms of its function. With further understanding of these processes, we may be able to develop new therapeutic targets for early gastric carcinoma, which would be of great benefit. These results suggest that LOC389332 exerts significant influences on the initiation and invasion of gastric carcinoma by interfering with these cell function process.

Conclusions

In conclusion, we demonstrated that the LncRNAs expression profile between EGC and gastritis tissues was significantly different and the ectopically expressed LncRNAs easily distinguished between EGC and gastritis tissues. Results of qRT-PCR validated that LOC389332 was overexpressed in gastric cancer, precancerous lesions, and gastric cancer cells. In addition, to the best of our knowledge, the present research is the first to identify that the neoteic LncRNA LOC389332 was overexpressed in gastric cancer tissues and cell lines. Functional study revealed that knockdown of LOC389332 could repress cell proliferation and migration in vitro, indicating its oncogenic role in gastric carcinoma. Furthermore, LncRNA expression microarray and GO enrichment assay on the LOC389332 knockdown cell model showed that LOC389332 may perform their function through altering cell membrane function, signal transmission, and cell adhesion. However, the detailed functional mechanisms of LncRNAs need further exploration.

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