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 HOXTIP 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 HOXTIP were not significantly different between gastric cancer tissues and nearby tissues (P value = 0.43). HOXTIP 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 circulating exosomes reflects their expression in the original tissues and can be used as diagnostic and prognostic tools in gastric cancer [9]. These circulating 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 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 spectrophotometer. 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             | Forward | Reverse |
|---------------|-----------------------------|---------|---------|
| B2M           | 5'-AGATGAGTATGCGCTGCGTG-3'  | Forward | Reverse |
|               | 5'-CAGGATCTTCAAACCTCCA-3'  |         |         |
| LINC-ROR      | 5'-TATAATGAGATAACCTCTTA-3'  | Forward | Reverse |
|               | 5'-AGAACTGTCAATACGTTC-3'   |         |         |
| MEG3          | 5'-TGGCATAGAGGAGGTGAT-3'    | Forward | Reverse |
|               | 5'-GGAGTGCTGCTGGAGAAATA-3' |         |         |
| HOTTIP        | 5'-AGCTCTTTCCCGACAGT-3'     | Forward | Reverse |
|               | 5'-CCCTACCAAGCGCTCTTC-3'   |         |         |
| HOXA-AS2      | 5'-GGCTTGAGTAATCGACTCTCT-3' | Forward | Reverse |
|               | 5'-TAGTCAAGCGGCTGAACTCAA-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 χ² 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 and nearby non-cancerous tissues.

| 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%  |
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

| IncRNAs   | 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).
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            |                      |                        |         |                        |                        |         |                        |                        |         |                     |                       |         |
| >50            | 10 (47.6%)           | 11 (52.4%)             | 0.64    | 6 (28.6%)              | 15 (71.4%)             | 0.63    | 7 (33.3%)              | 14 (66.7%)             | 0.67    | 3 (42.9%)           | 4 (57.1%)             |         |
| ≤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       |                        |                        |         |                        |                        |         |                     |                       |         |
| 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    |                        |                        |         |                        |                        |         |                     |                       |         |
| 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    |                        |                        |         |                        |                        |         |                     |                       |         |
| <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%)            |         |
| Histology grade | 0.52                | 1                      | 1       |                        |                        |         |                        |                        |         |                     |                       |         |
| 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%)             | 0.2     |
| 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 (37.3%)              | 8 (62.7%)              |         | 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    |                        |                        |         |                        |                        |         |                     |                       |         |
| 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%)            |         |
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 LAT52 [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. Over-expression 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 Oskoei: Analyzed and interpreted the data.
Bashdar Mahmood Hussen: Conceived and designed the experiments; Wrote the paper.
Soudeh Ghafori-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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