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LINC02688 and PP7080 as novel biomarkers in early diagnosis of gastric cancer

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ABSTRACT

Despite considerable progress in gastric cancer screening, prevention, and treatment, it remains a major cause of morbidity and mortality worldwide. Due to late diagnosis of the disease, early potential diagnostic biomarkers are needed. Accumulating evidence indicates that non-coding RNAs have potential applications as diagnostic and prognostic biomarkers in gastric cancer. Herein, we investigated the expression levels of two novel non-coding RNAs, long intergenic non-protein coding RNA 2688 (*LINC02688*) and *LOC25845* (*PP7080*) by real-time PCR for the first time in 47 gastric cancer patients. We found significant downregulation of LINC02688 and *LOC25845* (*PP7080*) with 3.44 and 2.2-fold decrease, respectively in tumoral tissues in comparison with their adjacent non-tumoral counterparts ($P < 0.0001$). Our data also indicates that more than 96% and 88% of patients showed unchanged or decreased expression of LINC02688 and *LOC25845* (*PP7080*), respectively. As most gastric cancer patients showed lower expression of these two lncRNAs, no significant association between clinicopathological features of the patients and the level of LINC02688 and *LOC25845* (*PP7080*) expression could be detected. Furthermore, ROC curve analysis indicated that LINC02688 and *PP7080* can serve as good predictive biomarkers for distinguishing tumoral tissues from their adjacent non-tumoral counterparts. Taken together, our findings suggested that these two novel tumor suppressor non-coding RNAs may act as novel diagnostic biomarkers for diagnosis of carcinogenesis event even at earlier stages of gastric adenocarcinoma.

1. Introduction

Gastric cancer (GC) is the fifth most frequently diagnosed cancer, and remains the third leading cause of cancer-related mortality worldwide, accounting for about 8.2% of all death cases [1]. Based on GLOBOCAN 2018 data more than 1,000,000 new cases and around 783,000 deaths from GC occurred in 2018 [1]. Both genetic and epigenetic factors as well as environmental determinants affect GC development [2–10]. Despite considerable progresses achieved in the treatment of GC, it remains a major clinical challenge due to late stage diagnosis and its poor prognosis [11–13]. Consequently, finding reliable prognostic biomarkers improves early diagnosis with an increased opportunity for designing strategies for the prevention and treatment of GC. It is now well established that long non-coding RNAs (lncRNAs), which are defined as transcripts with more than 200 nucleotides, have diverse roles in regulating gene expression, and can be used as biomarkers for cancer diagnosis and prognosis as well as potential targets for cancer therapy [14–17]. In a previous pilot study, we unveiled important novel lncRNAs that were down-regulated in GC using RNA-sequencing (RNA-seq) technology by comparing the transcriptome level of tumoral tissues with their normal counterparts (accepted manuscript and unpublished data). Here the expression levels of two novel lncRNAs that were significantly downregulated were evaluated using Taqman real time PCR among gastric cancerous and non-cancerous tissues.

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2. Materials and methods

2.1. Patients

A total of 94 specimens including 47 tumor and their adjacent non-tumoral tissues were collected from newly diagnosed gastric adenocarcinoma patients who underwent gastrectomy at hospitals in Mazandaran province, Iran during September 2015 to June 2018, and were enrolled into this study. All samples were snap-frozen in the liquid nitrogen after surgery, and stored at −80 °C for further analyzes. Written informed consent was obtained from all patients prior to enrolment in the study. This study was approved by the Ethics Committee of Babol University of Medical Sciences, and all procedures were performed in compliance with Helsinki declaration. Pathologic diagnosis of the tumoral and non-tumoral tissues have been performed by using hematoxylin and eosin staining, and infection status (*H. pylori*, CMV, HHV6, and EBV) was evaluated using PCR and real time PCR method as described previously [18].

2.2. RNA extraction

Approximately 10 mg of tumoral tissues and their adjacent non-tumoral counterparts were homogenized using liquid nitrogen. Subsequently, total RNA was extracted using RNX-plus reagent (Cinagen, Iran) according to the manufacturer's instructions. The purity and quantity of total RNA were measured by using NanoDrop 2000 (Thermo Scientific, USA), and 28S/18S ratio (28S and 18S ribosomal RNA bands) was evaluated by electrophoresis on 1.8% agarose gel to monitor the RNA integrity.

2.3. Quantitative real-time PCR analysis (qPCR)

Expression levels of two lncRNAs including LINC02688 and *LOC25845* (*PP7080*) were examined by qRT-PCR in 47 pairs of gastric adenocarcinoma tissues and their normal adjacent counterparts. First-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, USA) using a gene specific stem-loop primer [19,20]. QRT-PCR was performed in a StepOne Plus real time instrument (Applied Biosystems, USA) using HotStar-Taq DNA Polymerase kit (Qiagen, Germany) according to the manufacturer's instructions. Amplification program consisted of initial denaturation at 95 °C for 15 min, followed by 45 cycles of 15 s at 95 °C, 1 min at 60 °C. The expression levels of target RNAs were normalized to those of *RNU6*. All reactions were performed in triplicate. The sequences of forward and reverse primers along with universal Taqman probe are presented in Table 1. The sequence of Taqman probe was FAM 5′ TGGATGTGTCTGCGGCGTTTTATCAT 3′ BHQ-1, and the sequence of reverse primer was 5′ GTATCCAGTGCTGCGACCGT 3′.

Stem-loop sequence is underlined; target portion of mRNA is showed in red Bold.

2.4. Statistical analysis

Relative gene expression data was performed by StepOne software v2.3 and the $2^{-\Delta\Delta Ct}$ method. Comparisons of results between groups were performed by paired Student's *t*-test. Receiver operating characteristic curve (ROC) analysis was performed using Prism software. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Clinicopathological features

The median age of patients was 67 years (range, 34–85 years), at the time of diagnosis, and also the male-to-female ratio was 3:1. Overall, more than half of the tumors were found at proximal position of the stomach (cardia, fundus, and body), and poorly differentiated tumors were observed in 65% of patients. In general, two-third of tumors were in stage I and II. Family history was found in 12.7% of the patients. Approximately, 90% of patients had at least one infection (EBV or CMV or HHV6 or *H. pylori*).

3.2. Expression levels of LINC02688 and LOC25845 (PP7080)

As shown in Fig. 1, LINC02688 and *LOC25845* (*PP7080*) were significantly down-regulated by more than 3.44 and 2.2-fold in tumor samples in comparison with their adjacent non-tumoral tissues, respectively. Scatter plot analysis indicated that LINC02688 was significantly down-regulated in 32 out of 47 (68%) tumoral tissues in comparison with their adjacent non-tumoral counterparts. Also, out of 47 GC patients, 25 (53%) showed statistically significant down-regulation of *LOC25845* (*PP7080*) in tumoral tissues in comparison with their adjacent non-tumoral counterparts (Fig. 2). Interestingly, LINC02688 and *LOC25845* (*PP7080*) expressions were not detected in 2 and 1 tumoral tissue, respectively. There was no significant association between clinicopathological features of the patients and the level of LINC02688 and *LOC25845* (*PP7080*) expression (Fig. 3).

3.3. Association of the infection status and LINC02688 and LOC25845 expression level

Association between four studied infections including EBV, CMV, HHV6, and *H. pylor*i with the level of LINC02688 and *LOC25845* (*PP7080*) was investigated in GC patients. No significant difference in LINC02688 and *LOC25845* (*PP7080*) expressions level was detected between patients with and without viral infections. However, there was a significant difference for LINC02688 and *LOC25845* (*PP7080*) mRNA levels in *H. pylori* positive GC patients in comparison with *H. pylori* negative patients (Fig. 4) ($P = 0.0003$, and $P = 0.0001$, respectively).

Table 1
Sequences of primers used for evaluation of Non-coding RNAs expression.

Accession Number	Gene Name	Primers 5' → 3′
>NR_004394.1	*RNU6-1*	Specific forward primer: CTCGCTTCGGCAGCACATATAC RT-PCR primer GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACGAC**GTGTCATCCTTGC**
>NR_024158.1	*LOC25845*	Specific forward primer: GCCCTCGCCAGGTCTTTGAAC RT-PCR primer GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACGAC**TCCTGACTGGTG**
>NR_160890.1	*LINC02688*	Specific forward primer: GGTCTTGGTGTCCTCTGACTTCTG RT-PCR primer GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATGCACTGGATACGAC**CCAGCGACTCTC**

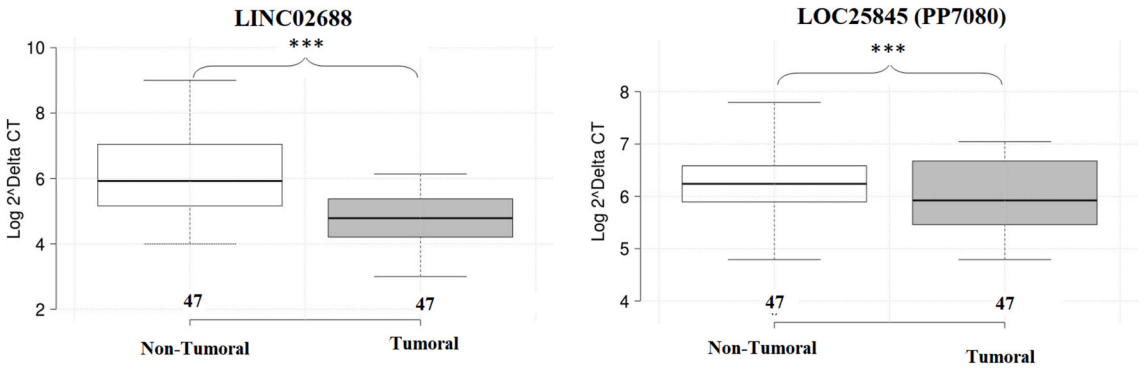

Fig. 1. *LINC02688* and *LOC25845* (*PP7080*) expression fold change in 47 patients (tumoral tissues in comparison with non-tumoral tissues). Analyzes were performed in triplicate, and data are shown as mean ± SD. *** indicates significant difference $P < 0.0001$.

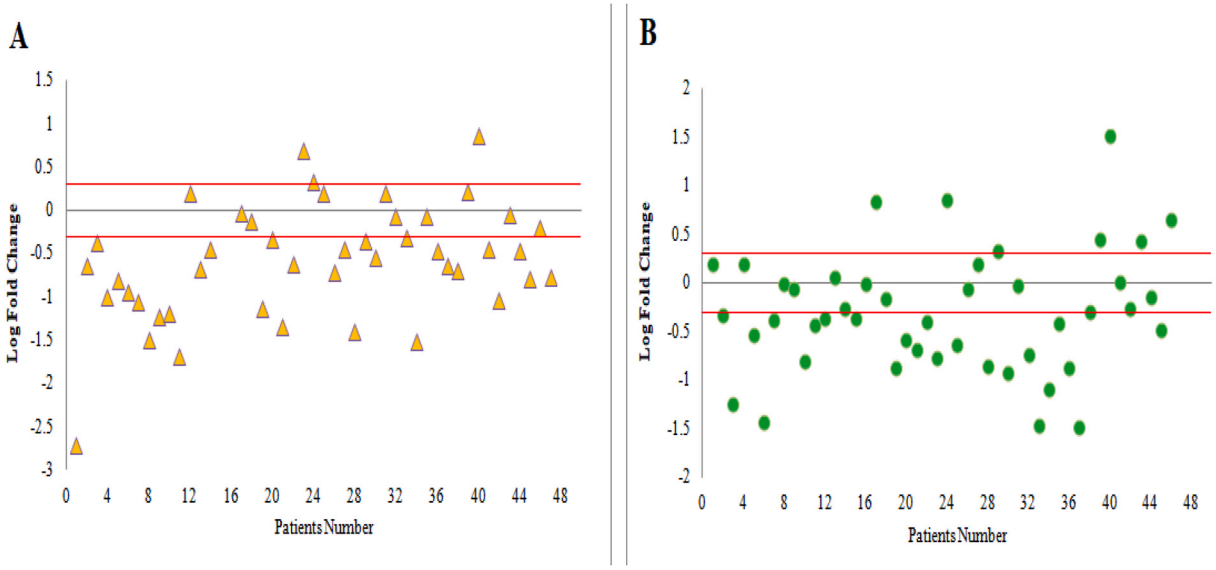

Fig. 2. Scatter plot of lncRNAs expression in tumoral and adjacent non-tumoral tissues . A) LINC02688; B) *LOC25845* (*PP7080*). Gene expression variations were considered in 47 gastric adenocarcinoma patients. Each plot represents a person's gene expression change in tumor tissue in comparison with adjacent normal tissue. Horizontal red lines represent cut-off values corresponding to log two-fold changes in expression. The upper and lower parts of the red lines indicate upregulation, and downregulation in the tumoral tissues in comparison with non-tumoral tissues, respectively.

3.4. Receiver operating characteristic (ROC) curve analysis

The prognostic power of the candidate lncRNAs was evaluated by calculating the area under the curve (AUC) of receiver operating characteristic (ROC) curve. The AUCs of *LINC0268* and *LOC25845* were 0.63 (sensitivity 71.11% and specificity 51.06%, $P = 0.02$) and 0.68 (sensitivity 80.43% and specificity 53.19%, $P = 0.002$), respectively in tumoral tissues (Fig. 5). Also, based on the AUC values, *LINC0268* and *LOC25845* (*PP7080*) had the best performance in differentiation of male from female patients (Fig. 5).

4. Discussion

The advent of high-throughput RNAseq witnessed a growing recognition for identifying thousands of unknown lncRNAs whose aberrant expression is closely associated with the initiation and development of cancers. Further findings demonstrated that many novel lncRNAs are dysregulated in GC, and closely related to tumorigenesis, metastasis, and prognosis or diagnosis. For instance, long intergenic non-protein coding RNA 941 (*LINC00941*) was found to be an independent predictor of tumor depth and distant metastasis in GC [21]. Also, long intergenic noncoding RNA 01296 was shown to mediate tumorigenesis through sponging miR-122 in GC [22]. It was also shown that lncRNA *SUMO1P3* which is a direct target of CCHC-type zinc finger nucleic acid binding protein (CNBP) is an independent predictor for invasion and drug resistance in GC [23]. LncRNA *SNHG3* is another regulatory RNA involved in the progression of GC by regulating mediator complex subunit 18 (*MED18*) gene [24]. In the present study, we evaluated for the first time the expression level of two novel lncRNAs including *LOC25845* (*PP7080*) and LINC02688 in 47 pairs of GC tumoral tissues and their adjacent non-tumoral counterparts. Although the two novel lncRNAs analyzed in the present study were significantly down-regulated in tumor tissues in comparison with their paired adjacent normal gastric tissues, no significant association has been found between their expression level and patient's gender, tumor stage, tumor grade, and lymph node metastasis, highlighting their potential application in diagnosing tumors even at early stages or low dedifferentiation levels in GC. Furthermore, ROC curves analysis was performed in order to evaluate the predictive ability of lncRNAs expression in GC. The LOC25845 (PP7080) and LINC02688 transcript levels had more than 70% specificity in this regard, indicating that these lncRNAs can serve as a potential biomarker in evaluating GC patients. Therefore, for suspicious samples at earlier stages of the disease, assessing the expression level of these lncRNAs could be useful to evaluating the malignancy of

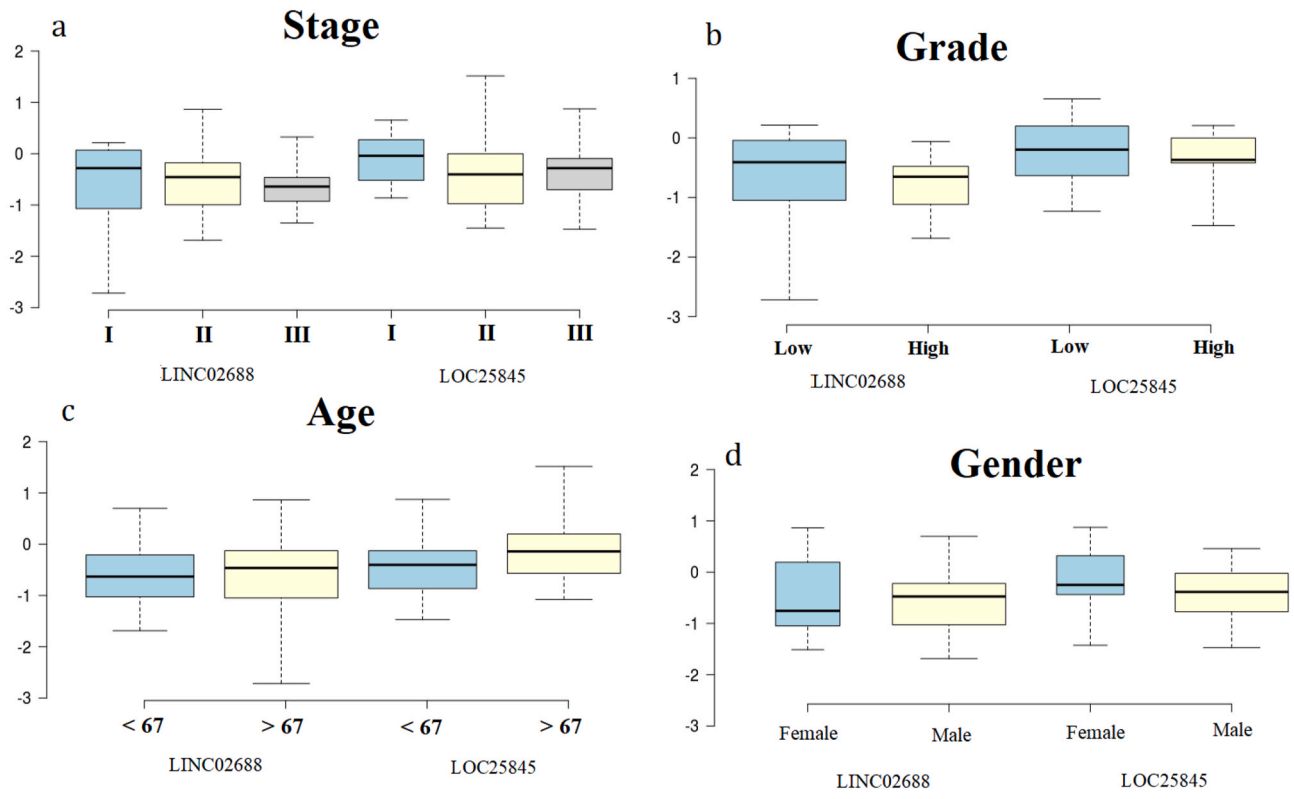

Fig. 3. Association between LINC02688 **and *LOC25845* (*PP7080*) expression and clinical characteristics of gastric cancer patients.** The expression level was represented by box plots using web (http://ualcan.path.uab.edu/index.html) for patients characteristics including (a) cancer stages, (b) tumor grade, (c) age, and (d) gender.

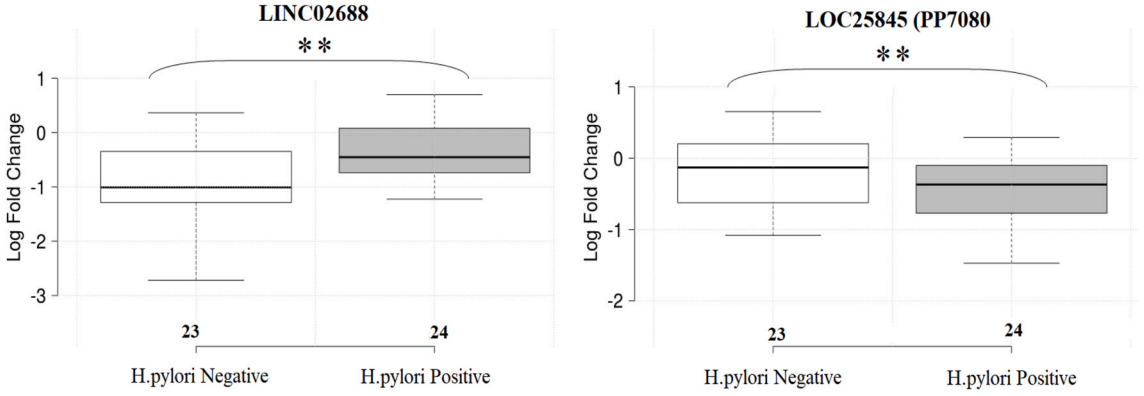

Fig. 4. Variations of lncRNAs according to *H. pylori* infection. LINC02688 and *LOC25845* (*PP7080*) mRNA expression in *H. pylori*-positive (N = 24) and *H. pylori* –negative (N = 23) gastric cancers
* indicates significant difference P < 0.01 and ** indicates significant difference P < 0.001.

the collected tissue. In line with our study, microarray data (GSE49355 and GSE62321) of human colon tumor indicated that *PP7080* was downregulated in liver metastasis of colon carcinoma [25]. Also, Huang et al. by analyzing TCGA database that included 460 patients with colon adenocarcinoma indicated that the expression of *PP7080* was dysregulated in colon adenocarcinoma [26].

5. Conclusion

The present study for the first time revealed that *LOC25845* (*PP7080*) and LINC02688 were downregulated in GC tissues, and their expressions were decreased from early to advanced stages of GC. Further studies with more clinical samples of different type of cancers, and different ethnic populations are necessary in order to investigate the exact roles of *LOC25845* (*PP7080*) and LINC02688 in development of cancers. These novel lncRNAs could be considered as a potential target for early diagnosis of GC in the future.

Ethics approval

This study was approved by the Ethics Committee of Babol University of Medical Sciences and all procedures were performed in compliance with Helsinki declaration.

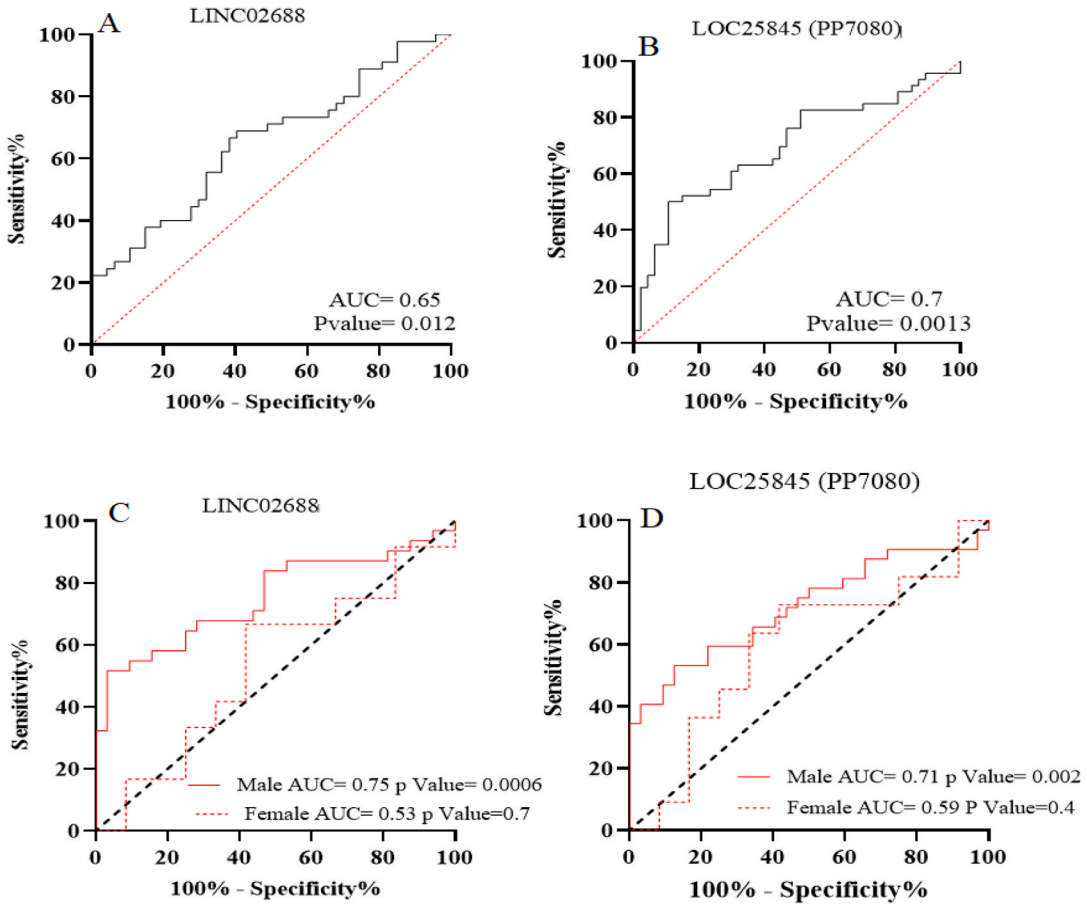

Fig. 5. The discriminatory ability of the candidate lncRNAs in gastric cancer patients. ROC curve analysis of the A) LINC02688 and B) *LOC25845* in tumoral tissues in comparison with their adjacent non-tumoral counterparts. ROC curves analysis of the (C) LINC02688 and (D) *LOC25845* (PP7080) in gender groups. AUC: area under the curve; ROC: receiver operating characteristic.

CRediT authorship contribution statement

Sadegh Fattahi: Methodology, Formal analysis, Investigation, Writing – original draft. **Novin Nikbakhsh:** Resources, Visualization. **Hassan Taheri:** Resources, Visualization. **Elham Ghadami:** Investigation, Resources. **Mohammad Ranaee:** Investigation, Visualization. **Haleh Akhavan-Niaki:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

Authors declare no conflict of interest.

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