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Original Article

Introduction of Four Long Non-Coding RNAs (LncRNAs) as Molecular Probes in Gastric Malignancy

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Abstract

Introduction: Gastric cancer, known as one of the most frequent types of cancer, is associated with extensive mortality. Early detection could help to increase the survival rate of patients. Long non-coding RNAs (lncRNAs) as newly considered cancer probes were evaluated regarding as potential diagnostic candidates.

Materials and Methods: LncRNAs *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1*, and *LINC00667* were selected for wet-lab analysis based on literature review and pathway enrichment *in-silico* analysis. Four candidate lncRNAs in 70 gastric tissue samples including gastric tumors (n = 35) and paired Adjacent Normal Gastric Tissues (ANGTs) were collected for quantitative analysis. In the following, the expression level of four candidate lncRNAs was analyzed in gastric cancer samples compared to ANGTs and patient's clinico-pathological data. Receiver Operating Characteristic (ROC) analysis was also performed to determine the diagnostic value of *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1* and *LINC00667* levels in tissue samples. Different pathways associated with *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1* and *LINC00667* lncRNAs were identified.

Results: The significantly elevated levels of all four lncRNAs were detected in GC tissue samples compared to ANGTs (P-value<0.05). The Aria Under Curve (AUC) of *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1*, and *LINC00667* in the ROC curve were 0.825, 0.928, 0.688, and 0.649, respectively.

Conclusions: Our results indicated that the expression levels of the four candidate lncRNAs were elevated in gastric tumors compared to AN GT samples. It might be helpful to illuminate the molecular mechanisms underlying gastric carcinogenesis which have been served as tumor-associated biomarkers for diagnosis.

Keywords: Gastric Cancer, LncRNAs, Expression Level, Molecular Probes

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Introduction

A class of transcribed RNA molecules that are longer than 200 nucleotides are called long noncoding RNAs (lncRNAs) and do not encode proteins. They are involved in the regulation of stem cell properties including differentiation, maintenance of normal cell and tissue homeostasis, cellular transformation, tumor progression and enrichment of a Cancer Stem Cell (CSC) population.[1] Various expressions of lncRNAs in several cancers could lead to a spectrum of clinical outcomes. Some studies have indicated that lncRNAs are major players in many types of cancer processes. This could suggest the lncRNAs as potential biomarker probes for diagnosis, medical treatment, and prognostics in human cancers such as gastric cancer.[2,3]

One of the most common malignant tumors and major cause of cancer-related mortality is gastric cancer.[4] Its early diagnosis could be treated with surgical techniques and the implementation of traditional radiotherapy, chemotherapy, and neoadjuvant therapy. This malignancy is associated with the late diagnosis and progression to advanced stages and metastasis, which the treatments will not be as effective as the early disease.[5] However, gastric cancer is seldom diagnosed early and therefore in most patients the disease hides by the time of clinical manifestation.[6] Thus, early diagnosis of gastric cancer is vitally important in ensuring an excellent prognosis.[7]

Related lncRNAs and their expression profiles in early stages in comparison with advanced gastric tumors or non-cancerous samples are developed by RNA-seq, microarray or quantitative-Polymerase Chain Reaction (qPCR).[8] Some lncRNAs involved in gastric cancer are known including *X inactive-specific transcript (XIST)*, *HOX antisense intergenic RNA (HOTAIR)*, *H19*, *LINC00152* etc.[9]

In this study, based on literature review and pathway enrichment *in-silico* analysis, we hypothesized that *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1*, *LINC00667* lncRNAs are involved in important cancer related molecular pathways. So, dysregulation of these four lncRNAs could have a great impact on GC progression and they have potential for serving as biomarker probes for the diagnosis of gastric cancer.

Materials and Methods

LncRNAs Selection and In-Silico Analysis

In order to candidate appropriate lncRNAs, firstly, a comprehensive literature review on lncRNAs in cancer revealed that *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1* and *LINC00667* lncRNAs are involved in the various cancer development.[9-13] However, the role and molecular mechanism of them in gastric cancer is not clear yet. To investigate the regulatory mechanism underlying the function of four lncRNAs in gastric cancer development by pathway enrichment analysis (Figure 1), at first, we applied the web server for RNA-RNA Interaction (http://rtools.cbrc.jp/cgi-bin/RNARNA/index.pl) to conduct gene set enrichment in order to obtain a list of predicted target genes for four candidate lncRNAs.

To find the biological pathways in which our four candidate lncRNAs were involved, a pathway enrichment analysis for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, was performed using the Enrichr, (RRID:SCR_001575), a comprehensive gene set enrichment analysis web server. *p*-values less than 0.05 were regarded as statistically significant.

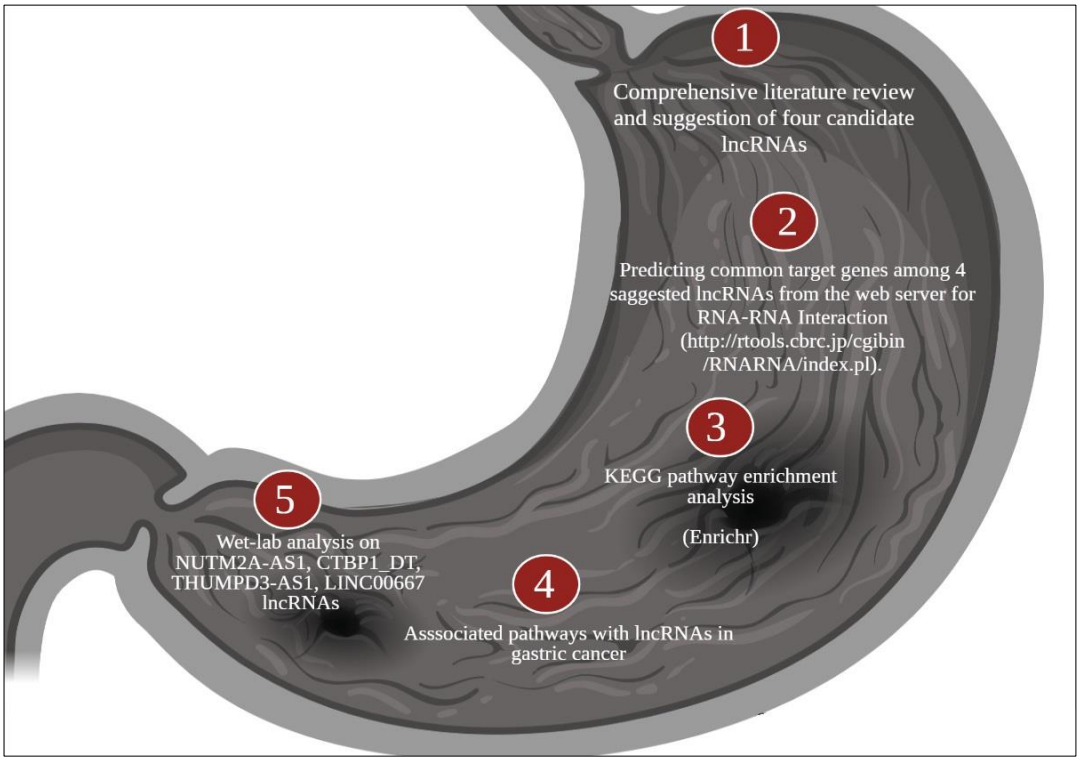

Figure 1. Flow Diagram of *In-Silico* Study Design.

Clinical Samples

During gastrectomy, 35 tissue samples were attained prior to receiving chemo/radiotherapy. Following samples were directly frozen and stored at -70 °C until expression analysis. Pathologist examined all specimens to prove the pathologic signs. The study protocol was approved by the Ethical Committee (IR.BMSU.REC.1398.281) in Baqiyatallah University of Medical Sciences. All subjects gave a written informed consent in accordance with the Declaration of Helsinki.

The expression levels of lncRNAs in all gastric tissue samples including gastric tumors (n = 35) and paired adjacent normal gastric tissues (ANGTs) (n = 35, histologically normal samples with at least 3 cm distance from the tumor) were then assessed. All demographic and pathologic information (age, sex, smoking, tumor grade, *Helicobacter pylori* (*H. pylori*) infection etc.) of participants and samples was recorded for extra subgroup analysis.

Quantification of LncRNAs

The AccuZolTM total RNA extraction kit (Bioneer Co., Korea) was applied to extract total RNAs from tissue samples (separately for tumor tissues and ANGT samples) according to the manufacturer's instructions. The cDNAs were synthesized from RNA by the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan). The qPCRs were performed with SYBR Green Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) in 15 µl final volume including 1.5 µl template cDNA mixed with 7.5 µl 2× SYBR Green PCR master mix and 25 pmol of each Lnc-specifc forward and reverse primer. The qPCRs were performed in duplicate according to the standard program on Rotor-Gene Q instrument (QIAGEN, Germany): 10 s at 95 °C, followed by two cycles of amplification (10 s at 95 °C, 30 s at 60 °C) and finally a dissociation curve step (ramp from 70 to 90 °C) to verify

amplification specificity. The GAPDH gene was considered as a housekeeping gene to normalize the output data. To certify the reaction specificity, a Non-Template Control (NTC) reaction was used for every primer set. The list of primer sequences used for RT-PCR analysis have been presented in the Table 1.

Table 1. The Sequence of Primers Used in This Study

Primer	Sequence
NUTM2A-AS1	**F**: 5′- TACCTCTAGTTCTTCCCGGC -3′ **R**: 5′- TTTTGCTTTTCTCCTGGCCC -3′
CTBP1_DT	**F**: 5′- CAAGGGCACTCAAAGGGCTA -3′ **R**: 5′- CAGGCAGGCAAACACAGAAC -3′
THUMPD3-AS1	**F**: 5′- TGGTGCCACCTATGTTGTGG -3′ **R**: 5′- ACACTTCAGCCAGCAGAGAC -3′
LINC00667	**F**: 5′- TGTGCGAGAAAGCCTACCTG -3′ **R**: 5′- GCCTGCATCAAAAAGTCGGG -3′
GAPDH	**F**: 5′- TGAACGGGAAGCTCACTGG -3′ **R**: 5′- TCCACCACCCTGTTGCTGT -3′

Statistical Analysis

The quantitative variables were presented as mean ± standard error of mean (SEM). Graph-pad Prism 7 (RRID:SCR_002798) software was used for statistical analyses. Data normality was checked by the Shapiro–Wilk test. The lncRNA expression levels between the two defined groups (tumor vs ANGTs) were measured by paired t-test. For evaluating the diagnostic value of such lncRNAs, ROC curve analysis was performed using MedCalc software version 18.5 (RRID: SCR_015044). *p*-values below 0.05 were considered to be statistically significant values.

Results

LncRNAs and In-Silico Analysis

The enriched KEGG pathways for target genes of *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1*, *LINC00667* are depicted in Figure 2. The results showed that the common target genes of four candidate lncRNAs have enriched in pathways implicated in gastric cancer pathogenesis (Supplementary S1). According to the important regulatory role of *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1*, *LINC00667* in pathways involved in cancer, we select them for wet-lab investigation.

Clinical Characteristics of Samples and LncRNA Expression Quantification

The present study enrolled a total of 70 tissue samples encompassing 35 gastric tumors and 35 ANGT samples. The clinical characteristics of the study participants are summarized in Table 2. In wet-lab analysis, the expression levels of *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1* and *LINC00667* lncRNAs were investigated. According to fold change and comparative *p*-value determined by paired t-test, a significant overexpression was observed for all investigated lncRNAs in tumor tissues compared to the ANGT samples (Table 3).

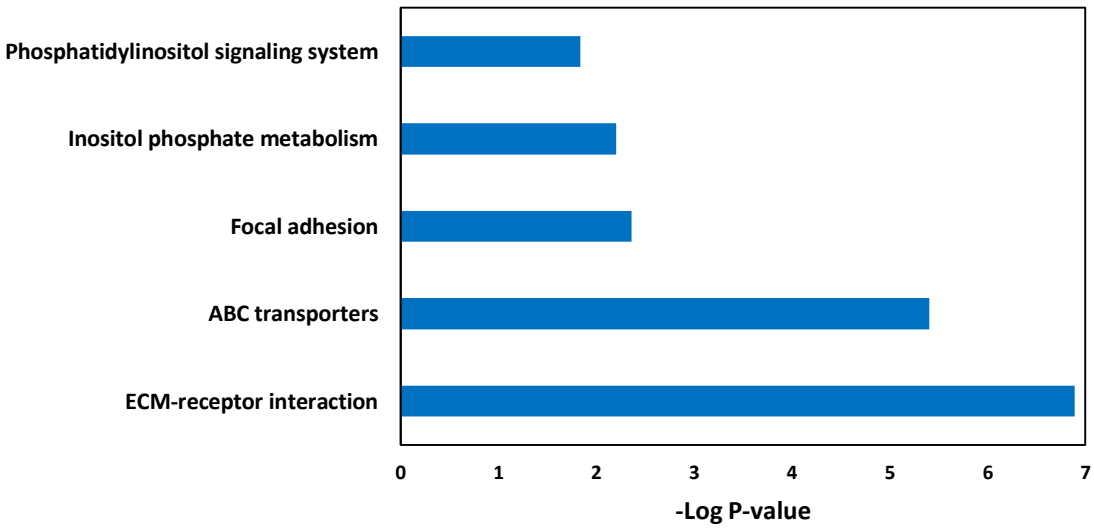

Figure 2. Pathways Enriched by Common Target Genes of NUTM2A-AS1, CTBP1-DT, THUMPD3-AS1 and LINC0066 LncRNAs Presented as a Bar Chart.

Relationship Between the Four-LncRNA Signature and Clinicopathological Features of Gastric Cancer Patients

Enrolled patients were classified based on their general information (Table 2). Then, the lncRNAs expression in different subgroups of cancerous samples was investigated (Table 4). Results showed a significant upregulation of *NUTM2A-AS1* (p = 0.01), *THUMPD3-AS1* (p = 0.04) and *LINC00667* (p = 0.01) in Lymph Node Metastasis (LNM) in positive samples. Advanced-grade samples and tumors with a larger size had also represented significant association with *NUTM2A-AS1* (p = 0.01) and *LINC00667* (p = 0.02) over-expression. The other features including age, gender and *H. pylori* infection, did not show any significant association with the studied lncRNAs.

Table 2. General Information of Enrolled Patients

Variables		Values[1]
Age (Years, mean range)		45.91 (30-65)
Sex	Male	23 (65.71%)
	Female	12 (34.29%)
Grade	I+II	17 (48.57%)
	III+IV	18 (51.43%)
LNM[2]	Negative	14 (40%)
	Positive	21 (60%)
Histological form	Diffuse	20 (57.14%)
	Intestinal	15 (43.86%)
Smoking	Non-smoker	10 (47.61%)
	Smoker	11 (52.39%)
H. pylori infection	Negative	18 (50%)
	Positive	17 (50%)

[1)] For each parameter, some parameters were nor recorded in the questionnaire, so there are a number of missing values., [2)] LNM: Lymph node metastasis

Table 3. According to Fold Change and *p*-values, Results Show a Significant Upregulation of Selected LncRNAs in GC Samples Compared to ANGTs[1] Samples

LncRNA	ΔΔCt Values (Mean)	Fold Change ($2^{-\Delta\Delta Ct}$)	*p*-value
NUTM2A-AS1	-2.353	5.10	< 0.0001
CTBP1-DT	-2.328	5.02	< 0.0001
THUMPD3-AS1	-1.353	2.55	0.0022
LINC00667	-1.262	2.39	0.0131

[1)] ANGT; Adjacent Normal Gastric Tissue

Assessment of Diagnostic Power of LncRNA

Detailed results of ROC analysis have been provided in Figure 3 and Table 5. The area under the curves (AUC) for *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1* and *LINC00667* were 0.825, 0.928, 0.688 and 0.649, respectively. The results showed the high sensitivity for *CTBP1-DT* and *THUMPD3-AS1* (97.14% and 94.29%, respectively). Except *THUMPD3-AS1*, all the other lncRNAs represented a specificity higher than 80%.

Discussion

Nowadays, gastric cancer is a major health problem and is known as a mortal disease due to ineffective therapeutic intermediation after detection in advanced stages.[4,14] Accordingly, finding proper biomarkers and developing new diagnostic procedures are necessary for early detection and treatment of this disease.[15] Lately, the role of lncRNAs in human physiological and pathological status including GC has been reported. Following this, the results of recent investigations indicate that lncRNAs may be stimulating molecular candidates for unresolved issues in cancers.[1,9]

Table 4. The LncRNAs Expression in Different Subgroups of Cancerous Samples (n = 35)

Feature	N	NUTM2A-AS1 Mean ± SEM	*p*-value	CTBP1_DT Mean ± SEM	*p*-value	THUMPD3-AS1 Mean ±SEM	*p*-value	LINC00667 Mean ± SEM	*p*-value
Age									
≤45	17	-2.41 ± 0.60	0.77	-2.03 ± 0.48	0.38	-1.68 ± 0.64	0.47	-1.55 ± 0.77	0.73
>45	18	-2.29 ± 0.70		-2.60 ± 0.34		-1.03 ± 0.51		-1.00 ± 0.65	
Gender									
Male	23	-2.05 ± 0.60	0.64	-2.16 ± 0.37	0.22	-1.12 ± 0.50	0.67	-0.99 ± 0.57	0.79
Female	12	-2.92 ± 0.67		-2.65 ± 0.47		-1.78 ± 0.70		-1.77 ± 0.88	
LNM									
Neg	14	-1.10 ± 0.79	0.03	-2.10 ± 0.39	0.84	-0.50 ± 0.53	0.04	-0.50 ± 0.67	0.01
Pos	21	-3.18 ± 0.48		-2.47 ± 0.42		-1.91 ± 0.55		-1.77 ± 0.65	
Grade									
I+II	17	-1.48 ± 0.68	0.01	-2.17 ± 0.35	0.63	-0.94 ± 0.44	0.07	0.00 ± 0.6	0.02
III+IV	18	-3.17 ± 0.57		-2.47 ± 0.47		-1.74 ± 0.67		-2.45 ± 0.60	
Size									
<5	17	-1.13 ± 0.67	0.02	-2.39 ± 0.36	0.96	-1.84 ± 0.59	0.34	-0.11 ± 0.64	0.02
≥5	18	-3.50 ± 0.50		-2.26 ± 0.46		-0.88 ± 0.54		-2.56 ± 0.61	
H. pylori									
Neg	18	-2.30 ± 0.58	0.96	-2.58 ± 0.43	0.52	-1.45 ± 0.61	0.66	-1.39 ± 0.74	0.76
Pos	17	-2.40 ± 0.72		-2.06 ± 0.40		-1.24 ± 0.54		-1.12 ± 0.62	

Table 5. The Detailed Parameters of ROC Curve Analysis

LncRNAs	Associated Criterion	AUC[1]	J[2]	Sensitivity	Specificity	*P*-value
NUTM2A_AS1	≤4.0447	0.825	0.4857	51.43	97.14	<0.0001
CTBP1-DT	≤6.9911	0.928	0.8000	97.14	82.86	<0.0001
THUMPD3_AS1	≤2.5657	0.688	0.3143	94.29	37.14	0.0031
LINC00667	≤0.2853	0.649	0.2571	40.00	85.71	0.0245

[1)] Area under the curve, [2)] Youden index

The role and molecular mechanism of *NUTM2A-AS1* in cancers including gastric cancer is not clear yet. Its oncogenic role has also been demonstrated by representing its upregulation in non-small cell lung carcinoma and hepatocellular carcinoma.[16,17] Our results showed that *NUTM2A-AS1* has strongly been overexpressed in tumor samples. Furthermore, we found that increased expression levels of *NUTM2A-AS1* is associated with advanced tumor grade, LNM positive specimens and larger size of tumors. Likewise, Wang et al.'s investigation on gastric cancer cells showed that overexpression of *NUTM2A-AS1* induces cell viability, invasion, tumorigenesis and drug resistance. Their results showed that *NUTM2A-AS1* targets miR-376a to induce tumorigenesis and drug resistance.[12] This could indicate that *NUTM2A-AS1* acts as a tumor promoter in GC.

CTBP1-DT is a newly discovered lncRNA with an unknown mechanism in cancer biology. In this study, we found a higher expression level of *CTBP1-DT* in tumor

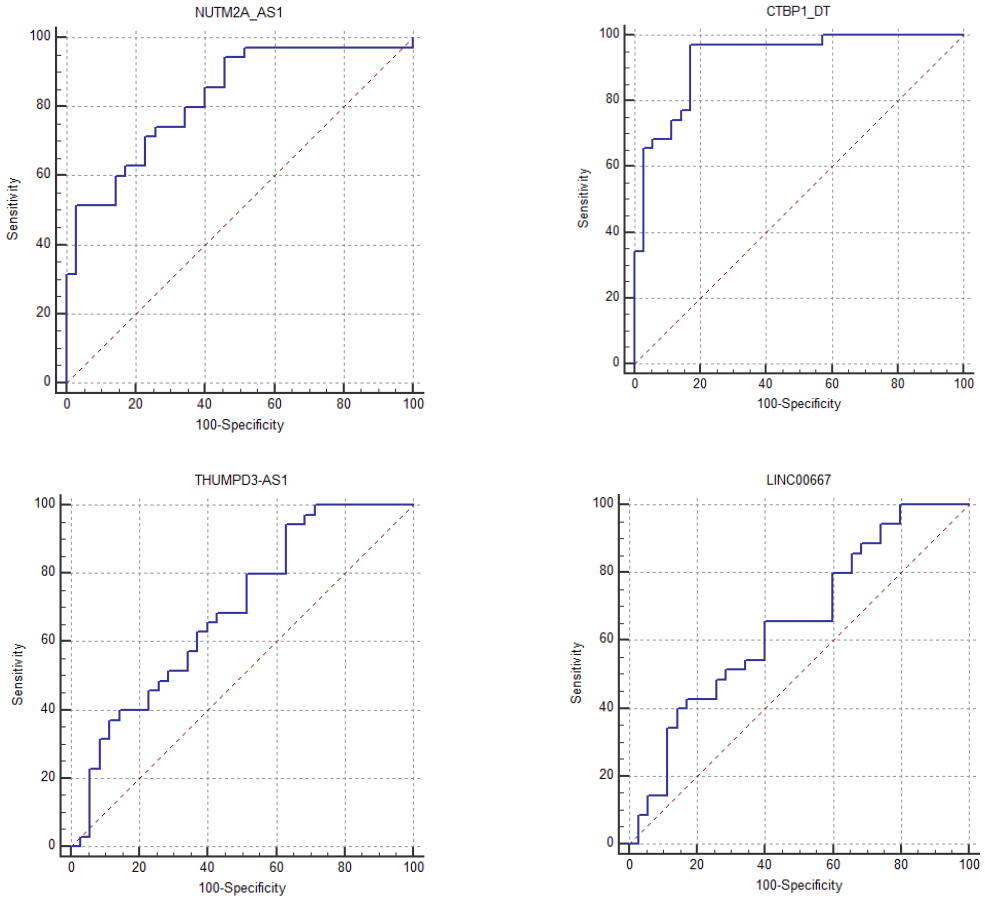

Figure 3. ROC Was Performed on Four LncRNAs in GC Tissues (n = 35) and ANGTs (n = 35) to Determine the Optimal Cutoff Value.

specimens compared to ANGTs, but we did not find any significant association between *CTBP1-DT* expression level and LNM positivity, tumor grade or tumor size. Similarly, Yang et al. reported an overexpression of *CTBP1-DT* in gastric cancer cells. They claimed that *CTBP1-DT* promotes gastric cancer cells and inhibits apoptosis by regulating the miR-139-3p/MMP11 molecular axis.[18] These results indicate the oncogenic role of *CTBP1-DT* in GC. However, the findings of the abovementioned study like the association of *CTBP1-DT* with clinicopathological features including involvement in increased tumor size and tumor grade, and also determining metastatic features, contradict the results of the present.[18] These findings are in contradictory with ours, but biological pathway redundancies in tumor biology as well as tumor heterogeneity could describe the above-mentioned contradictions.

Our results showed the remarkably higher expression level of *THUMPD3-AS1* in GC tumor specimens than ANGTs that were significantly associated with LNM positive specimens and marginally with higher-grade tumors. Aberrant expression of *THUMPD3-AS1* in various types of malignancies has been also reported so far.[10,19] In a study on Non-Small Cell Lung Cancer (NSCLC), it was represented that the overexpression of *THUMPD3-AS1* increases the cell proliferation and self-renewal of NSCLC cells by sponging miR-543 and dysregulating the *ONECUT2* gene. Furthermore, *THUMPD3-AS1* has been shown to block the apoptosis via down regulating miR-543.[10] The oncogenic role of *THUMPD3-AS1* has also been previously reported in breast cancer.[19]

The present results showed the upregulation of *LINC00667* in tumor samples, especially in higher grades, larger tumors, and LNM positive samples. Although the role of *LINC00667* in the proliferation and tumorigenicity of colorectal cancer cells have been represented by knock down studies, its clinical significance in GC has remained unclear. However, several studies reported the oncogenic role of *LINC00667* in a range of cancers.[11,20]

The pathway enrichment analysis showed that common target genes of *NUTM2A-AS1*, *CTBP1-DT*, *THUMPD3-AS1* and *LINC00667* are significantly enriched in pathways relevant to cancer including GC such as ECM-receptor interaction, ATP-Binding Cassette (ABC) transporters, focal adhesion, inositol phosphate metabolism, phosphatidylinositol signaling system, thyroid hormone signaling pathway, complement and coagulation cascades.

The role of ECM-receptor interaction has not been fully clarified in gastric cancer. The interaction of ECM components such as laminin, fibronectin and collagen and tumor which is

facilitated by adhesion receptors such as integrins, plays a vital role in tumor invasion and metastasis. Focal adhesion pathway as ECM-receptor interactions may be involved in cancer metastasis. It has been reported that numerous types of collagens are involved in the focal adhesion and ECM-receptor interaction pathways in gastric cancer.[21] Jang et al. reported that integrin-mediated signaling in epithelial gastric cancer cells could be affected by dense ECM in the tumor microenvironment which promotes cell invasion and proliferation via enhanced Focal Adhesion kinase (FAK) phosphorylation. This could lead to cell transformation into the mesenchymal phenotype and drug resistance.[22]

ABC transporters regulate intracellular organelles such as the mitochondrion, lysosome, endoplasmic reticulum and Golgi apparatus by having an effect on cellular levels of hormones, lipids, ions, xenobiotics and other small molecules by transporting molecules across cell membranes. Studies confirmed that lncRNAs could regulate ABC transporters in chemo-resistant cancers.[23] It was also reported that overexpression of the lncRNAs could induce the expression of critical genes of the ABC family in cancers.[24]

Phosphatidylinositol signaling system is a pathway that promotes cell growth, metabolism, survival, anti-apoptotic activities, metastatic cascade including proteolytic activity, cytoskeletal remodeling, and resistance to chemotherapy. It is also confirmed that enhanced activity of this pathway due to high expression levels of *HER2* could lead to poor prognosis of gastric cancer.[25]

Finally, we assessed the diagnostic power of four candidate lncRNAs in gastric cancer by using ANGTs as control. The ROC curve has been extensively used to measure diagnostic accuracy. The AUC for NUTM2A-AS1 and CTBP1-DT indicates that they may be considered as promising candidate biomarker probes for gastric cancer detection. Also, the sensitivity and specificity of lncRNAs expression as a diagnostic test were calculated. The results showed the high sensitivity for CTBP1-DT and THUMPD3-AS1 (97.14% and 94.29%, respectively). Totally, although four candidate lncRNAs are very effective in differentiating between two groups of tumor tissue and ANGT samples, but, CTBP1-DT has the best accuracy with a high AUC (0.928), sensitivity (97.14) and specificity (82.86). While this study tried to reveal the unknown dimensions of a limited number of lncRNAs in its dimensions, but its amazing results indicated a great call for more extensive and in-depth studies to discover important and unknown aspects of this field.

Conclusion

In conclusion, the results of our study identified several enriched pathways which are related to gastric cancer. Furthermore, we found a significant association between *NUTM2A-AS1*, *THUMPD3-AS1* and *LINC00667* lncRNAs with LNM condition, tumor grade and size of tumor as clinicopathological features. These findings suggest that *NUTM2A-AS1*, *THUMPD3-AS1* and *LINC00667* are potential candidates for monitoring the prognosis of gastric cancer patients. However, it should be pointed out that the results of this study were based on a relatively limited number of subjects and clinicopathological variables; thus, confirmation is required by conducting larger researches.

Authors' Contributions

All authors contributed to the study conception and design. The idea development, moderation of research projects and adjusting the draft performed by MH. Material preparation, data analysis and draft preparation were performed by AZ. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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