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ORIGINAL ARTICLE

Overexpression of the GClnc1 as a Diagnostic Biomarker in Gastric Cancer Patients and its Link with *H. Pylori* Infection

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SUMMARY

Background: **Gastric cancer (GC) is a complicated multifactorial neoplasm with a high fatality and prevalence rate around the globe that required the discovery of many unknown mechanisms involved in its inception and progression. The aim of this project was to investigate the alterations of GClnc1 expression in cancerous tissues relative to marginal non-cancerous tissues of patients with GC and its association with clinicopathological features.**

Methods: **In this research, the expression level of GClnc1 was assessed using the qRT-PCR. For this, 80 pairs of cancerous and marginal non-cancerous GC samples tissues were gathered. Then RNA isolation and cDNA synthesis were carried out. Eventually, the difference of GClnc1 expression levels in tumor tissue relative to marginal non-tumor tissue specimens of GC patients and its association with pathological characteristics, as well as biomarker's performance of GClnc1, was investigated.**

Results: **Expression data examination of GClnc1 indicates increased expression in GC tumor tissues relative to marginal non-cancerous tissues ($p < 0.0001$). GClnc1 overexpression was significantly linked with pathological features of patients with lymph node metastasis ($p = 0.037$) and *H. pylori* infection ($p = 0.029$). Based on ROC analysis, the GClnc1 as a biomarker has AUC, sensitivity%, and specificity% of 0.8228, 90%, and 61.67%, respectively ($p < 0.0001$).**

Conclusions: **Due to the GClnc1 increased expression in tumor tissues of GC patients, our research proposed that GClnc1 may be involved in the promotion and development of GC cells as a novel oncogene. Besides, in the molecular targeted therapies of GC patients, GClnc1 can be considered as a potential biomarker.**

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INTRODUCTION

Gastric cancer (GC) is one of the five most common cancers in the world with approximately 1 million newly identified cases in 2018. According to the classifications of GC pathology called Lauren, GC consists of two groups: diffuse and intestinal. Besides, the mentioned groups of GC have diverse clinical, morphological, and genetic characteristics [1-3]. A variety of risk fac-

tors, including the host genotype, environmental factors, and *H. pylori* infection can involve in the initiation and promotion of GC cells. *H. pylori* plays a critical role in the development procedure of GC by colonizing gastric epithelial cells and causing acute and chronic gastritis, ulceration, and inflammation [4-6]. Therefore, identifying the molecules and pathways involved in GC cell progression can be the most effective strategy to improve the prevention, recognition, and treatment methods of GC.

lncRNAs are RNA transcripts that are unable to encode proteins but participate in a wide range of biological activities as regulatory molecules. Many pivotal cellular functions of lncRNAs are regulated by their interaction with DNA, RNA, and proteins. Besides, they act as guide, signal, decoy, and scaffold molecules at chromatin levels to regulate gene expression. Also, disorder in the regulation of lncRNAs expression is associated with the pathology of several cancers [8-12]. Currently, many lncRNAs have been discovered to participate in the promotion of malignancies, including breast, gastric, and colorectal cancer, by serving as oncogene or tumor suppressor genes [13-16]. The GClnc1 (gastric cancer-associated lncRNA1) functions as a scaffold molecule through binding to WDR5 (an important element of the histone methyltransferase complex) and KAT2A histone acetyltransferase and coordinates their localization and pattern of histone modification on the target SOD2 gene. SOD2 is a gene in the superoxide dismutase family that encodes a mitochondrial protein and converts oxidative phosphorylation byproducts to hydrogen peroxide and oxygen. Mutations in this gene have been linked to various diseases, such as cancers [17,18]. Therefore, we designed this research to evaluate the GClnc1 expression level changes in cancerous tissues of GC patients as compared with marginal non-cancerous tissues and the relationship between these changes and patients' pathological features as well as biomarker performance of the GClnc1 gene.

MATERIALS AND METHODS

Clinical specimen collection

In this present work, 80 specimens pairs of tumor tissues and marginal non-tumor tissues of patients with GC were gathered who were undergoing endoscopy at Noor Nejat Hospital (Tabriz, Iran). All GC and marginal non-cancerous tissues were immediately put in RNase-free microtubes and frozen in liquid nitrogen to preserve the samples until RNA isolation. Then they were stored at -80℃. The pathologist confirmed the pathology report for each GC tissue sample. The content of the consent form was explained to all patients and the patients read and signed it. This research has been approved by the Ethics Committee of Tabriz University, Tabriz, Iran (approval number of IR. TABRIZU. REC. 1398.015).

Total RNA preparation and cDNA synthesis

In the procedure of total RNA preparation from tumor tissue and marginal non-tumor tissue of GC specimens, TRIZOL solution was used according to the manufacturer's instructions (Invitrogen, Waltham, MA, USA). Quantitative analysis of RNA extracted by Nanodrop spectrophotometer at 260/280 OD (Thermo Fisher Scientific, Waltham, MA, USA) and also a qualitative examination by 2% agarose gel electrophoresis was performed. After the extraction step, all RNA isolated samples were stored at -80℃. In the next step, the concentration of isolated RNA specimens was determined. Thereupon to eliminate possible DNA contamination DNaseI treatment was performed. All cDNA synthesis steps were carried out based on the kit protocol instructions (TaKaRa, Kusatsu, Japan), each reaction including extracted RNA, 3.5 μL of master mix, and 1.5 μL of DEPC-treated water. Then the obtained mixture for each specimen was incubated at 37℃ for 60 minutes and 85℃ for 5 seconds.

Primer design

The specific primer sequences utilized in this project for β-actin and GClnc1 genes were designed by Gene Runner software (version 6.5.52). Then, the specificity control of the sequences was evaluated in BLAST at the NCBI. The primers' sequences (Sinaclon, Tehran, Iran) are as follows:

GClnc1 primers forward:
5'-CCAGGGGAAGTACTGTTTGGGA-3'
and reverse:
5'-TATGTACCAGGCTTGATGCAG-3'
also β-actin primers forward:
5'-AGAGCTACGAGCTGCCTGAC-3'
and reverse:
5'-AGCACTGTGTTGGCGTACAG-3'.

Quantitative RT-polymerase chain reaction

Evaluation of GClnc1 expression levels in tumor tissue samples relative to marginal non-tumor tissues was performed through the qRT-PCR technique and using SYBR Green Master Mix (Amplicon, Odense, Denmark) with a Light Cycler® 96 Real-Time PCR system (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The β-actin gene was selected as the internal control for the expression data normalization and also each sample reaction was performed in duplicate. According to the kit instructions, each reaction in a total volume of 14 μL included: 7 μL of SYBR Green Master Mix (2x), 0.6 μL of specific primers for β-actin and GClnc1 (10 μM), 1 μL of cDNA (100 ng/μL), and 5.4 μL ddH2O. The process of thermal cycles included the following: step 1: 95℃ for 10 minutes, step 2: 40 cycles comprising 95℃ for 30 seconds and 60℃ for 30 seconds, and 72℃ for 30 seconds, and step 3: 72℃ for 5 minutes.

Table 1. Relationship between increased levels of GClnc1 expression and pathological characteristics in patients with gastric cancer.

Variable	Number of patients	Expression mean in tumoral tissue	Expression mean in marginal tissue	p-value	Non-significant differences
Age (years)				**0.875**	**non significant**
> 50	**41**	**3.8×10^{-3}**	**1.1×10^{-3}**		
< 50	**39**	**3.5×10^{-3}**	**1.2×10^{-3}**		
Gender				**0.478**	**non significant**
Male	**47**	**3.6×10^{-3}**	**1.2×10^{-3}**		
Female	**33**	**3.8×10^{-3}**	**1.1×10^{-3}**		
Size of the tumor (cm)				**0.250**	**non significant**
< 5 cm	**50**	**3.7×10^{-3}**	**1.1×10^{-3}**		
> 5 cm	**30**	**3.6×10^{-3}**	**1.3×10^{-3}**		
TNM stage				**0.324**	**non significant**
I	**14**	**4.5×10^{-3}**	**1.2×10^{-3}**		
II	**40**	**3.8×10^{-3}**	**1.1×10^{-3}**		
III	**17**	**3.1×10^{-3}**	**1.2×10^{-3}**		
IV	**9**	**2.9×10^{-3}**	**1.5×10^{-3}**		
Lymph node metastasis				**0.037**	
Absence	**28**	**3.5×10^{-3}**	**1.6×10^{-3}**		
Present	**52**	**3.8×10^{-3}**	**0.9×10^{-3}**		
H. pylori				**0.029**	
Positive	**46**	**3.7×10^{-3}**	**1.4×10^{-3}**		
Negative	**34**	**3.7×10^{-3}**	**1.0×10^{-3}**		

*** The underlined line numbers in the table were significant.**

Table 2. The ROC curve statistical analysis of the performance of GClnc1 as a biomarker in GC patients.

ROC curve data	Values
AUC	**0.8228**
Sensitivity	**90%**
Specificity	**61.67%**
Cutoff value	**> 0.001396**
Standard error	**0.03783**
95% CI	**0.7486 to 0.8969**
p-value	**< 0.0001**
The number of marginal non-tumor tissues samples	**80**
The number of cancerous tissues samples	**80**

Statistical analysis

The relative comparison of GClnc1 expression levels in GC cancerous tissues with marginal non-tumor tissues was calculated using the $2^{-\Delta Ct}$ obtained from qRT-PCR. Gene expression data of GClnc1 and its linked pathological characteristics were examined through the Mann-Whitney test. The one-way analysis (ANOVA) test is used to compare the means between two groups. Evaluation of the GClnc1 biomarker's performance and determination of the sensitivity%, specificity%, and cut-off value were carried out through the receiver operating characteristic (ROC) curve. Statistical analyses were

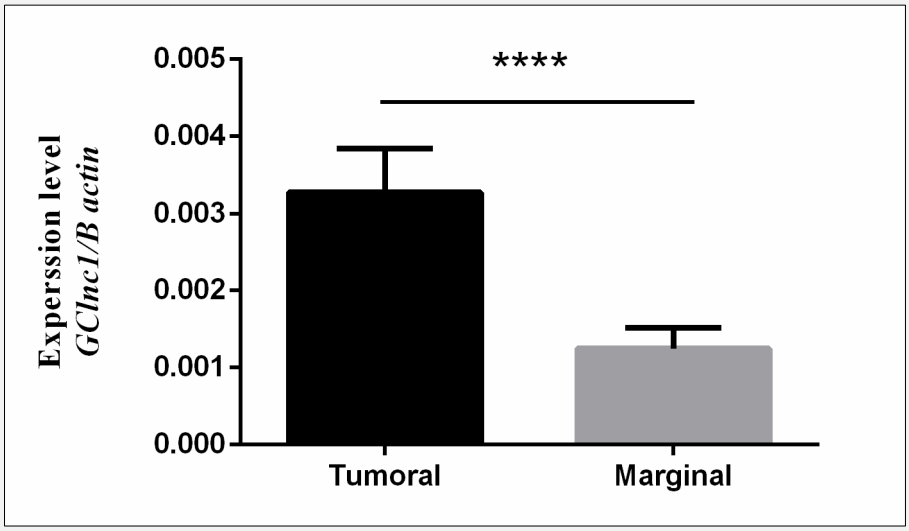

Figure 1. Increased GClnc1 expression in tumor tissues relative to marginal non-tumor tissues in patients with gastric cancer.

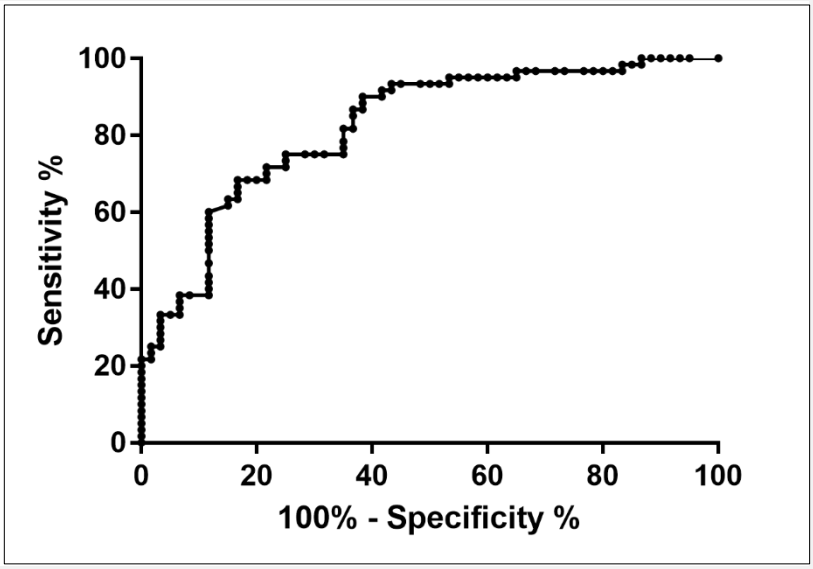

Figure 2. The ROC test analysis (AUC, sensitivity%, specificity%, and the cutoff value were 0.8228, 90%, 61.67%, and > 0.001396, respectively, CI 95% = 0.7486 to 0.8969).

performed using Excel, SPSS statistics (version 24), and GraphPad Prism 7 software. The confidence interval (CI) of 95% and p-value less than 0.05 were considered significant in all statistical analyses of the data.

RESULTS

Increased expression of GClnc1 in tumor tissue specimens of GC patients

According to the analysis of GClnc1 expression data, the expression level of GClnc1 was significantly increased in GC tissues as compared with marginal non-tumor tissue specimens (p-value < 0.0001; Figure 1).

The relationship between overexpression of GClnc1 and clinicopathological features

In examining the association with high expression of GClnc1 and the patients' clinicopathological characteristics this study represents a significant link with lymph node metastasis (p-value = 0.037) and patients infected with *H. pylori* (p-value = 0.029). Although no significant link was observed between overexpression of mentioned IncRNA and features such as age (p-value = 0.875), gender (p-value = 0.478), TNM stage (p-value = 0.324), and size of tumors (p-value = 0.250) (Table 1).

Biomarker's function of GClnc1

GClnc1 can function as a molecular marker to distinguish GC tissue from non-cancerous tissue in patients with GC malignancy. Also, this biomarker has the area under the curve (AUC), sensitivity%, and specificity% of 0.8228, 90%, and 61.67%, respectively (p < 0.0001; Figure 2) (Table 2).

DISCUSSION

Although in recent years there have been remarkable advances in the therapies of GC, including surgery combined with chemotherapy and radiotherapy, this aggressive and heterogeneous cancer still has a high death rate among patients. The 5-year survival rate in GC patients is less than 35%; also, the poor prognosis in GC patients is one of the most important reasons for the late detection of patients in advanced stages. In addition to an individual's genetic predisposition to GC, the diverse environmental risk factors comprising the use of alcohol and smoking, infection with *H. pylori*, and Epstein-Barr virus are involved in the promotion of GC. *H. pylori* in the process of chronic inflammation with its antigen components CagA and VacA function as carcinogen and can lead to changed cell proliferation, apoptosis, and epigenetic modifications of tumor suppressor genes [19-21]. So, by recognizing the molecules that participate in the onset and development of GC cells, it is possible to significantly reduce the incidence and mortality rate of GC patients through early diagnosis and treatment. LncRNAs, as important regulatory molecules, have a fundamental role in human malignancies. Different dimensions of gene expression regulation at transcriptional, post-transcriptional, and epigenetics levels are affected by their regulatory performances [22]. In the present research, the obtained results of GClnc1 expression data examination are indicative of a significantly high expression of the mentioned lncRNA in GC tissues as compared to marginal non-cancerous tissues. Besides, the GClnc1 overexpression was significantly associated with pathological features in GC patients that were infected with *H. pylori* and have lymph node metastasis. Also, the results proposed that GClnc1 has a potential performance in the targeted treatment of GC patients and may act as an oncogene in the promotion of GC.

The GClnc1 with 2,155 nucleotides in length has been located on 6 human chromosomes and their mechanisms of function have been investigated in the promotion of several cancers. In the study of bladder cancer, the significant overexpression of GClnc1 has occurred in tumor tissues. Besides, the level of expression was associated with low survival rates, the promotion of cell proliferation, the ability of metastasis, and invasion of cancer cells in these patients. In this regard, it was found that by binding to LIN28B, GClnc1 causes the increased expression of MYC proto-oncogene [23]. In a study of patients with ovarian cancer, a significant increase in GClnc1 in tumor tissues was observed. Also, high expression of GClnc1 is significantly associated with the proliferation and migration of ovarian cancer cells. In particular, GClnc1 regulates p53 signaling pathways and can play an effective role in the development of ovarian cancer by inhibiting p53 [24]. Examination of the mechanism of function of GClnc1 in colorectal cancer tissue revealed that a significant overexpression in this lncRNA occurred in tumor tissues relative to non-cancerous colorectal tissues. Also, colorectal cancer patients with the increased expression level of GClnc1 had poor overall survival rates. GClnc1 can proliferate and promote colorectal cancer cells with a reduction of P21 and BAX expression through the P53 signaling pathway [25]. Research on osteosarcoma patients to discover the functional mechanism of GClnc1 has indicated that the GClnc1 expression levels are significantly increased in osteosarcoma tissues, leading to the development of tumorigenesis of osteosarcoma cells. It also reduces BAX and P21 expression by suppressing P53 acetylation [26]. The above studies have revealed that GClnc1 can have a potential function as a therapeutic target in these malignancies. As a result of the overexpression of GClnc1 in the cancer tissue of GC patients and its significant relationship with pathological characteristics in patients infected with *H. pylori* and lymph node metastasis, we recommended that the mentioned lncRNA may be involved in the development and progression of gastric malignancy as an oncogene. Moreover, GClnc1 has potential functions as a molecular marker in gastric cancer tissue detection and can be utilized in the targeted therapies of patients suffering from gastric cancer.

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Availability of Data and Material:
The confirmed data in this study are available upon reasonable petition from the corresponding author.

Ethical Approval:
The Ethics Committee of Tabriz University approved this study.

Declaration of Interest:
The authors have no declared conflicts of interest.

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