

The Comprehensive Analysis of Competitive Endogenous RNA Networks And Tumor-Infiltrating Immune Cells In Intestinal and Diffuse Gastric Cancer

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Abstract

Background: Gastric cancer (GC) is one of the leading causes of cancer death worldwide. Increasing evidences have revealed different molecular characteristics between intestinal type GC and diffuse type GC.

Results: We constructed Lauren subtype-specific competitive endogenous RNA (ceRNA) networks and identified common ceRNA network by comprehensive bioinformatics methods including differential expression analyses, RNA-RNA interaction prediction, negatively correlated RNA pairs and weighted genes co-expression network analysis. Besides, we detected the fraction of 22 immune cell types in GC by CIBERSORTx and investigated the correlation of immune cells and markers of ceRNA network. Ultimately, diagnostic performance of lncRNAs and mRNAs in ceRNA network were estimated by support vector machine (SVM). SNHG14 was identified as ceRNA of hsa-miR-429/ ZFPM2, hsa-miR-429/ZEB1 and hsa-miR-200b-3p/ZEB1 axes in all GC. Mast cells and Macrophages significantly differed not only between the tumor and normal tissue, but also between the two subtypes. Mast cells and Macrophages are significantly associated with multiple components in ceRNA network. Our results demonstrated lncRNAs and mRNAs of ceRNA network in the whole group (AUC=0.9152), the intestinal group (AUC=0.9670) and the diffuse group (AUC=0.9737) showed good performance in tumor diagnosis.

Conclusion: Based on ceRNA networks and patterns of immune infiltration, our study provided a valid bioinformatics basis in order to explore the molecular mechanism and estimated diagnosis performance of RNAs in ceRNA network.

Background

Gastric cancer (GC) is the sixth most common cancer and the second leading cause of cancer-related deaths worldwide [1]. Worldwide mortality rates for GC have declined in the past 10 years, however the survival rate remains low [2]. Many clinical, molecular, and pathologic data suggest that GC is a heterogeneous disease [3]. So, investigating the molecular mechanism of GC needs us to classify the patients by various characteristics. GC has many classification systems, such as: the Lauren, and the World Health Organization (WHO) classification systems [4] [5]. Lauren classification mainly including intestinal type and diffuse type, the former was previously a well-defined, sequential precancerous histological change and the latter was also defined as a cancer with poor adhesion. Many studies proved that the significant differences existed in the two Lauren types [6] [7] [8].

Long non-coding RNAs (lncRNAs) have attracted great attention because of their new role in cancer [9, 10]. In the past decade, the discovery of oncogenes and cancer epigenetics has revealed that many epigenetic modifiers, such as lncRNAs, are involved in the progression of various cancers [9–11] and functioned through a variety of mechanisms. The competitive endogenous RNA (ceRNA) hypothesis was first proposed by Salmena L, Poliseno L, et al in 2011 [12]. The ceRNA network assumed that any RNA transcript containing microRNA (miRNA) response elements can bind to miRNAs to regulate the

expression of miRNA target messenger RNA (mRNA), thus playing a crucial role in tumor initiation as well as progression.

Increasing evidence suggests that lncRNA-miRNA-mRNA regulatory axes are highly correlated with GC [13, 14] [15]. In our study, we performed weighted genes co-expression network analysis (WGCNA) to differentially expressed lncRNAs and mRNAs in The Cancer Genome Atlas (TCGA) and screened out the malignant stage-related RNAs for constructing the ceRNA network in two Lauren types patients and all TCGA patients. In addition, the proportions of immune cells were qualified by CIBERSORTx algorithm. We assessed the association between immune cells and RNAs of ceRNA network and the prediction performance of them distinguishing the malignant tissue and normal tissue by supporting vector machine (SVM). The flowchart of this research is shown in Fig. 1. Systematically identifying and comparing lncRNA biomarkers acting as ceRNAs, could contribute to elucidate the similarities and differences of the molecular mechanisms between intestinal-type gastric cancer (IGC) and diffuse-type gastric cancer (DGC), thereby providing valuable clues for investigating molecular mechanism.

Result

2.1 Identification of differentially expressed RNAs: The gene expression profiles of 375 GC samples and 32 normal samples were obtained. We removed the miRNAs having zero expression more than 20% samples. The baseline characteristics of three patient groups available from the TCGA are described in Table 1. A total of 485 DElncRNAs, 2540 DEmRNAs and 185 DEmiRNAs in DGC were detected. A total of 1499 DElncRNAs, 3570 DEmRNAs and 90 DEmiRNAs in IGC were detected. A total of 558 DElncRNAs, 1373 DEmRNAs in comparable group of DGC vs IGC were detected.

Table 1
Basic characteristics of the groups.

Variables	the whole group	the intestinal group	the diffuse group
	n = 375	n = 163	n = 63
Age (Mean ± SD)	65.83 ± 10.64	65.82 ± 10.12	61.65 ± 11.09
Gender			
Male	241(64.27%)	106(65.03%)	38(60.32%)
Female	134(35.73%)	57(34.97%)	25(39.68%)
Stage			
I	53(14.13%)	23(14.11%)	7(11.11%)
II	111(29.60%)	36(22.09%)	19(30.16%)
III	150(40.00%)	77(47.24%)	29(46.03%)
IV	38(10.13%)	24(14.72%)	5(7.94%)
Unknown	23(6.13%)	3(1.84%)	3(4.76%)
T category			
T1	19(5.07%)	10(6.13%)	0(0.00%)
T2	80(21.33%)	31(19.02%)	17(26.98%)
T3	168(44.80%)	77(47.24%)	27(42.86%)
T4	100(26.67%)	45(27.61%)	19(30.16%)
Unknown	8(2.13%)	0(0.00%)	0(0.00%)
N category			
N0	112(29.87%)	43(26.38%)	14(22.22%)
N1	97(25.87%)	40(24.54%)	21(33.33%)
N2	75(20.00%)	43(26.38%)	13(20.63%)
N3	74(19.73%)	32(19.63%)	15(23.80%)
Unknown	17(4.53%)	5(3.07%)	0(0.00%)
Grade			
G1	10(2.67%)	6(3.68%)	1(1.59%)
G2	137(36.53%)	84(51.53%)	1(1.59%)
G3	219(58.40%)	69(42.33%)	58(92.06%)

Variables	the whole group	the intestinal group	the diffuse group
	n = 375	n = 163	n = 63
G4	0(0.00%)	0(0.00%)	0(0.00%)
GX	9(2.40%)	4(2.45%)	3(4.76%)

2.2 WGCNA: In order to select tumor-related RNAs, we did WGCNA to DElncRNAs and DErnRNAs of two groups. Nine modules related to pathological stage and T stage with $p < 0.05$ in IGC and five modules in DGC were included in subsequent investigation (Fig. 2A and 2B).

2.3 Construction of the ceRNA network: Based on the ceRNA theory, ceRNA triplets consisting of negatively correlated lncRNA-miRNA and miRNA-mRNA competing pairs were selected. The ceRNA networks were further screened through the DERNAs from WGCNA. Finally, we constructed ceRNA network in IGC (Fig. 3A), including 24 lncRNA-miRNA pairs and 38 miRNA-mRNA pairs and one in DGC (Fig. 3B), including 34 lncRNA-miRNA pairs and 42 miRNA-mRNA pairs. The common 9 lncRNA-miRNA and 9 miRNA-mRNA were gained (Fig. 3C), including: 5 lncRNAs (PWAR6, SNHG14, AC007392.3, RP11-389G6.3 and ADAMTS9-AS2), 6 miRNAs (hsa-miR-106b-5p, hsa-miR-15a-5p, hsa-miR-196a-5p, hsa-miR-19a-3p, hsa-miR-200b-3p and hsa-miR-429) and 8 mRNAs (CFL2, KANK2, FGF2, TMEM100, PALLD, PRICKLE2, ZEB1 and ZFPM2).

2.4 Functional enrichment analysis: To gain insights into the different biological features implicated with mRNAs of ceRNA network, functional enrichment analyses were respectively performed for mRNAs identified in the above three ceRNA networks. The mRNAs in intestinal-specific ceRNA network were significantly enriched in Proteoglycans in cancer, Breast cancer, Regulation of actin cytoskeleton, MicroRNAs in cancer, Bacterial invasion of epithelial cells, Estrogen signaling pathway, Axon guidance and Focal adhesion (Fig. 4A). Comparatively, the mRNAs in diffuse-specific ceRNA network were primarily implicated in Signaling pathways regulating pluripotency of stem cells, Transcriptional mis-regulation in cancer and Regulation of actin cytoskeleton (Fig. 4B). Then, mRNAs in the common ceRNA network were significantly enriched in Regulation of actin cytoskeleton and MicroRNAs in cancer (Fig. 4C).

2.5 Differential immune cells and correlation analyses: Tumor-infiltration immune cells had been shown to play a role in tumorigenesis, progression, metastasis and drug resistance. Immune cells estimated by CIBERSORTx algorithm are displayed in Table S1. The differences of immune cells by Wilcoxon rank-sum test were depicted in Fig. 5. Some immune cells (Macrophages M0, Macrophages M1 and Mast cells resting) were commonly differing in three groups, which may play important role in GC. But Macrophages M0 and Mast cells resting immune cells are also different between the two Lauren sub-types (Fig. 5D). To investigate the potential relationship immune cells with the RNAs of Lauren classification specific ceRNA network, the correlation analyses were implemented (Fig. 6). Mast cells resting was evidently correlated with multi-markers of ceRNA network in three groups.

2.6 Diagnostic significance of lncRNA and mRNA biomarkers: Increasing evidence has demonstrated that lncRNAs and mRNAs can be used as biomarkers to guide decision on cancer diagnosis and therapy. The common RNAs of ceRNA network could represent the common molecular characteristics of two Lauren gastric cancer. Here, lncRNA and mRNA signatures were developed to distinguish tumor with normal tissues by SVM. The results in the test cohort showed that identification effect of SVM models was pretty excellent, whether distinguishing tumor in IGC (AUC = 0.9670) (Fig. 7A), DGC (AUC = 0.9737) (Fig. 7B) or all tumor samples (AUC = 0.9152) (Fig. 7C).

Discussion

It was reported that lncRNAs promotes tumorigenesis and development by competitively combining the shared miRNAs with the target gene sponge through the ceRNA mechanism [13, 16, 17]. Tumor-infiltration immune cells, which are an important part of tumors, had been revealed to play a role in tumorigenesis, progression, metastasis and drug resistance by many studies [18] [19]. GC is a heterogeneous disease. Herein, we divided GC samples into the whole group, the intestinal group and the diffuse group, which allowed us to study the common mechanisms and specific mechanisms between two Lauren classification. In our study, we constructed Lauren classification specific ceRNA network and GC ceRNA network based on differential expression, WGCNA, negatively correlated RNAs pairs. Then, correlation analyses markers of ceRNA networks with immune cells were carried out. The high AUC values of these markers of ceRNA networks identifying tumors proved their clinical application.

Based on ceRNA theory and supplementary theory [12, 20, 21], We use $p < 0.01$ and $\log_2FC > 1$ to identify differentially expressed RNAs because this minimizing interferential RNA pairs and avoiding missing important RNA pairs. WGCNA can make us to further screen out disrelated RNAs with tumor. Then, we performed differential expression analysis between two Lauren classifications and many cancer-related and immune-related KEGG enrichment pathways (corrected $p < 0.05$) indicated that there are also differences in these pathways between IGC and DGC (detailed pathways results in Table S2). The differences in cancer-related pathways prove that there are differences in many cancer characteristics between two sub-types, and also support the strong heterogeneity of GC. The DERNAs between two sub-types were took into consideration to further identify subtype-specific network and common network. we thought that even the common networks in two subtypes must have differences in the level of expression and subtype-specific networks were even more so. The violin plot illustrated some immune cells, such as T cells gamma delta, NK cells resting, Macrophages M0, Macrophages M2 and Mast cells activated, differed in IGC and DGC. The Lauren classification should be considered into immune therapy of GC.

The mRNAs in common ceRNA network were enriched in Regulation of actin cytoskeleton and MicroRNAs in cancer, which manifested that targeted mRNAs of common network were regulated by relevant miRNAs. In the ceRNA network, CFL2, FGF2 and ZEB1 were reported to be miRNA's target associated with GC [22] [23] [24]. ZFPM2 and ZEB1 consisting in "MicroRNAs in cancer" pathway had common upstream lncRNA, namely SNHG14. It was reported to contribute to GC development through targeting miR-145/SOX9 axis [25]. However, it is possible that there are other regulation axes.

In the IGC-specific ceRNA network, hsa-miR-106b-5p, hsa-miR-17-5p and hsa-miR-93-5p have many targets, indicating their important roles. They were primarily sponged by three lncRNAs (HAND2-AS1, SNHG14 and PWAR6). So, HAND2-AS1, SNHG14 and PWAR6 deserve further investigation to verify their effect in ceRNA network of IGC. In the DGC-specific ceRNA network, hsa-miR-148b-3p and hsa-miR-16-5p respectively have seven and four targets. But lncRNAs sponging miRNAs only have SNHG14 and AC007392.3, also proving SNHG14's vital function in GC.

It was reported that Mast cells and Macrophages made effect in GC development[26] [27]. Mast cells and Macrophages significantly differed not only between the tumor and normal tissue, but also between the two subtypes. Like the expression level of two subtypes' cancer-related pathways, a similar pattern exists for two types of immune cells. And these two kinds of immune cells are significantly associated with multiple components in ceRNA network. Further studies on these two types of immune cells are necessary, which can help us to discover new therapeutic method or mechanism of GC.

LncRNAs and mRNAs were reported to be prognostic and diagnostic biomarkers of many cancers. Our results demonstrated lncRNAs and mRNAs of ceRNA network in all three groups showed good performance in tumor diagnosis. Perhaps they also guide clinical decision-making.

It is important to note that our results have not been validated by well-designed experiment and that is our prospective work. The public data we use are western demographic data, a conclusion should be made with caution in Asian countries. The number of DGC was relatively small, rendering the results less reliable.

Conclusion

we constructed Lauren subtype ceRNA networks and common network based on differential expression analyses, negatively correlated RNAs pairs and WGCNA. SNHG14, Mast cells and Macrophages maybe have crucial function in GC. LncRNAs and mRNAs had excellent effect in tumor diagnosis.

Methods

5.1 Data collection and differential gene expression analysis: GC gene expression data of fragments per kilobase of transcript per million mapped reads (FPKM) and count were obtained from TCGA database by using the "TCGAbiolinks" package in R [28], a total of 375 GC samples and 32 adjacent tissue samples. The RNA-seq data was loaded with FPKM, which were converted to transcripts per million (TPM) after removing duplicated genes and zero expression genes. We matched and selected lncRNA and mRNA using GENCODE Release 22 (https://www.gencodegenes.org/human/release_22.html) [29] as gene annotation, which consisted with the official pipeline of TCGA data portal. Based on the Lauren classification information, all GC patients were divided into the whole group, the intestinal group and the diffuse group. The miRNA data transferred by $\log_2(\text{Reads per million mapped reads} + 1)$ was downloaded

in UCSC Xena (<http://xena.ucsc.edu/>) [30]. We removed the miRNAs having zero expression more than 20% samples.

The Limma package in R was utilized to screen the differentially expressed miRNAs (DEmiRNAs) [31] and the DESeq2 and EdgeR package in R was used to identify the differentially expressed lncRNAs (DElncRNAs) and mRNAs (DEmRNAs) between tumor and adjacent tissues [32]. DESeq2 and EdgeR algorithm were used for identifying DElncRNAs and DEmRNAs between IGC and DGC. The final differentially expressed RNAs (DERNAs) were obtained based on the two algorithm results. The lncRNAs and mRNAs with the false discovery rate (FDR) $P < 0.01$ and the log (fold change) > 1.0 or < -1.0 were only regarded as differentially expressed RNAs and the miRNAs with false discovery rate (FDR) < 0.05 and the \log_2 fold change (\log_2FC) > 1.0 or < -1.0 as differentially expressed miRNAs.

5.2 WGCNA based on DElncRNAs and DEmRNAs: The lncRNAs and mRNA data profiles identified above were merged into one profile. Weighted genes co-expression network analysis was performed using the WGCNA R package [33]. We set minModuleSize as 30 and no mixModuleSize. Firstly, we removed the significant outliers and screened out appropriate soft threshold power. Then, the Pearson's correlation matrices were calculated for all the paired RNAs and a weighted adjacency matrix was constructed. Finally, we performed the correlation analysis to the module eigengenes as the first principal component and clinical traits including pathologic stage and tumor stage (T stage). All processes above were carried out in intestinal group and diffuse group.

5.3 Prediction of lncRNA-miRNA and miRNA-mRNA interactions: The lncRNA-miRNA interaction pairs were predicted by DEmiRNAs in LncBase Predicted v.2 with the threshold of 0.9 [34]. The miRNA-mRNA interaction pairs were predicted by DEmiRNAs in miRTarBase [35] and TarBase V.8 [36] in which there are experimental validation miRNA-mRNA interaction pairs. The correlation analyses were performed on all DElncRNA-DEmiRNA pairs and DEmiRNA-DEmRNA pairs, the reason is that their expression is negatively related. We thought the RNA-RNA pairs with $R < -0.3$ and $p < 0.05$ are negatively related and taken in consideration for subsequent analyses. Then, we took the intersection of DElncRNAs and DEmRNAs of tumor and normal tissue, DElncRNAs and DEmRNAs of intestinal group and diffuse group, RNAs related with stage from WGCNA and negatively related RNA interaction pairs. Finally, the filtered RNA-RNA interaction pairs were obtained. All processes above were carried out in intestinal group and diffuse group. The constructed ceRNA networks were visualized by Cytoscape V3.7.2 [37].

5.4 Functional enrichment analysis: To illustrate the functional annotations implicated with the DEmRNAs of ceRNA networks between two Lauren classifications, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using KOBAS 3.0 online database [38]. Pathways with adjusted $p < 0.05$ were considered significantly. Top 10 pathways were showed using circle pots.

5.5 CIBERSORTx estimation: The CIBERSORTx algorithm with the B-mode of batch correction mode, relative mode and 1000 permutations was used to estimate the fraction of 22 immune cell types in all GC

and adjacent normal tissues [39]. The Wilcoxon test was used to look for significantly differential immune cells between adjacent normal tissues and tumor.

5.6 Correlation analysis: We merged lncRNA and mRNA profiles of ceRNA network into one profile. Then, the significantly differential immune cell profiles were added into above profile. The correlation analysis of lncRNAs, mRNAs and immune cells with Pearson was performed in R.

5.7 Performance of distinguishing tumor of members in the ceRNA network: In order to avoid overfitting of predicting model, the samples were divided into two groups on the basis of 7:3, one in front was named as the train cohort and one in back was named as the test cohort. We trained classifiers in the train cohort using lncRNAs and mRNAs of ceRNA networks and examined the performance of classifiers in the test cohort. The receiver operating characteristic curves (ROC) were used to visualize the prediction effect and the area under curve (AUC) was estimated.

Abbreviations

gastric cancer = GC, the World Health Organization = WHO, competitive endogenous RNA = ceRNA, long noncoding RNA = lncRNA, microRNA = miRNA, messenger RNA = mRNA, weighted genes co-expression network analysis = WGCNA, The Cancer Genome Atlas = TCGA, support vector machines = SVM, intestinal-type gastric cancer = IGC, diffuse-type gastric cancer = DGC, fragments per kilobase of transcript per million mapped reads = FPKM, transcripts per million = TPM, differentially expressed miRNAs = DE miRNAs, differentially expressed lncRNAs = DE lncRNAs, differentially expressed mRNAs = DE mRNAs, differentially expressed RNAs = DE RNAs, false discovery rate = FDR, \log_2 fold change = \log_2FC , tumor stage = T stage, Kyoto Encyclopedia of Genes and Genomes = KEGG, the receiver operating characteristic curves = ROC, the area under curve = AUC

Declarations

Availability of data and materials: Raw stomach cancer RNA-seq data can be obtained at The Cancer Genome Atlas (TCGA) and named as TCGA-STAD. The miRNA data of stomach cancer was downloaded in UCSC Xena (<http://xena.ucsc.edu/>) and named as GDC TCGA Stomach Cancer (STAD). We downloaded RNA annotation (GENCODE Release 22) from GENCODE (https://www.encodegenes.org/human/release_22.html).

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Figures

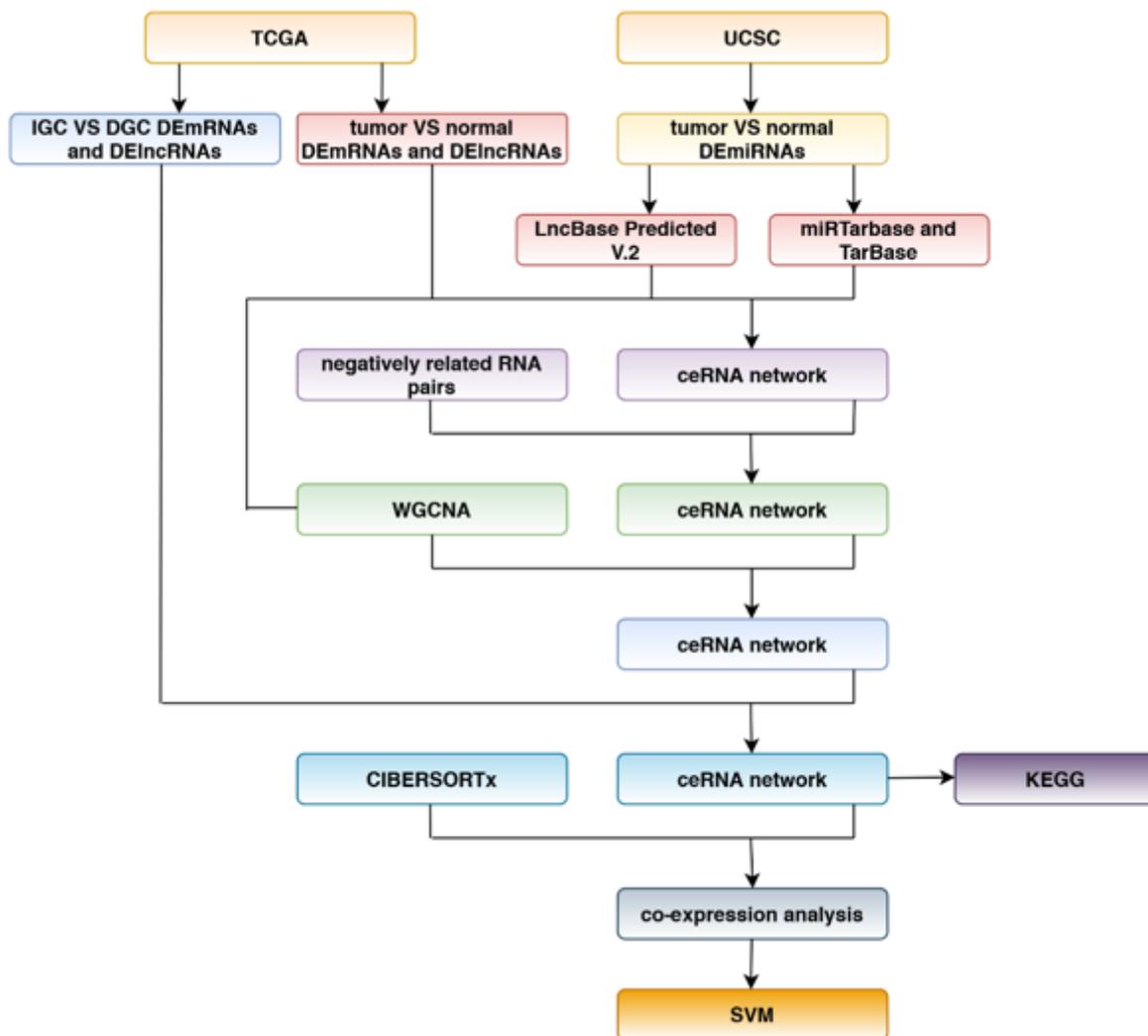


Figure 1

Workflow of our bioinformatics analyses.

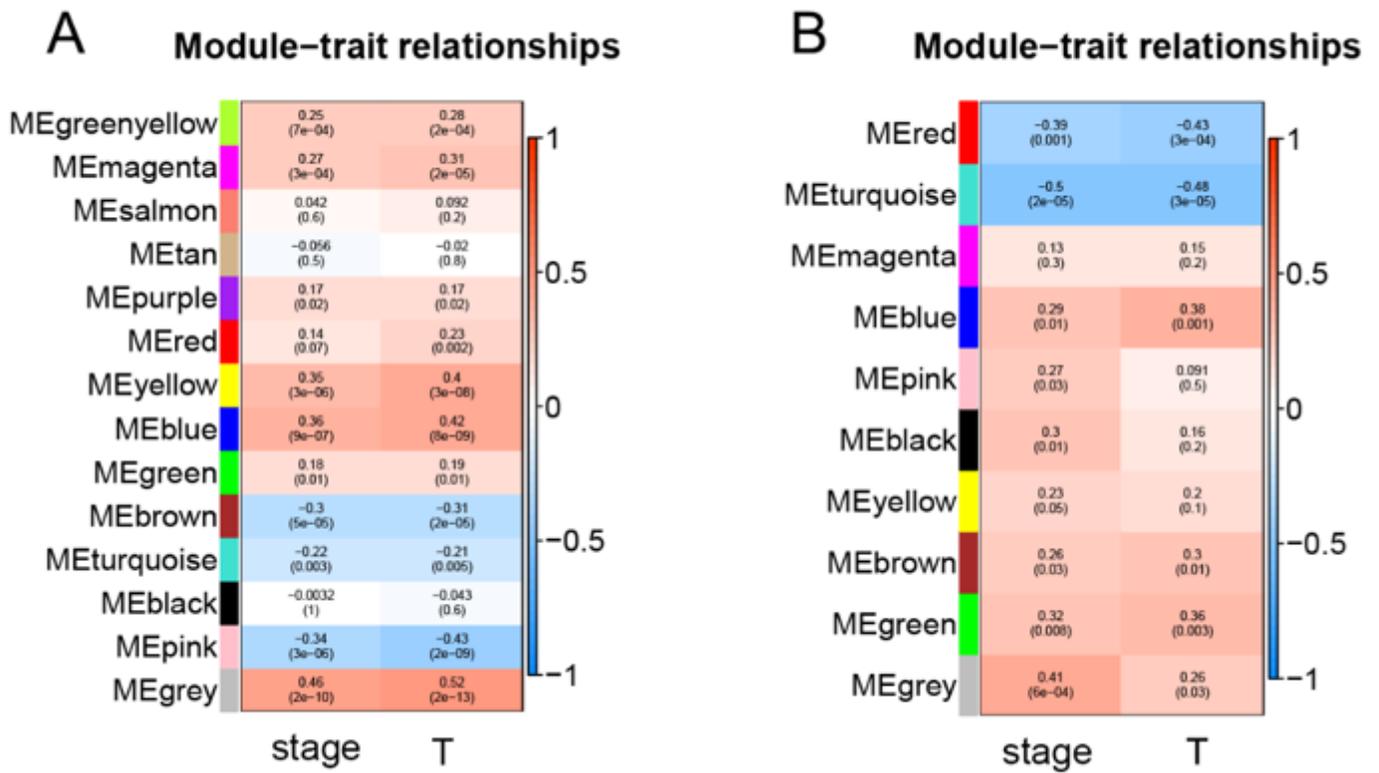


Figure 2

Weighted genes co-expression network analysis. (A) in the intestinal group. (B) in the diffuse group.

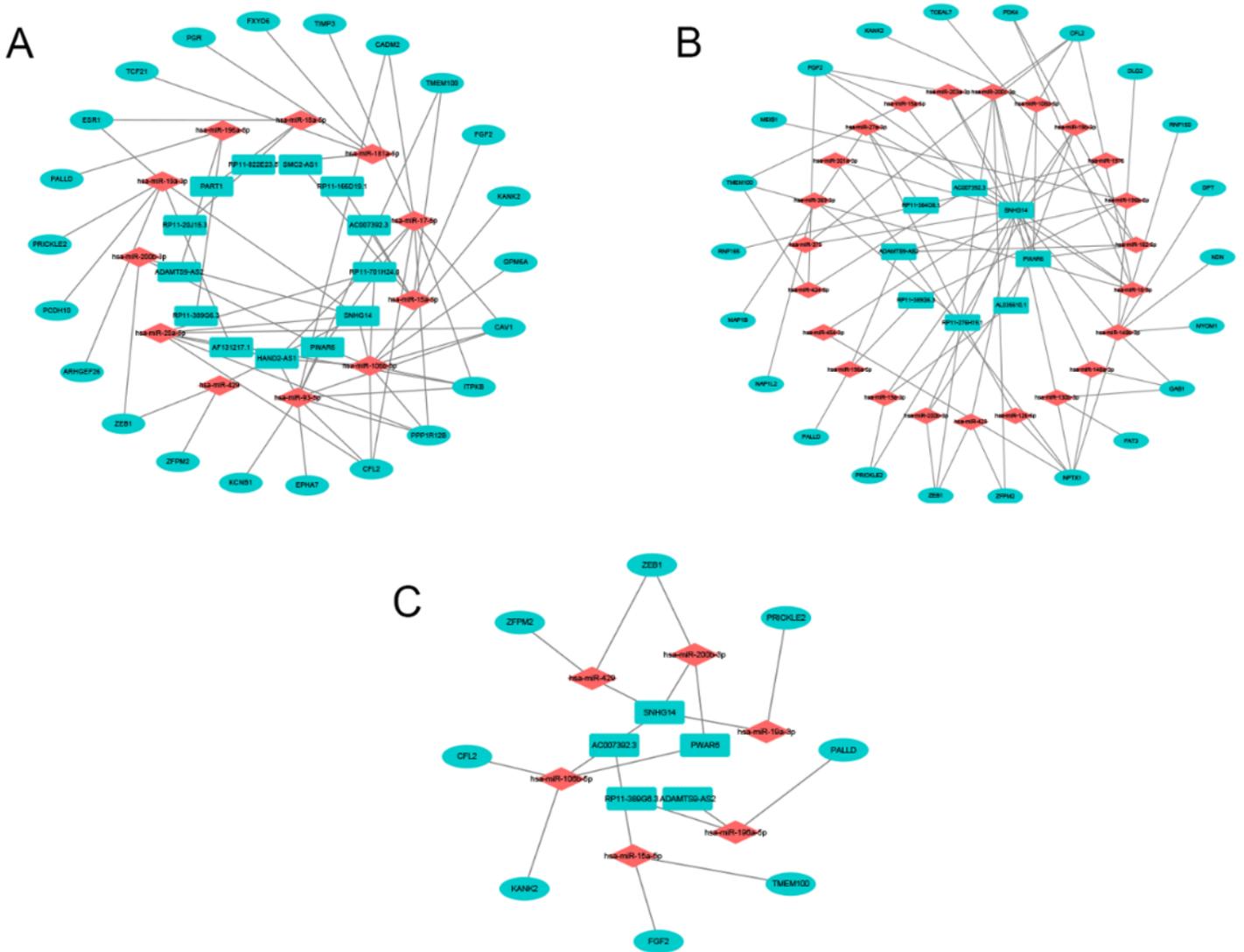


Figure 3

The ceRNA network. (A) in in the intestinal group. (B) in the diffuse group. (C) in the whole group. The red nodes indicate upregulated expression, whereas the blue ones stand for downregulated expression. Circle stands for the mRNAs, while square is indicative of the lncRNAs, diamond suggests the miRNAs, and the gray edges represent the lncRNA-miRNA-mRNA interactions.

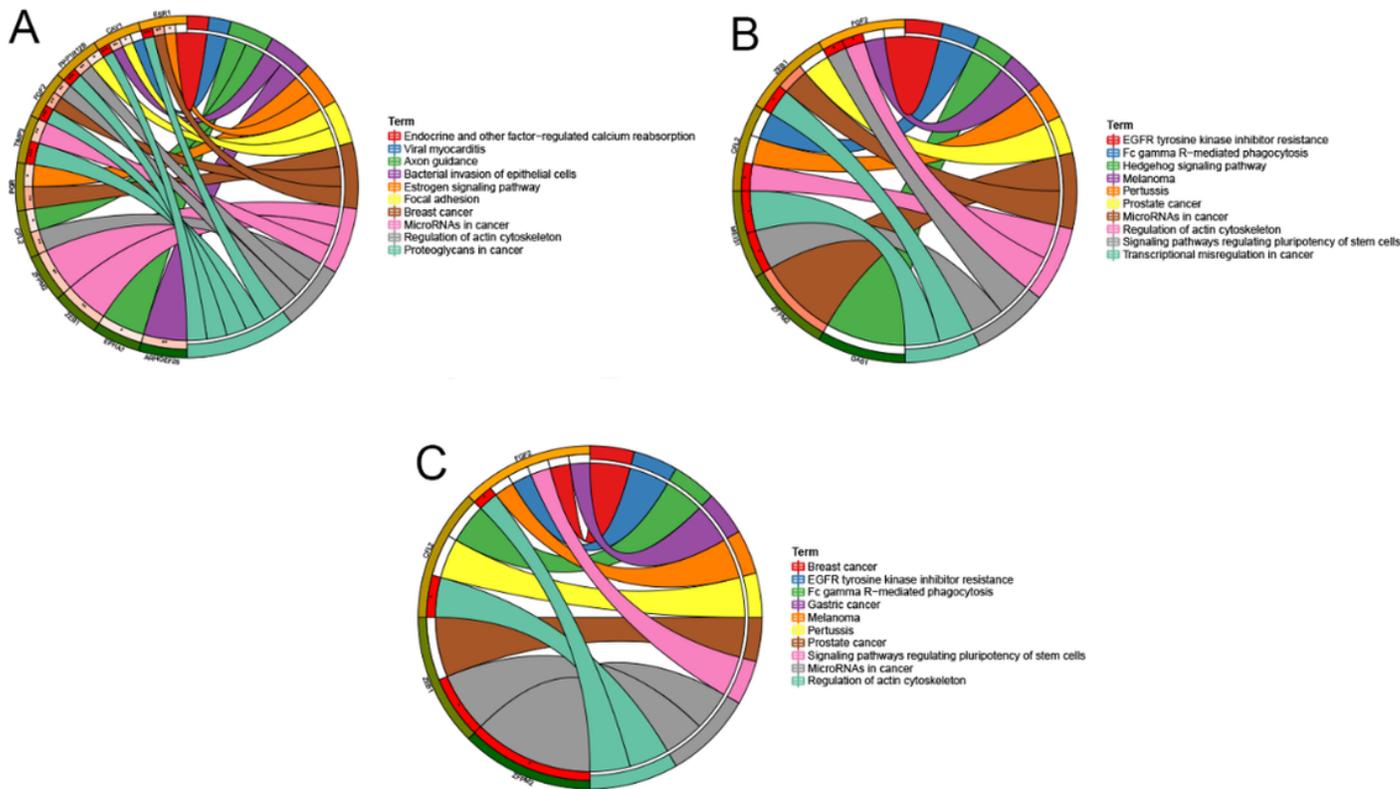
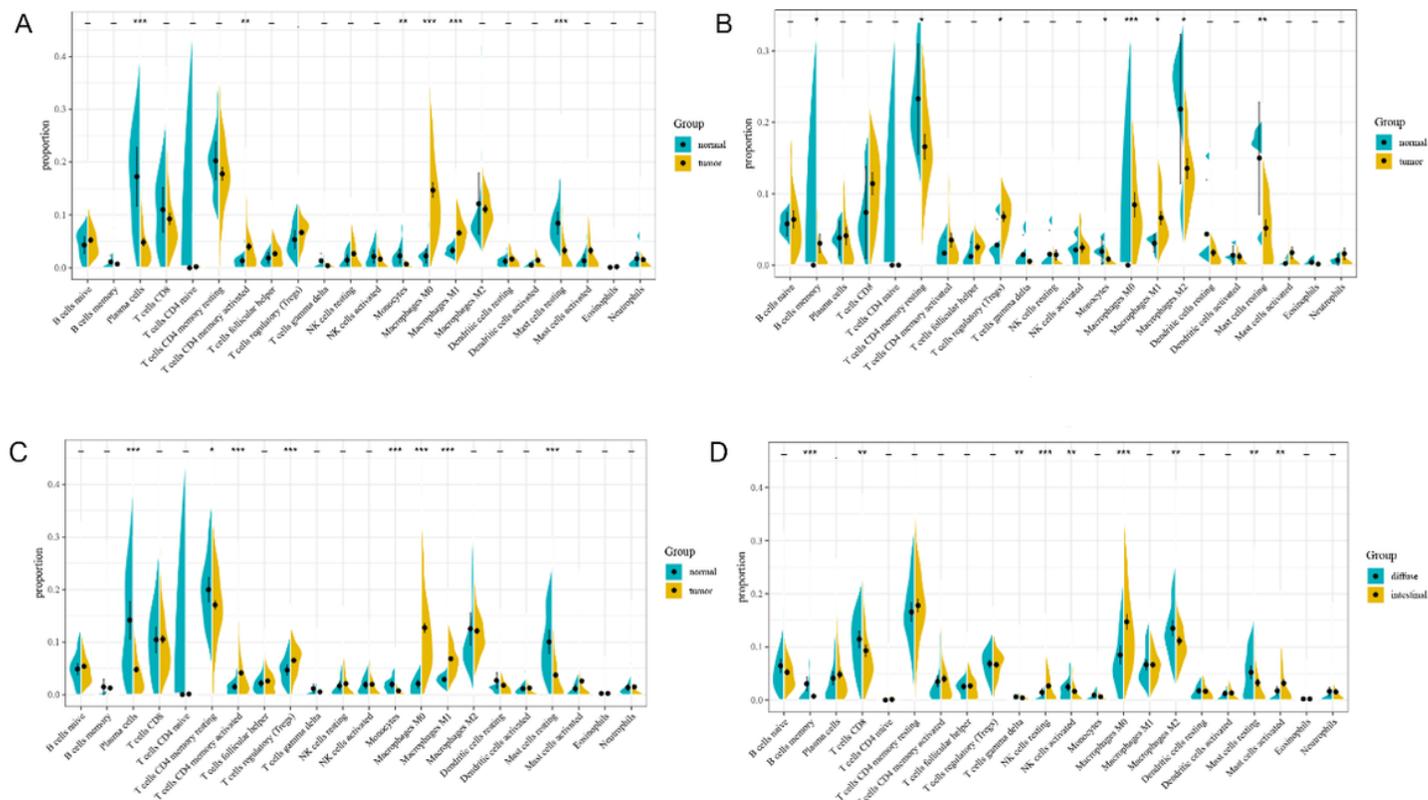


Figure 4

KEGG terms enrichment analyses of mRNAs in the ceRNA network. (A) in the intestinal group. (B) in the diffuse group. (C) in the whole group.



diffuse group. (C) in the whole group.

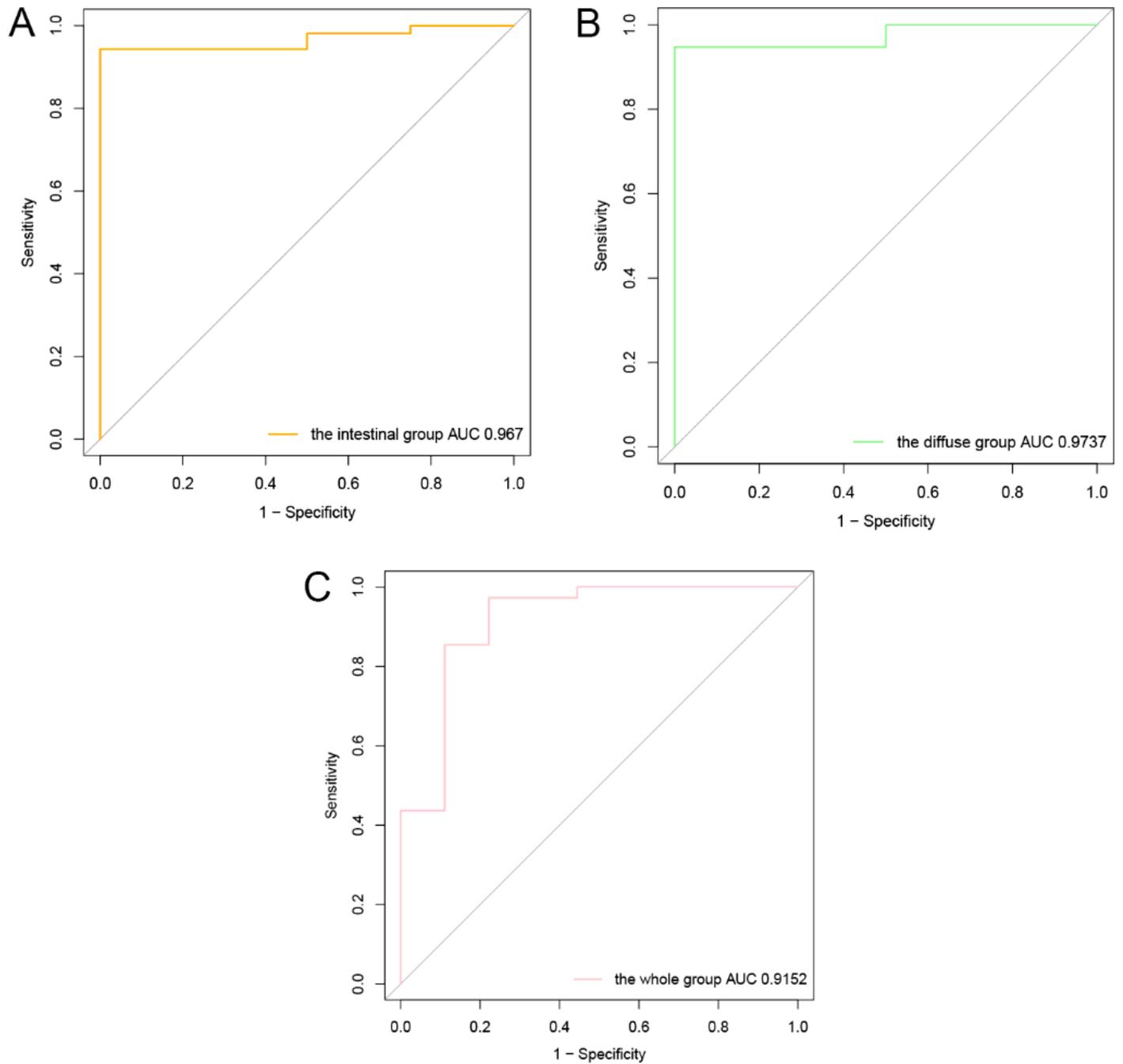


Figure 7

The areas under the receiver operating characteristic curves of lncRNAs and mRNAs in ceRNA networks. (A) RNAs in intestinal subtype-specific ceRNA network. (B) RNAs in diffuse subtype-specific ceRNA network. (C) RNAs in common ceRNA network.

Supplementary Files

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- [TableS1.xlsx](#)
- [TableS2.xlsx](#)