Inconsistency in Expression Pattern of a Five-IncRNA Signature As a Potential Diagnostic Biomarker for Gastric Cancer Patients in Bioinformatics and in Vitro

Mahmoud Ghanei
Mashhad University of Medical Sciences

Arash Poursheikhani
Mashhad University of Medical Sciences

Azadeh Aarabi
Mashhad University of Medical Sciences

Negin Taghechian
Ferdowsi University of Mashhad

Mohammad Reza Abbaszadegan (  abbaszadeganmr@mums.ac.ir)
Mashhad University of Medical Sciences

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Abstract

**BACKGROUND:** Due to the diagnosis of gastric cancer in advance stages as well as its poor prognosis, finding biomarkers is essential.

**OBJECTIVE:** In this study, using the TCGA RNAseq data of gastric cancer patients, we evaluated the diagnostic value of IncRNAs which had differential expression.

**METHODS:** we evaluated P value, FDR, log fold change for whole transcripts. Next, by comparison of the RNAseq gene names with total known IncRNA names, we identified differential expressed IncRNAs. Following, we calculated specificity and sensitivity for IncRNAs came from previous step. For more confirmation, we predict target genes and performed GO and KEGG signalling pathway analysis. At the end, we examined the reliability and consistency of expression of this signature in three gastric cancer cell lines and one of them in twenty tumoric and tumor adjacent normal tissue samples using qRT-PCR.

**RESULTS:** Five IncRNAs had proper sensitivity and specificity and had target genes involved in cancer-related signaling pathways; however, they showed different expression pattern in TCGA data and in vitro.

**CONCLUSIONS:** The results of our study demonstrated that the five IncRNAs PART1, UCA1, DIRC3, HOTAIR, and HOXA11AS require more investigation to be confirmed as a diagnostic biomarker in gastric cancer.

1. Background

Gastric cancer (GC) is one of the most important leading causes of cancer-related death worldwide (1). According to the report of Global Cancer Observatory (GLOBOCAN) in 2018, gastric cancer is fifth in incidence and third in mortality among cancers (both sexes and all ages) across the world, but it is the second and the fifth in incidence and mortality in Iran, respectively (2). Despite advancements in treatment of gastric cancer patients based on surgical approaches and targeted drug chemotherapy, poor prognosis and late manifestation of symptoms act as obstacles on early diagnosis of these patients; therefore, identifying patients at advanced stages of the disease can make most patients untreated (3, 4). Early detection of patients, prediction of outcomes of treatments effectively, identification of new therapeutic targets, a better understanding of tumorigenesis and progression processes are crucial keys for improving the survival rate of GC patients. Therefore, the discovery and development of prognostic and diagnostic biomarkers are essential for the facilitation of early diagnosis, effective prediction of prognosis, resulting in better outcomes in GC patients.

Long noncoding RNAs (lncRNAs) are attributed as a class of noncoding RNAs (ncRNAs) of greater than 200 nucleotides in length, which have a specific expression in various tissues and diseases such as cancers (5–9). LncRNAs are involved in different biological processes include of cell development and differentiation, cell cycle arrest, apoptosis, autophagy, cell senescence, chromosome remodeling, X chromosome inactivation, alternative splicing, RNA decay, embryonic stem cells, cancer cell metastasis, drug resistance, etc. (7, 10–14). According to some studies, lncRNAs have a more important function than protein-coding genes in translational and clinical oncology (11, 15). On the other hand, a group of these noncoding transcripts act as a tumor suppressor or oncogene and be dysregulated in various types of cancers (16, 17). Other studies have shown that lncRNAs are correlated with cancer recurrence and poor prognosis although has not been fully elucidated yet in gastric cancer (18, 19). Since IncRNAs participate in various processes in cancers and also easy detection of them, they can be chosen as valuable biomarkers in the diagnosis and prognosis of cancers (20, 21). Several studies have explored the expression of some lncRNAs in GC and introduced several lncRNAs as biomarkers; however, a fewer number have proposed a lncRNA signature, and certainly, there are further lncRNAs that have to investigated and can be used as practical biomarkers after passing the laboratory steps (22, 23).

In this study, we attempted to perform a comprehensive study using transcriptomic data analysis of patients with gastric cancer that are freely available in The Cancer Genome Atlas database (TCGA) ([https://cancergenome.nih.gov/](https://cancergenome.nih.gov/)) and to provide a new and effective signature for the diagnosis of gastric cancer patients. With conducting data mining in RNAseq data of GC patients and thereafter determination of differentially expressed genes and calculating AUC of ROC curve, we identified a five-lncRNA signature ([HOTAIR, PART1, DIRC3, HOXA11AS, UCA1]) as a novel potential biomarker for diagnosis of GC patients. We also examined the expression of this signature in three gastric cancer cell lines and one of them in twenty tumoric and tumor adjacent normal tissue samples.

2. Materials And Methods

2.1. RNAseq data mining and finding of differentially expressed lncRNAs

The RNAseq data and clinical characteristics were downloaded from TCGA database to the address [https://cancergenome.nih.gov/](https://cancergenome.nih.gov/). After the data was received, the primary trimming was done and redundant columns and rows were deleted. The RNAseq dataset (STAD) contained 450 transcriptomes, of which 415 were tumoric and 35 were tumor-adjacent normal tissue samples.

The RNAseq data processing was continued using R program. In first, we performed secondary data trimming by virtue determination of the third data quantile and omission of the transcripts that had read counts fewer than 25 in the three quarters of the data. Applying this command resulted in the removal of 4639 transcripts from the original data set with 20531 transcripts. Then, by calling the edger library, logFC, logCPM, P-value and FDR were calculated for each transcripts. In the next step, by applying appropriate filtration (P-value < 0.05, FDR < 0.05, 1 < logFC < -1) on these components, the list of genes with differential expression was obtained. This list included all types of transcripts with differential expression, from which lncRNAs were to be identified. For this purpose, a list of all identified IncRNAs was obtained from the HUGO Gene Nomenclature Committee (HGNC) website and aligned with the list of differential expression genes to identify the IncRNAs with differential expression. The result was 14 IncRNAs, which were nominated for the next step of the analysis.

2.2. ROC curve
This step was done by SPSS software used to determine the sensitivity and specificity of the lncRNAs from previous step, which ultimately led to the selection of 5 lncRNAs as a potential diagnostic biomarker.

### 2.3. Target genes prediction and functional enrichment analysis

The target genes of the five lncRNA signature were predicted by usage of two online tools include of LncRRIsearch and lncRNA2Target. The sum of the identified target genes for these 5 lncRNAs were 238 by the LncRRIsearch after the removal of duplicates and 115 by the lncRNA2Target. Furthermore, the functional analysis of the GO annotation, DO and KEGG signaling pathways were performed. P-value less than 0.01 was considered statistically significant.

### 2.4. Protein–protein interaction network

To predict the relationships between the lncRNAs target genes products in network, the ID number of the lncRNAs target genes and STRING online tool were used. The highest confidence score (a combined score > 0.900) was considered significant. Globe-shaped proteins have been characterized based on their association with other proteins. The target genes with multiple connections to other target genes appear to play important roles in the protein-protein interaction network.

### 2.5. Statistical analysis

The data were presented as Mean ± SD. ROC curve analysis was conducted by IBM SPSS statistics v25. A p-value < 0.05 was considered statistically significant.

### 2.6. Samples and cell lines

Normal tissue samples as a control group were obtained from 20 non-cancerous individuals that had been scanned for gastroesophageal diseases via upper endoscopy in Imam Reza hospital, Mashhad, Iran. Healthy control tissue samples were confirmed by pathological examination. The fresh specimens immediately were transferred to RNA later solution and then stored at -70°C before RNA extraction. Written informed consent was obtained from all participants in this study before tissue sampling.

Three gastric cancer cell lines, MKN-45, AGS, and EPG were obtained from the National Cell Bank of Iran, Tehran. For all used cell lines, STR profiling was performed for the determination of cell lines identity; the report was compared with standard cell lines by the usage of matching criteria based on an algorithm that compares the number of shared alleles between two cell lines, expressed as a percentage. Cell lines with ≥ 80% match are considered to be related; derived from common ancestry. Cell lines with between a 55–80% match require further analysis for authentication of relatedness that all three cell lines had an acceptable resemblance.

### 2.7. RNA extraction and real-time PCR

Total RNA was extracted with RNX-Plus solution (SinaClon, Cat. No. RN7713C, Iran) according to manufacturer’s protocol. Next, the purity of the extracted RNAs was evaluated by Spectrophotometer, Biwave II (Biochrom, UK). We also used from Agarose gel electrophoresis to ensure RNA integrity. Next, for eliminating of DNA pollution, we treated the extracted RNAs by DNase1 (Thermo Fisher Scientific, Cat. No. EN0521). Reverse transcription reactions were performed in two steps using the NG dART RT kit (EURx, Cat. No. E0801-03) according to the manufacturer’s protocol. QRT-PCR analysis of the expression of the five IncRNAs was performed on a Light Cycler 96 (Roche Life Science, Roche Diagnostics GmbH, Germany) using the suitable primers (Table 1) and RealQ Plus (AMPLIQON, Germany) according to manufacturer’s protocol. All reactions were conducted in duplicate form and the expression of the five IncRNAs was normalized to the expression level of the housekeeping gene GAPDH.

| Target   | Forward Primer | Reverse Primer       | Product Size |
|----------|----------------|----------------------|--------------|
| DIRC3    | CTCATCTGTCCGACGAAGCA | CCCTACTGTCTTTGCAGAGA | 73           |
| HOTAIR   | GGAAGCGAAGGGGGTTGTGA | GCCTAGGGGCTTTCACTTT | 175          |
| HOXA11AS | TTTAGAGGGCGTGACATCCG | CTCAGTCGGGGTTCTCCACCG | 89           |
| PART1    | TCCAGAGCGAGCGTTACACT | TGCCCTTTCTCCCTCAGACA | 181          |
| UCA1     | GCCAGCCTAGCTTATCCA | CCCTGTTGCTAAGCCGATGA | 151          |

### 3. Results

#### 3.1. Differentially Expressed Genes

The data demonstrated that 3722 genes (1840 up-regulated and 1882 down-regulated) were differentially expressed in STAD. Moreover, 14 IncRNAs (139 up-regulated and 49 down-regulated) were identified that were most deferentially expressed in patients. The data are presented in Tables 2 and 3.
Table 2
Top 20 up and down-regulated Genes.

| Gene   | logFC  | logCPM    | PValue       | FDR       |
|--------|--------|-----------|--------------|-----------|
| ENPP7  | -7.24658 | 2.654383 | 4.6E-114     | 7.3E-110  |
| SLC2A7 | -6.4681  | -2.81146 | 1.04E-89     | 2.37E-86  |
| S100G  | -6.41206 | 0.514534  | 4.73E-56     | 2.21E-53  |
| FLG    | -6.36187 | 3.289874  | 5.1E-111     | 4.1E-107  |
| GIP    | -6.23295 | 1.270187  | 1.89E-38     | 3.8E-36   |
| CLDN22 | -6.1317  | -3.15007 | 2.71E-19     | 5.83E-18  |
| KRT1   | -6.10628 | 1.746563  | 6.56E-80     | 7.45E-77  |
| MEP1B  | -6.04667 | 3.490235  | 2.25E-65     | 1.79E-62  |
| ZG16   | -6.00036 | 2.506113  | 1.57E-60     | 9.99E-58  |
| AQP10  | -5.97356 | 2.003138  | 7.84E-74     | 7.33E-71  |
| SLC2BA1| -5.88603 | 0.267127  | 3.46E-85     | 6.88E-82  |
| ATP4B  | -5.85136 | 5.777434  | 5.54E-37     | 9.89E-35  |
| G6PC   | -5.65078 | 0.41213   | 5.26E-43     | 1.39E-40  |
| ATP4A  | -5.57913 | 5.920505  | 1.5E-27      | 9.08E-26  |
| CRNN   | -5.52021 | 8.116779  | 4.7E-13      | 4.38E-12  |
| CRCT1  | -5.49336 | 4.642107  | 9.88E-21     | 2.54E-19  |
| CA7    | -5.47359 | -0.32813  | 2.15E-98     | 6.83E-95  |
| ACER1  | -5.3863  | -0.21548  | 2.1E-32      | 2.34E-30  |
| KPRP   | -5.35025 | 1.760696  | 1.46E-18     | 2.87E-17  |
| GYS2   | -5.29783 | 0.208704  | 1.46E-43     | 3.94E-41  |

| Gene   | logFC  | logCPM    | PValue       | FDR       |
|--------|--------|-----------|--------------|-----------|
| ENPP7  | -7.24658 | 2.654383 | 4.6E-114     | 7.3E-110  |
| SLC2A7 | -6.4681  | -2.81146 | 1.04E-89     | 2.37E-86  |
| S100G  | -6.41206 | 0.514534  | 4.73E-56     | 2.21E-53  |
| FLG    | -6.36187 | 3.289874  | 5.1E-111     | 4.1E-107  |
| GIP    | -6.23295 | 1.270187  | 1.89E-38     | 3.8E-36   |
| CLDN22 | -6.1317  | -3.15007 | 2.71E-19     | 5.83E-18  |
| KRT1   | -6.10628 | 1.746563  | 6.56E-80     | 7.45E-77  |
| MEP1B  | -6.04667 | 3.490235  | 2.25E-65     | 1.79E-62  |
| ZG16   | -6.00036 | 2.506113  | 1.57E-60     | 9.99E-58  |
| AQP10  | -5.97356 | 2.003138  | 7.84E-74     | 7.33E-71  |
| SLC2BA1| -5.88603 | 0.267127  | 3.46E-85     | 6.88E-82  |
| ATP4B  | -5.85136 | 5.777434  | 5.54E-37     | 9.89E-35  |
| G6PC   | -5.65078 | 0.41213   | 5.26E-43     | 1.39E-40  |
| ATP4A  | -5.57913 | 5.920505  | 1.5E-27      | 9.08E-26  |
| CRNN   | -5.52021 | 8.116779  | 4.7E-13      | 4.38E-12  |
| CRCT1  | -5.49336 | 4.642107  | 9.88E-21     | 2.54E-19  |
| CA7    | -5.47359 | -0.32813  | 2.15E-98     | 6.83E-95  |
| ACER1  | -5.3863  | -0.21548  | 2.1E-32      | 2.34E-30  |
Table 3
The Differentially expressed lncRNAs.

| LncRNAs | logFC   | logCPM  | PValue  | FDR     |
|---------|---------|---------|---------|---------|
| BCAR4   | 5.410524| -0.55488| 5.09E-06| 1.76E-05|
| CDKN2BAS| -2.45409| 0.65801 | 2.32E-19| 5.05E-18|
| DIRC3   | -2.05778| -1.47014| 2.71E-18| 5.12E-17|
| DLX6AS  | 3.012512| -0.69193| 1.99E-06| 7.39E-06|
| DSCR4   | 6.162059| -1.17853| 2.5E-06  | 9.13E-06|
| FAM27B  | 2.800521| -2.89405| 0.029757 | 0.048477|
| CDH19   | -2.7026 | 1.429299| 6.03E-15 | 7.21E-14|
| HOTAIR  | 5.983826| 1.520031| 1.99E-22 | 6.36E-21|
| HOXA11AS| 3.541999| 1.706383| 1.99E-10 | 1.26E-09|
| HULC    | 6.653741| -0.35465| 2.83E-07 | 1.19E-06|
| IGF2AS  | 3.058415| -1.21374| 5.47E-06 | 1.88E-05|
| PART1   | -2.41557| 2.244247| 9.07E-17 | 1.38E-15|
| TTTY14  | -2.1316 | -1.34436| 2.58E-07 | 1.09E-06|
| UCA1    | 3.323226| 4.911589| 3.04E-08 | 1.47E-07|

3.2. ROC curve indicates good performance for the suggested diagnostic signature

For evaluation of the diagnostic power of the fourteen-lncRNA achieved from the RNAseq data analysis by R program, we calculated the AUC of the ROC curve. The higher AUC represents a better performance and an AUC more than 0.7 is acceptable and is considered as good efficiency. P-value less than 0.05 was considered as significant level. According to AUC and p-value, eventually the five lncRNAs (PART1, UCA1, D IRC3, HOTAIR and HOXA11AS) were selected as the candidate diagnostic signature. These selected lncRNAs had good potential sensitivity and specificity for the diagnosis of gastric cancer patients (Fig. 1). The expression pattern of these lncRNAs have been shown in Fig. 2.

3.3. The target gene prediction

The target genes of the diagnostic lncRNAs signature were predicted by LncRRisearch (24) and LncRNA2Target (25) online tools. The number of the target genes identified for lncRNAs PART1, UCA1, D IRC3, HOTAIR and HOXA11AS by the LncRRisearch database were 100, 100, 33, 100 and 100 respectively (this database represents the top 100 genes identified for the lowest sum of energy) and by the LncRNA2Target database were 0, 55, 0, 68 and 10, respectively. In total, 433 target genes from the LncRRisearch database and 133 target genes from the LncRNA2Target database were identified for this signature. After deleting duplicates and merging two lists, a list with 353 target genes was generated.

3.4. GO annotation, Disease Ontology and KEGG signaling pathways

For more investigation about the potential biological function and the mechanism of the suggested diagnostic signature, we took advantage of GO annotation, Diseases ontology and KEGG signaling pathway analysis for 353 target genes from the previous step. The GO annotation, Diseases ontology, and KEGG signaling pathway analysis were conducted and visualized by R software (ggplot2 package). P-value less than 0.01 was considered as cut off point. From the examination of these results, we found many functions that are related to EMT process, RNA silencing mechanisms, chromatin remodeling, molecular binding, gene transcription and cell proliferation. Therefore, it can be deduced that this suggested diagnostic signature may be related to gene expression and molecular and cellular functions. The results of KEGG signaling pathway analysis and disease ontology (DO) indicate activation of cancer-related pathways such as gastric cancer, prostate cancer, small cell lung cancer, pancreatic cancer, breast cancer, bladder cancer, colorectal cancer, melanoma, glioma, connective tissue cancer, bone cancer, renal carcinoma, etc. (Fig. 3).

3.5. Functional predictions and PPI network construction

To predict and visualize the protein-protein interactions among the target genes of the five-lncRNA signature, we used from STRING (27) software. For this purpose, to obtain protein-protein interaction (PPI) data, the target genes were first introduced in the STRING website. Next, the cut off greater than 0.900 for a combined score of PPIs was considered as appropriate criteria for selection and to construct PPI networks. In this network, unconnected nodes are not displayed. Of the 353 target genes identified, 252 genes were involved in constructing nodes of this network (Fig. 4). The genes with connections/interactions
more than 7 (degree > 7) were filtered. STRING data demonstrated that these lncRNAs contributed to several canonical signaling pathways related proteins such as cyclins and related CDKs, TWIST1, CDH1, MMPs, TP53, RB1, WNT7A, GSK3B, ATM, PTEN, BCR, ERBB2, VEGFR, SMAD, TGFβ2, MAPK1, FGF1, AKT1 and HIF1A.

3.6. Functional analysis via co-expressed target genes

At the beginning of this study and before selecting the appropriate lncRNAs as biomarkers, a list of genes with differential expression of TCGA data was obtained and then a list of target genes was predicted by online tools for this signature. In order to probe the functional value of the signature in the gastric cancer (stomach adenocarcinoma), we searched the predicted target genes that were co-expressed with our suggested signature and we found a list of 212 genes with this characteristic. In this new list, there are many master genes such as CDKs, TWIST1, CDH1, MMPs, TP53, RB1, WNT7A, GSK3B, ATM, PTEN, BCR, ERBB2, VEGFR, SMAD, TGFβ2, MAPK1, FGF1, AKT1 and HIF1A that involved in cancer-related pathways and therefore, dysregulated in many cancers.

3.7. Real-time PCR verification

We investigated the expression level of the suggested five-lncRNA signature in the three gastric cancer cell lines and 20 healthy tissue samples. We couldn’t confirm the similar expression pattern for these lncRNAs in the cell lines and TCGA RNAseq data of gastric cancer patients using real-time PCR. We used from paired t-test as a proper statistical analysis for the compare expression level of the five-lncRNA signature in three gastric cancer cell lines with 20 healthy tissue samples. We found that PART1 and DIRC3 had no detectable expression in none of the three cell lines and normal tissue samples. In the other hand, UCA1 was expressed in whole samples. Two lncRNAs, HOTAIR and HOXA11AS, were similarly expressed only in the EPG cell line and normal tissue samples. We also examined the expression level of UCA1 in twenty tumor and tumor-adjacent normal tissue samples with confirmed expression level greater than 2 (logFC > 2) for TWIST1. This study showed that there is no significant correlation between expression level of UCA1 and TWIST1 in tissue samples which is in contrast to our expectations for UCA1 expression.

4. Discussion And Conclusions

In our investigation, we used the TCGA RNAseq data to introduce a lncRNA signature which can be utilized as diagnostic biomarker. Our bioinformatics data was illustrated that lncRNAs such as PART1, UCA1, DIRC3, HOTAIR and HOXA11AS have more differential expression in the tumor tissues vs to the normal counterpart margins. Moreover, ROC curve analysis showed that these lncRNAs have significant sensitivity and specificity (diagnostic) values.

Furthermore, we investigated the role of the lncRNAs through the enrichments and real-time PCR.

It has been demonstrated that down-regulation of lncRNA PART1 blocked cell proliferation and promotes apoptosis in bladder cancer (28). Furthermore, exosome-mediated transfer of lncRNA PART1 can induced chemotheraphy resistance in ESCC by competing endogenous miRNA (29). PART1 promotes tumorigenesis by miR-143 in colorectal cancer (30). Leung GK et al. declared that a long non-coding RNA signature can predict prognosis of the glioblastoma multiform patients (31).

Overexpression of lncRNA UCA1 has been suggested that increases cell growth and chemo-resistance by inhibiting miR-513a-5p in retinoblastoma cells (32). In addition, UCA1 overexpression is associated with poor survival rate in patients with digestive system malignancies (33). Ma C et al. demonstrated that high expression of serum UCA1 can be consider as a potential biomarker for clinical diagnosis of gastric cancer (34). It has been suggested that UCA1 confers endocino-therapy resistance through EZH2/p21 axis and the PI3K/AKT signaling pathway in breast cancer (35). UCA1 can promote carcinogenesis by Wnt signaling pathway in papillary thyroid carcinoma (36). Downregulation of UCA1 has been showed that enhances the radio-sensitivity and inhibits cell migration by suppression of EMT in colorectal cancer cells (37). Moreover, Yuan D et al. found that the Knockdown of UCA1 increases cisplatin sensitivity in tongue squamous cell carcinoma cells (38).

Previous investigations have reported that lncRNA DIRC3 expression had impact on carcinogenesis. This lncRNA has been regulated by MITF-SOX10 in melanoma tumor (39). Shao Y et al. showed that DIRC3 and near NABP1 genetic polymorphisms are associated with poor prognosis in laryngeal squamous cell carcinoma patient (40).

LncRNA HOTAIR has been demonstrated that can be serve as a prognostic and diagnostic biomarker in cancers. It promotes tumor progression via sponging miR-217-6PC5 axis in gastric cancer (41). HOTAIR mediates the switching of histone H3 lysine 27 acetylation to methylation, to promote EMT process in gastric cancer (42). Moreover, it has been reported that HOTAIR rs17720428 SNP is correlated with the risk and prognosis of gastric cancer in the Chinese Han population. HOTAIR modulates KLF12 to regulate gastric cancer progression via PI3K/ATK signaling pathway by sponging miR-618 (44). It has been reported that lncRNAs such as H19, HOTAIR, UCA1 and PVT1 could be serve as potential diagnostic and prognostic biomarkers in patients with gastric cancer (45). Chen LC et al. demonstrated that high HOTAIR expression promote proliferation and metastasis in gastric cancer via miR-126/CXCR4 axis (46).

Chen P et al. exhibited that lncRNA HOXA11-AS acted as a ceRNA to promote cisplatin resistance of human LUAD cells via the miR-454-3p/Stat3 axis(47). Li W et al. showed which increased expression of HOXA11-AS is a risk factor for poor clinical outcomes in numerous tumors and may act as a novel biomarker for poor prognosis and metastasis in cancers(49). Hu XF et al. demonstrated the oncogenic role of HOXA11AS in breast cancer, providing novel clues for the future clinical diagnosis and treatment of early stage breast cancer patients(50). Hu CP et al. showed that up-regulation of lncRNA HOXA11-AS predicted a poor prognosis and lncRNA HOXA11-AS promoted cell epithelial-mesenchymal transition (EMT) by inhibiting miR-200b expression in NSCLC(51). Sun M et al. declared that HOXA11-AS not only could promote GC cells migration and invasion in vitro, but also promotes GC cells metastasis in vivo, at least in part, by regulating β-catenin and KLF2(52). LncRNA HOXA11-AS was showed that able to distinguish CRC tissue from non-cancerous tissue, and CRC tissue with lymph node metastasis from CRC without lymph node metastasis (53).
To clarify the molecular function of this suggested five-lncRNA signature, we predicted the target genes and the corresponding pathways using GO annotation, disease ontology (DO) and KEGG signaling pathway analysis. The results showed the participation of this signature in essential biological processes such as cell division, transcription regulation, change in expression of growth factors and enriched KEGG pathways including PI3K-Akt signaling pathways, p53 signaling pathways and pluripotent stem cell signaling pathways. In order to further investigation of the proposed IncRNA signature, the protein-protein interactions of the target genes were predicted.

The current study indicated inconsistency in the results of real-time PCR in the cell lines and TCGA RNAseq data. LncRNAs PART1 and DIRC3 had no detectable expression in any cell lines. These two lncRNAs were down-regulated in tumoric TCGA RNAseq samples vs normal margin; lack of expression of them in the cell lines is probably due to very low or no expression of them which emanated from cumulative mutations that cell lines bear in passing time, presence of different subclones in culture and so, their complex and unclear interactions, different circumstance of cells in culture vs body, and other causes that are still unclear. The function of lncRNAs PART1 and DIRC3 is unclear in gastric cancer; therefore, future studies should focus on these lncRNAs and clarify their function in gastric cancer. In the other hand, IncRNA UCA1 was expressed in all three cell lines; however, its expression was differnt from TCGA data. Of the probable reasons for its expression in all three cell lines, could be its more important role in gastric carcinogenesis, more stability and more expression; however, deviation in the expression pattern with TCGA data is among the cases that are very difficult to comment on. Nevertheless, HOTAIr and HOXA11AS were expressed just in EPG cell line and had no detectable expression in other two cell lines. This can be explained with role of these lncRNAs in EPG cell line but in no others, because of different genetic context of three cell lines. Overall, the exact interpretation of these discrepancies between the results of bioinformatics and laboratory studies requires further study, and here we have only stated a series of hypotheses.

The activation of PI3K-Akt pathway is reported in many malignancies. The inhibition of the PI3K-Akt pathway can induce apoptosis and decrease cell division via negative regulation of Plk1 both in vitro and in vivo (56, 57). One of the most important gene involved in this pathway is TWIST1. This gene normally is overexpressed in many cancers and promote metastasis using activation of EMT process. TWIST1 was predicted as an important target gene for UCA1 and also was existed in differential expression genes list resulted from TCGA data analysis. In order to examination of correlation between TWIST1 and UCA1, the lncRNA that had been expressed in all three cell lines, we performed real-time PCR for UCA1 in twenty tumoric and tumor-adjacent normal tissue samples with confirmed over-expression of TWIST1 (logFC > 2). This examination showed that UCA1 was upregulated and down regulated in 8 and 3 samples, respectively. In other 9 tissue samples, UCA1 did not show altered expression. Altogether, evaluation of UCA1 using paired t-test did not show a significant statistical relationship in twenty tissue samples; This was another important contradiction that we encountered during this study. Discrimination in sampling (selection of samples with high TWIST1 expression) seems to be one of the reasons for this unexpected result; However, based on bioinformatics analysis, our sampling was correct and the results should have been different.

In total, like any other studies, our study had its limitations. First, the data used for this study to introduce a diagnostic signature were extracted from a single database (TCGA); the use of more databases such as GEO certainly makes the results more reliable. Second, due to the use of cell lines in this study for investigation of suggested signature expression and the differences between these cells in terms of growth conditions and cumulative mutations, as well as the low number of cell lines used, it is necessary to examine the expression of these 5 lncRNAs in and more tissue samples.

In conclusively, we could not present a five-lncRNA signature with diagnostic potential for gastric cancer because of some identified contradictions in bioinformatics and laboratory study. More investigations should be performed for ultimate validation or rejection of this signature; investigations that cover our faults using large sample size, examination of these result in tissue samples and also further exploration in biological and molecular mechanism of suggested five-lncRNA signature in gastric cancer progression.

**Declarations**

- Ethics approval and consent to participate: The ethics committee of Mashhad University of Medical Sciences approved the study and the committee's reference number is 1398.522
- Consent for publication: Not applicable
- Availability of data and materials: The datasets generated and analysed during the current study are available in the FireBrowse repository via link: [http://gdac.broadinstitute.org/runs/standard_2016_01_28/data/STAD/20160128/gdac.broadinstitute.org_STAD.Merge_rnaSeq_illuminaHiSeq_rnaSeq].
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- Authors' contributions:

**CONCEPTION:** Ghanei M., Poursheikhani A., Abbaszadegan MR.

**INTERPRETATION OR ANALYSIS OF DATA:** Ghanei M., Poursheikhani A., Abbaszadegan MR., Aarabi A., Taghehchian N.

**PREPARATION OF THE MANUSCRIPT:** Ghanei M., Poursheikhani A.

**REVISION FOR IMPORTANT INTELLECTUAL CONTENT:** Abbaszadegan MR.

**SUPERVISION:** Abbaszadegan MR.

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Figures

Figure 1
ROC curve analysis of the IncRNAs. A. HOTAIR, B. HOXA11AS, C. UCA1, D. D IRC3, E. PART1.

Figure 2
The expression pattern of suggested IncRNA signature.
Figure 3
GO, DO and KEGG pathway enrichment analysis of the differentially expressed genes (Top 20 GO enrichment are presented). A. GO, B. DO, C. KEGG pathway

Figure 4
Protein-protein interaction (PPI) network of the differentially expressed genes of STAD (score > 0.9000).