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Research article

Down-regulation of *LINC-ROR*, *HOXA-AS2* and *MEG3* in gastric cancer

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been identified as modulators of gastric carcinogenesis. Evaluation of expression amounts of these transcripts is a primary but essential step for recognition of the role of lncRNAs in the carcinogenesis. Therefore, we compared expressions of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples and nearby non-cancerous samples. Expression levels of *LINC-ROR*, *HOXA-AS2* and *MEG3* lncRNAs have been lower in gastric cancer samples compared with nearby non-cancerous samples (Expression ratios = 0.26, 0.37 and 0.36; P values = 0.021, 0.015 and 0.032, respectively). However, expression levels of *HOTTIP* were not significantly different between gastric cancer tissues and nearby tissues (P value = 0.43). *HOTTIP* expression was associated with tumor size (P value = 0.04). In addition, *MEG3* expression was associated with site of primary tumor (P = 0.0003). Expressions of *LINC-ROR* and *HOXA-AS2* were not associated with any clinical or pathological parameter. ROC curve analysis revealed that *HOXA-AS2* and *LINC-ROR* could significantly differentiate between gastric cancer samples and nearby non-cancerous tissues (AUC values = 0.68 and 0.64; P values = 0.01 and 0.04, respectively). Taken together, the current investigation provides clues for contribution of *LINC-ROR*, *HOXA-AS2* and *MEG3* lncRNAs in gastric carcinogenesis and warrants further mechanistical assays.

1. Introduction

Gastric cancer is regarded as an important neoplasm throughout the world being responsible for 26,560 new cases in 2021 and approximately 11,180 demises in the United States [1]. The pathoetiology of this kind of cancer signifies a typical model of gene-environment interactions [2]. Chronic infection with *Helicobacter pylori* (*H. pylori*) is regarded as the main basis of noncardia tumors, with nearly all cases resulting from this kind of infection [3]. Consumption of alcohol, tobacco smoking, and salt-preserved food are other risk factors for gastric cancer [4].

Genetic factors participate in gastric tumorigenesis through changing expression patterns of genes and the resultant malignant transformation [5]. The most prevalent genetic aberrations in this type of cancer are activation of β-catenin and K-ras oncogenes, amplification of the c-erbB2 and c-met genes, mutations in p53 and E-cadherin as well as microsatellite instability [2]. Meanwhile, epigenetic changes such as hypermethylation of promoter CpG islands, particularly in hMLH1 and p16 genes have been reported in gastric cancer [2].

This type of cancer has also been associated with abnormal expression of several long non-coding RNAs (lncRNAs) [6]. LncRNAs are one of the principal regulatory mechanisms in the human genome. They have sizes more than 200 nt and share several features with mRNA coding genes, yet they normally do not have open reading frames [7]. These transcripts have been shown to influence genome stability, cell cycle progression, apoptotic pathways and angiogenic processes, thus affecting gastric carcinogenesis from different points [6]. Recent studies have identified

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several cancer-related lncRNAs in bio-fluids of cancer patients proving these transcripts as particularly valuable tools for cancer diagnostic methods [8]. Moreover, detection of lncRNAs has been used as a strategy for prediction of prognosis of patients with different types of cancers [8]. Most notably, lncRNAs have been found in cancer-derived exosomes. The amount of these transcripts in the circulatory exosomes reflects their expression in the original tissues and can be used as diagnostic and prognostic tools in gastric cancer [9]. These circulatory particles can also promote metastasis of gastric cancer [9].

Due to inter-population heterogeneity in gastric cancer risk factors and course, expression analysis of lncRNAs in each population is a prerequisite for design of diagnostic panels for each population. *HOXA distal transcript antisense RNA* (*HOTTIP*) is an lncRNA which controls the activity of several 5′ *HOXA* genes encoding critical regulators of development [10]. Expression of this lncRNA has been shown to be elevated in gastric cancer samples in a cohort of Chinese patients [11]. LincRNA-Regulator of Reprogramming (LINC-ROR) is another lncRNA whose abnormal expression has been associated with cell proliferation, invasiveness, and cancer progression [12]. Moreover, this lncRNA participates in DNA damage response [13]. *HOXA cluster antisense RNA 2* (*HOXA-AS2*) is an oncogenic lncRNA that promotes malignant features of glioma through modulating RND3 [14]. Finally, *maternally expressed 3* (*MEG3*) is an lncRNA known to affect several aspects of carcinogenesis ranging from apoptosis and proliferation to invasiveness and epithelial-mesenchymal transition [15]. In the current investigation, we compared expression levels *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs between gastric cancer samples and nearby non-cancerous samples.

2. Materials and methods

2.1. Patient samples

The study included 30 patients. Thirty pairs of gastric cancer tissues and nearby non-cancerous tissues were purchased from tumor bank of National Cancer Institute, Imam Khomeini Hospital, Tehran, Iran. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1398.218).

2.2. Expression analyses

Total RNA was isolated from gastric tissue specimens using TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration and purity of the extracted RNA was assessed by photospectrometer. The absorbance of RNA samples was measured at 260 and 280 nm. After treatment with DNase I, RNA samples were subjected to cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). *B2M* was selected as the reference gene. Each run consisted of a negative control sample (no template control). All experiments were run in duplicate with similar amounts of the template from each sample. LncRNA quantification was performed using SYBR-Green. The sequences of primers are shown in Table 1. Primers were similar to a previous study [16].

Table 1. Primers used for expression assays.

Gene	Primer sequence	
B2M	Forward	5′-AGATGAGTATGCCTGCCGTG-3′
	Reverse	5′-CGGCATCTTCAAACCTCCA-3′
LINC-ROR	Forward	5′-TATAATGAGATACCACCTTA-3′
	Reverse	5′-AGGAACTGTCATACCGTTTC-3′
MEG3	Forward	5′-TGGCATAGAGGAGGTGAT-3′
	Reverse	5′-GGAGTGCTGTTGGAGAATA-3′
HOTTIP	Forward	5′-AGCTCTTTTCCCCGACAGTG-3′
	Reverse	5′-CCTTCACCAAGCTCCCTCTG-3′
HOXA-AS2	Forward	5′-GGCTTGAGATACTTGACCTTGC-3′
	Reverse	5′-TATGTCAGCCGTCAGAATCCAA-3′

2.3. Statistical methods

Relative expressions of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples versus nearby tissues were measured using the Relative Expression Software Tool-RG-version 3 (QIAGEN, Qiagen Germany Bloomberg, Korea). The mathematical model in this tool is based on the PCR efficiencies and the mean crossing point deviation between sample and control group. Then, the expression ratios are examined for significances by a randomisation test. The statistical significance was appraised using the Student paired t test. The association between clinical/pathological parameters and relative expressions of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* was judged using the $\chi 2$ test. The correlation between relative expressions of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* was measured using the regression model. Diagnostic power of lncRNAs in differentiating between cancerous and non-cancerous tissues was appraised by plotting the receiver operating characteristic (ROC) curves.

3. Results

3.1. Demographic data of patients

Mean age (± standard deviation) of patients recruited for this study was 42.53 (±10.1). Other clinical data of these patients are demonstrated in Table 2.

3.2. Expression assays

Expression levels of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples and nearby non-cancerous samples are depicted in Figure 1.

Expression levels of *LINC-ROR*, *HOXA-AS2* and *MEG3* lncRNAs have been lower in gastric cancer samples compared with nearby non-cancerous samples (Expression ratios = 0.26, 0.37 and 0.36; P values = 0.021, 0.015 and 0.032, respectively). However, expression levels of *HOTTIP* were not significantly different between gastric cancer tissues

Table 2. General data of recruited patients.

Parameters	Groups	Values
Gender	Male	78.6%
	Female	21.4%
Site of primary	Cardia	41.4%
	Antrum	31%
	Body	27.6%
Histology grade	2	37.5%
	3	58.3%
	4	4.2%
Lymphatic invasion	Yes	82.8%
	No	17.2%
Vascular invasion	Yes	82.8%
	No	17.2%
Peritoneal invasion	Yes	62.1%
	NO	37.9%
TNM stage	I	3.4%
	II	31%
	III	44.8%
	IV	20.8%
Histological form	Intestinal	46.7%
	Diffuse	53.3%
H. pylori Infection	Positive	50%
	Negative	50%
Smoking	Non-Smoker	50%
	Smoker	13.6%
	Ex-Smoker	36.4%

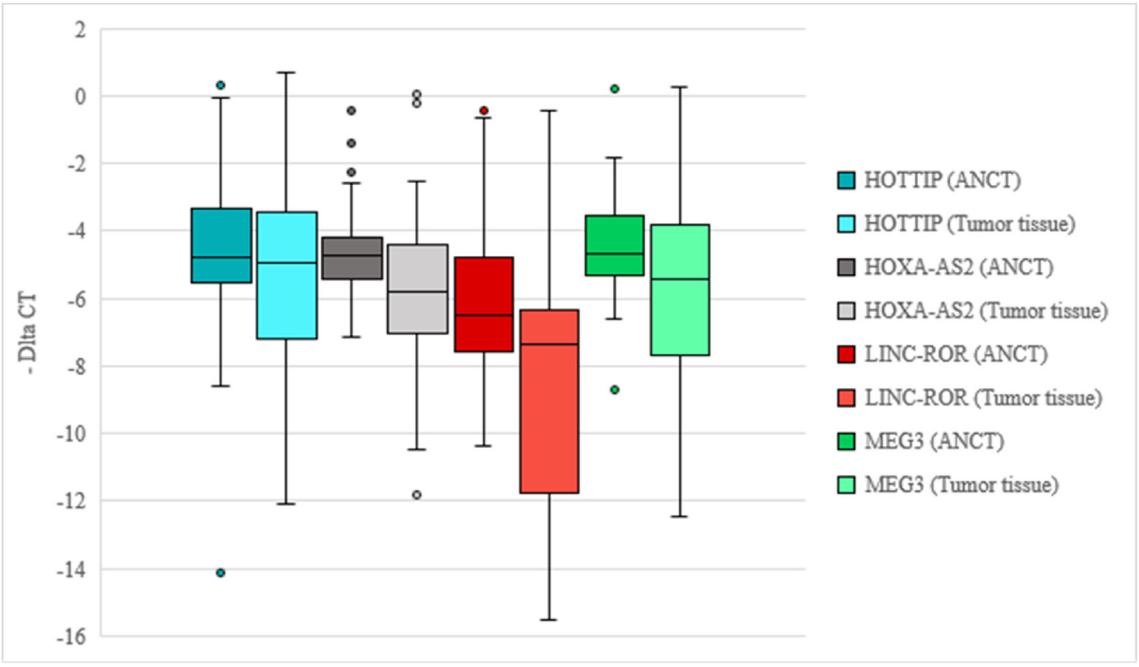

Figure 1. Expression levels of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples and adjacent non-cancerous tissues (ANCTs). Maximum, minimum and mean values as well as interquartile range are shown. Outliers are shown by circles. The statistical significance was appraised using the Student paired t test. Level of significance was set at $P < 0.05$.

and nearby tissues (P value = 0.43). Table 3 shows the statistical parameters of expression assays.

Expression levels of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs were correlated with each other in both gastric cancer samples and nearby non-cancerous samples (Table 4). The most robust correlations were detected between *HOTTIP* and *MEG3* (r = 0.94) and between *HOTTIP* and *HOXA-AS2* (r = 0.91) in gastric cancer tissues.

HOTTIP expression was associated with tumor size (P value = 0.04). In addition, *MEG3* expression was associated with site of primary tumor (P = 0.0003). Expressions of *LINC-ROR* and *HOXA-AS2* were not associated with any clinical or pathological parameter (Table 5).

ROC curve analysis revealed that *HOXA-AS2* and *LINC-ROR* could significantly differentiate between gastric cancer samples and nearby non-cancerous tissues (AUC values = 0.68 and 0.64; P values = 0.01 and 0.04, respectively) (Figure 2).

Combination of expression levels of *HOXA-AS2* and *LINC-ROR* enhanced the diagnostic power (P value = 0.009). Table 6 shows the detailed statistical parameters of ROC curve analysis.

4. Discussion

LncRNAs have appreciated roles in the carcinogenic processes [6]. These transcripts regulate cancer stem cells properties, cell cycle progression, epithelial-mesenchymal transition and cell apoptosis/proliferation [17]. Therefore, assessment of expression of these transcripts would provide important mechanistical clues in cancer research. In the current project, we compared expressions of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples and nearby non-cancerous samples. Expression levels of *LINC-ROR*, *HOXA-AS2* and *MEG3* lncRNAs have been lower in gastric cancer samples compared with nearby non-cancerous samples. Yu et al. have reported down-regulation of *LINC-ROR* in gastric cancer tissues compared with their nearby non-tumor tissues. Notably, expression of this lncRNA has been associated with tumor differentiation [18]. *LINC-ROR* expression has been previously assessed in a cohort of Iranian patients with diverse types of cancers revealing its up-regulation in esophageal, ovarian, and cervical cancers, while being down-regulated in breast, sarcoma, colon, and melanoma patients [19]. Although we detected down-regulation of this lncRNA in tumoral samples, we could not detect any association between its levels and histopathological parameters. A recent overview of *LINC-ROR* function in diverse cancers has indicated close relation between dysregulation of this lncRNA and advanced clinicopathological features showing a poor clinical outcome [20]. Thus, lack of association between expression of this lncRNA and clinical data in the current study might be explained by small sample size of the study.

HOXA-AS2 has been previously reported to be an oncogenic lncRNA in glioma, as its silencing has inhibited cell proliferation and

Table 3. Expression levels of *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples (n = 30) and paired non-cancerous tissues (n = 30). The statistical significance was appraised using the Student paired t test. Level of significance was set at $P < 0.05$.

lncRNAs	Parameters	Values
HOTTIP	Expression ratio	0.658
	P-value	0.43
HOXA-AS2	Expression ratio	0.373
	P-value	0.015
LINC-ROR	Expression ratio	0.265
	P-value	0.021
MEG3	Expression ratio	0.36
	P-value	0.032

Table 4. Correlation between expression levels *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* lncRNAs in gastric cancer samples (n = 30) and paired non-cancerous tissues (n = 30) (Spearman's correlation coefficients are shown. **P values < 0.01).

		MEG3	LINC-ROR	HOXA-AS2
HOTTIP	Tumor tissues	0.94**	0.74**	0.91**
	Non-tumor tissues	0.61**	0.54**	0.36**
HOXA-AS2	Tumor tissues	0.91**	0.77**	
	Non-tumor tissues	0.71**	0.62**	
LINC-ROR	Tumor tissues	0.76**		
	Non-tumor tissues	0.84**		

Table 5. Association between relative expression of expression levels *LINC-ROR*, *HOXA-AS2*, *MEG3* and *HOTTIP* and clinical data (Chi-square test was used for detection of associations. Level of significance was set at $P < 0.05$).

	HOTTIP up-regulation	*HOTTIP* down-regulation	P value	*HOXA-AS2* up-regulation	*HOXA-AS2* down-regulation	P value	*LINC-ROR* up-regulation	*LINC-ROR* down-regulation	P value	*MEG3* up-regulation	*MEG3* down-regulation	P value
Age			1			0.64			0.63			0.67
>50	10 (47.6%)	11 (52.4%)		6 (28.6%)	15 (71.4%)		7 (33.3%)	14 (66.7%)		7 (33.3%)	14 (66.7%)	
≤50	3 (42.9%)	4 (57.1%)		1 (14.3%)	6 (85.7%)		1 (14.3%)	6 (85.7%)		3 (42.9%)	4 (57.1%)	
Gender			0.37			0.62			1			1
Female	4 (66.7%)	2 (33.3%)		2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)	
Male	9 (40.9%)	13 (59.1%)		5 (22.7%)	17 (77.3%)		6 (27.3%)	16 (72.7%)		8 (36.4%)	14 (63.6%)	
Site of primary tumor			0.07			0.22			0.45			0.003
Cardia	4 (33.3%)	8 (66.7%)		1 (8.3%)	11 (91.7%)		2 (16.7%)	10 (83.3%)		1 (8.3%)	11 (91.7%)	
Antrum	7 (77.8%)	2 (22.2%)		4 (44.4%)	5 (55.6%)		4 (44.4%)	5 (55.6%)		7 (77.8%)	2 (22.2%)	
Body	2 (25%)	6 (75%)		2 (25%)	6 (75%)		2 (25%)	6 (75%)		2 (25%)	6 (75%)	
Tumor size (cm)			0.04			0.39			0.48			0.19
<4	4 (80%)	1 (20%)		2 (40%)	3 (60%)		2 (40%)	3 (60%)		3 (60%)	2 (40%)	
4-7	9 (47.4%)	10 (52.6%)		5 (26.3%)	14 (73.7%)		6 (31.6%)	13 (68.4%)		7 (36.8%)	12 (63.2%)	
>7	0 (0%)	5 (100%)		0 (0%)	5 (100%)		0 (0%)	5 (100%)		0 (0%)	5 (100%)	
Histology grade			0.52			1			1			0.2
2	6 (66.7%)	3 (33.3%)		2 (22.2%)	7 (77.8%)		3 (33.3%)	6 (66.7%)		2 (22.2%)	7 (77.8%)	
3	7 (50%)	7 (50%)		3 (21.4%)	11 (78.6%)		4 (28.6%)	10 (71.4%)		6 (42.9%)	8 (57.1%)	
4	1 (100%)	0 (0%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)		1 (100%)	0 (0%)	
TNM Staging			0.08			0.35			0.24			0.28
I	0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)		0 (0%)	1 (100%)	
II	3 (33.3%)	6 (66.7%)		2 (22.2%)	7 (77.8%)		4 (44.4%)	5 (55.6%)		2 (22.2%)	7 (77.8%)	
III	9 (69.2%)	4 (30.8%)		5 (38.5%)	8 (61.5%)		4 (30.8%)	9 (69.2%)		7 (53.8%)	6 (46.2%)	
IV	1 (16.7%)	5 (83.3%)		0 (0%)	6 (100%)		0 (0%)	6 (100%)		1 (16.7%)	5 (83.3%)	
Smoking			0.72			0.27			0.15			0.6
Non-Smoker	6 (54.5%)	5 (45.5%)		3 (27.3%)	8 (72.7%)		4 (36.4%)	7 (63.6%)		4 (36.4%)	7 (63.6%)	
Smoker	2 (66.7%)	1 (33.3%)		2 (66.7%)	1 (33.3%)		2 (66.7%)	1 (33.3%)		2 (66.7%)	1 (33.3%)	
Ex- Smoker	3 (37.5%)	5 (62.5%)		1 (12.5%)	7 (87.5%)		1 (12.5%)	7 (87.5%)		2 (25%)	6 (75%)	
H. pylori Infection			1			0.14			0.21			1
Positive	7 (46.7%)	8 (53.3%)		4 (26.7%)	11 (73.3%)		6 (40%)	9 (60%)		5 (33.3%)	10 (66.7%)	
Negative	7 (46.7%)	8 (53.3%)		3 (20%)	12 (80%)		2 (13.3%)	13 (86.7%)		5 (33.3%)	10 (66.7%)	

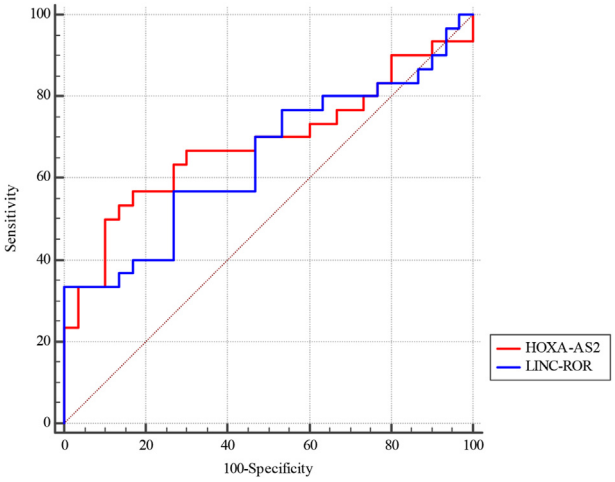

Figure 2. ROC curves showing diagnostic power of *HOXA-AS2* and *LINC-ROR* in distinguishing between gastric cancer tissues and nearby non-cancerous tissues (True positive rate is plotted against the false positive rate at various threshold settings. Level of significance is set at $P < 0.05$).

Table 6. Detailed statistical parameters of ROC curve analysis (Level of significance is set at $P < 0.05$, Youden's J statistic captures the performance of expression levels of genes as a dichotomous diagnostic test).

	Estimate criterion	AUC	J	Sensitivity	Specificity	P-value
HOXA-AS2	>5.52	0.684	0.4	56.7	83.7	0.01
LINC-ROR	>10.37	0.648	0.33	33.3	100	0.04
MEG3	>5.32	0.63	0.3	50	80	0.08
Combination of *HOXA-AS2* and *LINC-ROR*	>0.5	0.688	0.4	63.3	76.7	0.009

invasiveness, and induced cell apoptosis [14]. Moreover, this lncRNA has an oncogenic role in acute myeloid leukemia through binding with EZH2 and decreasing expression of LATS2 [21]. The current investigation proposes a different role for this lncRNA in gastric carcinogenesis and suggests that *HOXA-AS2* might have tissue-specific functions. Such tissue-specific roles have been formerly proposed for *LINC-ROR* [19].

Our data regarding expression pattern of *MEG3* in gastric cancer tissues is in line with the previously reported function for this lncRNA in this tissue [22], since *MEG3* has been shown to inhibit gastric carcinogenesis through regulation of epithelial-mesenchymal transition [22]. Consistent with these studies, another study has indicated the role of *MEG3* in inhibition of proliferation and metastasis of gastric cancer cells through modulating expression of miR-21 [23]. We also reported association between *MEG3* expression and site of primary tumor.

ROC curve analysis revealed that *HOXA-AS2* and *LINC-ROR* could significantly differentiate between gastric cancer samples and nearby non-cancerous tissues. The obtained AUC value for *LINC-ROR* in the current study is comparable with Yu et al. study [18], yet the specificity of this marker in our study is far beyond their study [18]. However, the AUC value obtained for combination of two lncRNAs was not high enough.

We also detected robust correlations between *HOTTIP* and *MEG3* and between *HOTTIP* and *HOXA-AS2* in gastric cancer tissues which might imply their coordinated function in the development of this kind of cancer.

We did not detect any significant difference in expression of *HOTTIP* between gastric cancer samples and nearby non-cancerous samples. Yet, expression of this lncRNA was associated with tumor size. Overexpression of *HOTTIP* has been formerly shown to be linked with some determinants of gastric cancer invasiveness such as greater tumor size, deep tumor penetration, lymph node involvement, high TNM stage, and shorter overall survival [24]. Moreover, a recent review about the role of this lncRNA in gastrointestinal cancers has suggested superiority of *HOTTIP* expression levels over currently used diagnostic markers for these types of cancers [25]. However, data regarding the expression pattern of this lncRNA in gastric cancer tissues versus nearby tissues are not consistent [26]. The observed similar levels of *HOTTIP* between cancerous and non-cancerous tissues in this study and the former inconsistencies cast doubt on the appropriateness of this lncRNA as diagnostic marker for gastric cancer. Moreover, these data indicate the necessity of conduction of expression profiling experiments in different ethnic groups to find the best cancer biomarkers in each population.

Taken together, the current investigation provide clues for contribution of *LINC-ROR*, *HOXA-AS2* and *MEG3* lncRNAs in gastric carcinogenesis and warrants further mechanistical assays. Our study has some limitations, namely small sample size and lack of validation of results in an independent cohort.

Declarations

Author contribution statement

Shahrad Soghala and Mohammad Taheri: Conceived and designed the experiments.

Kiana Harsiny, Parto Momeni and Mahsa Hatami: Performed the experiments.

Vahid Kholghi Oskooei: Analyzed and interpreted the data.

Bashdar Mahmud Hussen: Conceived and designed the experiments; Wrote the paper.

Soudeh Ghafouri-Fard: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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