Comprehensive analysis of expression profiles of long non-coding RNAs with associated ceRNA network involved in gastric cancer progression

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Abstract. Long non-coding RNAs (lncRNAs) play critical roles in the development and progression of cancers. The present study aimed to identify novel lncRNAs and associated microRNAs (miRNAs) and mRNAs in gastric cancer. Differentially expressed lncRNAs (DElncRNAs) and differentially expressed mRNAs (DEmRNAs) of 6 paired gastric cancer and normal tissues were identified using microarray. The DEmRNAs between gastric cancer and the normal control tissues were identified using miRNA-seq data from Cancer Genome Atlas. Common DElncRNAs from the Cancer RNA-Seq Nexus database and circRNA.net database were analyzed. A DElncRNAs-DEmRNAs-DEmRNAs network was constructed by target prediction. Functional enrichment analysis was employed to predict the function of DEmRNAs in the network. The correlation between the expression of DElncRNAs and DEmRNAs in the network was analyzed. A total of 1,297 DElncRNAs, 2,037 DEmRNAs and 171 DEmiRNAs were identified. Among the 4 lncRNAs common to the 3 datasets, prostate androgen-regulated transcript 1 (PARD1) was selected for further analysis. The analysis identified 5 DEmiRNAs and 13 DEmRNAs in the PARD1-mediated ceRNA network. The DEmRNAs in the ceRNA network were markedly enriched in cancer-related biological processes (response to hypoxia, positive regulation of angiogenesis and positive regulation of endothelial cell proliferation) and pathways (cAMP signaling pathway, cAMP signaling pathway and proteoglycans in cancer). Out of the 13 DEmiRNAs, 11 were positively associated with PARD1. The downregulation of PARD1, myosin light chain 9 (MYL9), potassium calcium-activated channel subfamily M alpha 1 (KCNNMA1), choleric receptor muscarinic 1 (CHRM1), solute carrier family 25 member 4 (SLC25A4) and ATPase Na+/K+ transporting subunit alpha 2 (ATP1A2) expression levels in gastric cancer was validated by RT-qPCR. On the whole, the current study identified a novel lncRNA and associated miRNAs and mRNAs that are involved in the pathogenesis of gastric cancer that may serve as potential therapeutic targets for the treatment of gastric cancer.

Introduction

Gastric cancer is one of the most commonly diagnosed and lethal types of cancer, corresponding to an estimated 951,600 new gastric cancer cases and 723,100 deaths worldwide in 2012 based on GLOBOCAN data (1). In Europe, gastric cancer is the fourth leading cause of cancer-related mortality and was associated with 107,000 (6.1%) cancer-related deaths in 2012 (2). Therefore, there is an urgent need for the elucidation of the molecular mechanisms underlying gastric cancer and for the development of effective therapeutic strategies.

A large proportion of the genome in human cells is transcribed into RNA molecules that lack protein-coding capacity (3,4). Of these, long non-coding RNAs (lncRNAs) are transcripts with lengths >200 nt (5) that play important roles in tumorigenesis and cancer progression (6-8). A growing number of gastric cancer-related lncRNAs have been identified in recent years. For example, the expression of the lncRNA BRAF-activated non-coding RNA (BANCR) was found to be upregulated in gastric cancer and to be associated with the patient survival rate (9). The expression of lncRNA LINC00857 has also been shown to be associated with a poor survival rate of patients with gastric cancer, and the downregulation of LINC00857 inhibits gastric cell proliferation (10). The downregulated expression of lncRNA LINC01939 has been shown to be associated with gastric cancer progression and a poor survival (11). According to the competing endogenous RNA (ceRNA) hypothesis (12), ceRNAs compete with mRNAs for binding to the same miRNA.
response elements (13). Recent studies have identified an increasing number of IncRNAs that function as ceRNAs, and play critical roles in the pathogenesis and progression of gastric cancer. For instance, IncRNAs, HOTAIR (14), SNHG6 (15), and NNT-AS1 (16), have been shown to be significantly upregulated in gastric cancer tissues and to function as oncogenes by competitively sponging miR-331-3p, miR-101-3p and miR-424 to regulate human epidermal growth factor receptor 2 (HER2), Zinc finger E-box-binding homeobox 1 (ZEB1) and E2F transcription factor 1 (E2F1), respectively. However, further studies are required to identify additional cancer-related IncRNAs, miRNAs and mRNAs.

In the present study, we analyzed the expression profiles of IncRNAs and mRNAs in gastric cancer tissues and adjacent normal tissues using microarray and analyzed miRNA expression profiles in gastric cancer and normal samples from The Cancer Genome Atlas (TCGA) by miRNA-seq. We identified differentially expressed IncRNAs, mRNAs and miRNAs (designated as DElncRNAs, DEMRNAs and DEMiRNAs, respectively). Gastric cancer-related DElncRNAs that were common to the Cancer RNA-Seq Nexus (CRN) database and circRnaRNA.net database were identified. A DElncRNA-DEMIRNA-DEMIRNA ceRNA network was constructed by target prediction based on the ceRNA hypothesis (12). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were also performed to predict the functions of the identified DEMiRNAs. In addition, the correlation between the expression of DElncRNAs and DEMiRNAs in the ceRNA network was analyzed. The expression levels of DElncRNAs and DEMiRNAs were validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Taken together, the present study identified novel gastric cancer-related IncRNAs, miRNAs and mRNAs and provided insight into the diagnosis and treatment of gastric cancer.

Materials and methods

Datasets and data preprocessing. We analyzed microarray data from our previous study [Vu et al (17)], which was generated from the global profiling of IncRNAs and protein-coding transcripts in 6 gastric cancer tissues and 6 pair-matched adjacent normal tissues using Arraystar Human LnRNA Microarray v2.0 (Arraystar, Rockville, MD, USA). The demographic and pathological characteristics of the patients are presented in Table I. This study was approved by Medical Ethics Committee of Ruijin Hospital Luwan Branch Shanghai Jiaotong University School of Medicine. All patients provided written informed consent.

RT-qPCR validation of DElncRNAs and DEMiRNAs. A total of 15 cases of gastric cancer and adjacent tissues were used for RT-qPCR validation. The demographic and pathological characteristics of all the patients are presented in Table I. The reactions and procedures were as follow: 1 µl Random Primer, 1 µg Total RNA and RNase-Free ddH2O was added to 12 µl total volume and was incubated at 65°C for 5 min, then 4 µl 5X Buffer, 2 µl dNTP Mix, 1 µl Protector RNase Inhibitor, and 1 µl Transcriptase added. The expression levels of the selected DElncRNAs and DEMiRNAs were validated by RT-PCR using the SYBR-Green PCR master mix kit (Applied Biosystems, Inc.). RT-qPCR was performed on an ABI QuantStudio™ 6 Flex System (Applied Biosystems, Inc.). Primer sequences for the amplification of target genes and the reference gene GAPDH are presented in Table II. The PCR protocol was: 95°C for 10 min, 45 cycles of 95°C for 15 sec and 60°C for 60 sec. To establish the melting curve, after the amplification reaction was completed, the reaction was performed at 95°C for 10 sec, 60°C for 60 sec, and 95°C for 15 sec, and slowly heating from 60°C to 99°C (+0.05°C/s). The relative expression levels of the genes were measured based on 3 independent experiments and calculated using the 2^(-∆∆cq) method (19).

Statistical analysis. Statistical analyses were performed using SPSS 21.0 software (IBM Corp.). All experimental data are
presented as the mean values ± standard deviation. Differences
between the gastric cancer group and normal group were deter-
mind using the Student's t-test. The comparisons between the
clinicopathological characteristics of the patients used in the
microarray and those used for RT-qPCR were carried out using
the Chi-square test. Values of P<0.05 and P<0.001 were consid-
ered to indicate statistically significant and highly statistically
significant differences, respectively.

Results

Identification of DEIncRNAs, DEMRNAs and DEMiRNAs.
A total of 1,297 DEIncRNAs and 2,037 DEMRNAs were
identified between the gastric cancer tissues and matched
normal control tissues with the cut-off values FC ≥2 and
P-value <0.05. A total of 171 DEMiRNAs were identified
between the gastric cancer and normal control samples

Table I. Demographic and clinicopathological information of the patients.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Group (n=21)</th>
<th>Microarray assay (n=6) (%)</th>
<th>Reverse transcription-quantitative PCR (n=15) (%)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Age</td>
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<td></td>
<td></td>
<td>0.94</td>
</tr>
<tr>
<td>&lt;60</td>
<td>3 (14.3)</td>
<td>9 (42.9)</td>
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<td></td>
</tr>
<tr>
<td>≥60</td>
<td>3 (14.3)</td>
<td>6 (28.5)</td>
<td></td>
<td></td>
</tr>
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<td>Sex</td>
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</tr>
<tr>
<td>Female</td>
<td>2 (9.5)</td>
<td>5 (23.8)</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>4 (19)</td>
<td>10 (47.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td>Low</td>
<td>2 (9.5)</td>
<td>6 (28.5)</td>
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</tr>
<tr>
<td>Middle and high</td>
<td>4 (19)</td>
<td>9 (42.9)</td>
<td></td>
<td></td>
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<tr>
<td>TNM</td>
<td></td>
<td></td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td>T1+T2</td>
<td>1 (4.8)</td>
<td>5 (23.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3+T4</td>
<td>5 (23.8)</td>
<td>10 (47.6)</td>
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<tr>
<td>Stage</td>
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<td>0.73</td>
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<tr>
<td>I+II</td>
<td>3 (14.3)</td>
<td>8 (38.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3 (14.3)</td>
<td>7 (33.3)</td>
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<tr>
<td>Metastasis</td>
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<tr>
<td>No</td>
<td>4 (19)</td>
<td>9 (42.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2 (9.5)</td>
<td>6 (28.5)</td>
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Table II. Primer sequences used for reverse transcription-quantitative PCR.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>Annealing temperature (˚C)</th>
<th>Aim band length (bp)</th>
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<td>B2M</td>
<td>F: 5'-CTCTTTTCTGGCCTGGAGGCTAT-3'</td>
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<td>135</td>
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<tr>
<td></td>
<td>R: 5'-AGTCAACTTCAATGTCGGAATGAT-3'</td>
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</tr>
<tr>
<td>PART1</td>
<td>F: 5'-CAAACGCTGAGTGCTGCTG-3'</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGTAAAGGGAAGTTCAATACACA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYL9</td>
<td>F: 5'-GCCTCGCGTGGTTCATCC-3'</td>
<td>60</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGGTACATCTCGTCCACTCTCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCNMA1</td>
<td>F: 5'-GGACTTAGGGAATGTTGCTG-3'</td>
<td>60</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGTTTGGTATGACATACTCCCTTTT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHRM1</td>
<td>F: 5'-TGGGAACATGCAAATCAGCTG-3'</td>
<td>60</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTCTCCAGCGGCTGCTTACA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC25A4</td>
<td>F: 5'-CTATGATAGTGGAGGATG-3'</td>
<td>60</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TACATAATATCGGCCCCTTTTCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP1A2</td>
<td>F: 5'-TGGCAATGATGACCACAAAG-3'</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAGGTCCACTTGGATTTGCG-3'</td>
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F, forward; R, reverse.
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from the TCGA dataset with the cut-off values FC ≥2 and FDR <0.05. The heatmaps for the total DElncRNAs and DEMRNAs are presented in Fig. 1A and B, respectively. A total of 4 IncRNAs [LOH12CR2, prostate androgen-regulated transcript 1 (PART1), HOTAIR and UCA1] were common among the microarray data, CRN data and cirClncRNA.net data (Fig. 1C). LOH12CR2 and PART1 were markedly downregulated, whereas HOTAIR and UCA1 were significantly upregulated in the gastric cancer tissues. The heatmap showing the 4 overlapping IncRNAs is presented in Fig. 1D. Raw microarray data were deposited in the Gene Expression Omnibus database under the accession number GSE53137.

Pan-cancer expression analysis of lncRNA PART1. Literature mining identified HOTAIR and UCA1 to be associated with gastric cancer. PART1 has been demonstrated to play important roles in other types of cancer. In the TCGA datasets, the PART1 expression levels were markedly downregulated in various cancers, including bladder urothelial carcinoma (BLCA), breast invasion carcinoma (BRCA), head and neck squamous cell carcinoma (HNSC) and stomach adenocarcinoma (STAD) (Fig. 2), thereby suggesting its important role in tumorigenesis. However, the expression of LOH12CR2 exhibited no marked differences between the cancer and adjacent tissue (Fig. S1). Thus, PART1 was selected for subsequent analysis.

Figure 1. DElncRNAs and DEMRNAs between gastric cancer tissues and normal tissues. Heatmaps of (A) DElncRNAs and (B) DEMRNAs in gastric cancer. (C) The lncRNAs common among microarray, Cancer RNA-Seq Nexus (CRN), and cirClncRNA.net data. (D) Heatmap showing the expression of the common IncRNAs. The red and green colors indicate upregulation and downregulation, respectively. DE, Differentially expressed.
Construction of PART1-DEmiRNA-DEmRNA network. The PART1-mediated ceRNA network was constructed based on target prediction. As shown in Fig. 3, PART1 was found to interact with 5 upregulated DEmiRNAs, including hsa-miR-4728-5p, hsa-miR-1304-3p, hsa-miR-708-5p, hsa-miR-1229-3p and hsa-miR-130b-3p. A total of 79 downregulated DEmRNA nodes were included in the network.

Functional enrichment analysis of the PART1-DEmiRNA-DEmRNA network. GO and pathway enrichment analyses were
performed to predict the functional role of the DE genes in the PART1-mediated ceRNA network and analyze the DE mRNAs in the ceRNA network. The top 10 GO terms and top 10 pathway terms are presented in Fig. 4A and B, respectively. In particular, 4 pathways, namely the cGMP-PKG signaling pathway, cAMP signaling pathway, proteoglycans in cancer and regulation of actin cytoskeleton, were significantly enriched. The 13 identified DE mRNAs were enriched in the top 10 pathway terms (Fig. 4C).

Correlation analysis of PART1 and DE mRNAs. Expression correlation analysis was performed between PART1 and 13 DE mRNAs in the top 10 pathway terms. The results revealed that the expression levels of 11 out of 13 DE mRNAs were positively associated with PART1 expression levels in gastric cancer (Fig. 5). However, the expression of Wnt family member 6 (WNT6) and cholinergic receptor muscarinic 1 (CHRM1) exhibited no correlation with PART1 in gastric cancer (Fig. S2).

RT-qPCR validation of PART1 and associated DE mRNAs. To validate the reliability of the microarray results and the above-mentioned analysis, the expression levels of PART1, myosin light chain 9 (MYL9), potassium calcium-activated channel subfamily M alpha 1 (KCNMA1), CHRM1, solute carrier family 25 member 4 (SLC25A4) and ATPase Na+/K+ transporting subunit alpha 2 (ATP1A2) were confirmed by RT-qPCR. Microarray results revealed that the PART1 lncRNA and 5 candidate DE mRNAs were downregulated in the gastric cancer tissues compared with the adjacent normal tissues (Fig. 6A). Consistent with these findings, the results of RT-qPCR revealed that the relative expression levels of PART1, MYL9, KCNMA1, CHRM1, SLC25A4 and ATP1A2 were significantly lower in the gastric cancer tissues compared with the adjacent normal tissues (Fig. 6B). Moreover, another 7 downregulated DE mRNAs including G protein subunit alpha Z (GNAZ), G protein subunit alpha Ol (GNAO1), related...
raS viral (R-Ras) oncogene homolog (RRAS), syndecan 2 (SDC2), fibroblast growth factor 2 (FGF2), sarcoglycan alpha (SGCA) and desmin (DES) in gastric cancer tissues relative to the adjacent normal tissues was confirmed by rT-Pcr (Fig. S3).

**Discussion**

In the present study, we identified 1,297 DElncRNAs, 2,037 DEMRNAs, and 171 DEMiRNAs between gastric cancer tissues and matched normal tissues. Two upregulated lncRNA PART1 and associated DEMRNAs were confirmed by RT-qPCR (Fig. S3).
(HOTAIR and UCA1) and two downregulated lncRNAs (LOH12CR2 and PART1) were common between the microarray, CRN and circInRNA-Net results. Several studies have previously reported that HOTAIR is an oncogene that plays a crucial role in the progression of various types of cancer (20), including gastric cancer (21,22), lung cancer (23), colorectal cancer (24), liver cancer (25), gallbladder cancer (26), breast cancer (27), cervical cancer (28), ovarian cancer (29), and prostate cancer (30). The oncogene UCA1 is known to be involved in various types of cancer, such as gastric cancer (31), breast cancer (32), bladder cancer (33) and colorectal cancer (34). In this study, GEPIA analysis of the TCGA data revealed that PART1 expression was significantly downregulated in various types of cancer, including gastric cancer. Thus, PART1 was selected for further analysis.

The PART1-mediated PART1-DEmiRNA-DEmRNA ceRNA network was constructed. PART1 was found to directly interact with 5 DEMiRNAs (hsa-miR-4728-5p, hsa-miR-1304-3p, hsa-miR-708-5p, hsa-miR-1229-3p and hsa-miR-130b-3p) and to indirectly interact with 79 DEMiRNAs via DEMiRNAs. miR-1229 expression was significantly upregulated in breast cancer and has been demonstrated to promote tumor growth by activating the Wnt/β-catenin signaling pathway (35). miR-130b-3p upregulation is mediated by a mechanism that is dependent on the cAMP-response element binding protein and is known to promote thyroid adenomas development (36). miR-4728-5p expression has been shown to be significantly upregulated in patients with gastric cancer who are positive for HER2, which is overexpressed in gastric cancer and serves as a clinical target for gastric cancer therapy (37,38). miR-708-5p is involved in various processes associated with tumorigenesis and acts as an oncogene in various cancers, including bladder cancer (39,40) and colorectal cancer (41). Taken together, the above-mentioned findings indicate that PART1 plays crucial roles in cancer.

Additionally, DEmiRNAs in the generated PART1-mediated ceRNA network were markedly enriched in cancer related biological process, such as response to hypoxia, positive regulation of angiogenesis and the positive regulation of endothelial cell proliferation. KEGG pathway analysis revealed that the identified DEmiRNAs were significantly enriched in the cGMP-PKG signaling pathway, cAMP signaling pathway, proteoglycans in cancer, and the regulation of actin cytoskeleton. Among these DEmiRNAs, 13 miRNAs were involved in the top 10 pathways; in addition, 11 out of these 13 miRNAs exhibited positive expression correlations with lncRNA PART1 expression. RT-qPCR results validated the downregulated expression levels of PART1, MYL9, KCNMA1, CHRM1, SLC25A4 and ATP1A2 in the gastric cancer compared with the adjacent normal tissues. PART1 expression has also been shown to be markedly decreased in the glioma tissues compared with normal brain tissues and its low expression was negatively associated with overall patient survival (42,43). PART1 is located in chromosome 5q, which is deleted in prostate cancer patients and cell lines and potentially contains cancer suppressor genes for prostate cancer, suggesting its crucial role in inhibiting prostate carcinogenesis (44,45). The downregulation of PART1 has been shown to inhibit the cell proliferation of prostate cancer and to promote cell apoptosis by modulating the Toll-like receptor pathway (46). Bioinformatics analysis of oral squamous cell carcinoma expression profiles from the GEO database has revealed that PART1 is associated with the pathogenesis of oral squamous cell carcinoma (47). The downregulation of PART1 and its role in gastric carcinogenesis have not yet been fully elucidated. However, to the best of our knowledge, this study was the first to illustrate the PART1-enriched lncRNA-mRNA interaction network and the enriched pathway in gastric cancer. These analyses are of great significance to understanding the role of PART1 in the occurrence and development of gastric cancer. MYL9 expression has been shown to be significantly downregulated in prostate cancer tissues and its decreased expression has been shown to be negatively associated with patient survival (48). A previous study demonstrated that low MYL9 expression levels were associated with the development and metastasis of non-small cell lung cancer (49). KCNMA1 expression has been shown to be downregulated in gastric cancer cells compared with normal gastric epithelial cells and has been found to function as a tumor suppressor gene (50). SLC25A4 expression has also been shown to be downregulated during external beam radiation therapy relative to baseline and has been shown to be significantly associated with cancer-related fatigue in prostate cancer patients. SLC25A4 expression is low in proliferating cells (51). ATP1A2 expression has also been shown to be downregulated in breast cancer (52). Another study also demonstrated that ATP1A2 expression was downregulated in bone marrow-infiltrating neuroblastoma cells and was strongly associated with patient outcome (53). PART1 and many of its associated DEmiRNAs and DEmRNAs are involved in the progression of gastric cancer or other types of cancer, thereby suggesting that PART1 plays important roles in gastric cancer development.

In conclusion, the findings of this study revealed that PART1 is a DEmiRNA between gastric cancer tissues and normal tissues. In this study, we generated and analyzed a PART1-mediated ceRNA network. The results indicated that PART1 interacts with DEmiRNAs (hsa-miR-4728-5p, hsa-miR-1304-3p, hsa-miR-708-5p, hsa-miR-1229-3p and hsa-miR-130b-3p) and 13 DEmRNAs. Of these, the expression levels of 11 DEmRNAs were found to be positively correlated with the PART1 expression levels. The expression levels of PART1, MYL9, KCNMA1, CHRM1, SLC25A4 and ATP1A2 were validated by RT-qPCR. The current findings of several genes, including IncRNAs, miRNAs and miRNAs, provide insight into the mechanisms underlying the pathogenesis of gastric cancer.

However, this study also had several limitations. First of all, the downregulation of PART1 needs to be verified in gastric cancer cell lines and the function of PART1 needs to be confirmed by cell function assay. Secondly, the association between candidate DEmiRNAs genes and PART1 in the gastric carcinogenesis needs to be confirmed by RNA-RNA interaction assay. Despite these shortcomings, the findings of this study may prove to be of great significance for the better understanding of the role and mechanisms of action of IncRNAs in gastric cancer.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
MJH designed the experiments, WG, JHR, XZ and XYH performed the experiments, WG and JHR analyzed the data, WG prepared the manuscript and MJH revised the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of Ruijin Hospital Luwan Branch Shanghai Jiao tong University School of Medicine. All patients provided written informed consent.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


