

Construction of ceRNA co-expression network and screening of molecular targets in colorectal cancer

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Research article

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Abstract

Objective to screen some RNAs that correlated with colorectal cancer (CRC). Methods Differentially expressed miRNAs, lncRNAs, and mRNAs between cancer tissues and normal tissues in CRC were identified using data from the Gene Expression Omnibus (GEO) database. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and protein-protein interactions (PPIs) were performed to do the functional enrichment analysis. And a lncRNA-miRNA-mRNA network was constructed which correlated with CRC. RNAs in this network were subjected to analyze the relationship with the patient prognosis.

Results A total of 688, 241, and 103 differentially expressed genes (diff-mRNA), diff-lncRNA, and diff-miRNA were obtained between cancer tissues and normal tissues. A total of 315 edges were obtained in the ceRNA network. lncRNA RP11-108K3.2 and mRNA ONECUT2 correlated with prognosis.

Conclusion The identified RNAs and constructed ceRNA network could provide great sources for the researches of therapy the CRC. And the lncRNA RP11-108K3.2 and mRNA ONECUT2 may serve novel prognostic predictor of CRC.

Introduction

Colorectal cancer (CRC) is a common malignant tumor in the gastrointestinal tract^[1]. The initial syndrome of CRC are not conspicuous, but with the development of the tumor, the patient will show changes in bowel habits, blood in the stool, diarrhea, alternating diarrhea and constipation, local abdominal pain and other symptoms. Patients with advanced symptoms often show syndromes such as anemia and weak^[2]. CRC is the third most common malignant tumor around the world and its mortality rate is extremely high^[3]. Considering the great threat to human health of CRC, a series of new diagnostic and therapeutic methods have emerged. CRC is a complex disease involving the expression and structure of genes^[4]. More and more studies have shown that miRNA can play a crucial aspect in cancer progression by regulating its related targets, including mRNA, lncRNA, etc^[5].

lncRNA is a non-coding RNA with a length of more than 200 nucleotides, which acts a pivotal part in abounding actions such as dose compensation effect, epigenetic regulation, cell differentiation regulation and cell cycle regulation^[6, 7]. It has been reported that upregulation of lncRNA FOXD3-AS1 suggests a lower survival rate in CRC patients. Experimental results showed that FOXD3-AS1 was overexpressed in CRC tissues and cells. Down-regulation of FOXD3-AS1 expression in vitro can promote cell proliferation, invasion and migration, and promote apoptosis^[8]. lncRNA-ATB and lncRNA-CCAT have strong accuracy in distinguishing CRC patients from healthy individuals^[9]. miRNAs are endogenous non-coding RNAs with managerial actions, with a length of approximate 22 nucleotides that are involving post-transcriptional gene expression regulation in animals and plants^[10]. miRNA-149 can be used as a target miRNA for identifying single bases in the serum of healthy and cancer patients. This method is direct and sensitive and can be used as an early diagnostic tool for colorectal cancer^[11]. The expression of miR-139-5p was down-regulated in the CRC cell line compared to the ordinary human colonic

mucosal epithelial cell line NCM460. The study subsequently also demonstrated that overexpression of miR-139-5p in colon tumor cell lines significantly inhibited proliferation of cells *in vivo* and *in vitro*. The final study found that miR-139-5p regulates chronic inflammation by inhibiting NF- κ B activity, thereby inhibiting cell proliferation and invasion in CRC [12].

A series of studies have shown that miRNAs can silence gene by binding to mRNA, and lncRNA can regulate gene expression level relying on competitively binding miRNAs [13, 14]. At this study, a lncRNA-miRNA-mRNA co-expression network was constructed using two GEO datasets to screen RNAs that may be associated with CRC, and supply a novel method for the diagnosis and therapy of CRC (Figure 1).

Materials And Methods

Data collection

The lncRNA/mRNA profile data GSE126092 was downloaded from Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/gds/?term> =). And the extracted data were produced by the platform of Agilent-074348 Human LncRNA v6 4 180K (GPL21047, Probe Name Version). This dataset contained the data of lncRNA/mRNA expression profiles in 10 colorectal cancer (CRC) tissues and their corresponding normal-appearing tissues (NATs). The miRNA profile data GSE126093 was also extracted from GEO database. The data were produced by the platform of Exiqon miRCURY LNA microRNA array, 7th generation (GPL18058, miRBase v18, condensed Probe_ID version). This dataset contained ten cases of CRC tissues and their corresponding NATs.

Data preprocessing and screening of differentially expressed mRNA, lncRNA and miRNA (diff-mRNA, diff-lncRNA, diff-miRNA)

Limma package was used to preprocess the downloaded raw data and screen the differentially expressed mRNA, lncRNA and miRNA. The preprocess process included background correction, normalization, and concentration prediction. The matrix data was combined with the chip platform annotation file to map the probe to the symbol. For multiple probes corresponding to a equal symbol, the final expression was decided by the mean of probes. Differential expression analysis of tumor vs. control was performed on the samples using classical Bayesian test, and corrected with Benjamini/Hochberg. P-value 0.05 and $\log_2 FC$ 2 were taken as the cuts-off for the screening of diff-mRNA, diff-lncRNA, diff-miRNA.

The heatmaps of diff-mRNA, diff-lncRNA, diff-miRNA were performed using heatmap (Version: 1.0.10 <https://cran.r-project.org/web/packages/pheatmap/index.html>).

Analysis of protein-protein interactions (PPIs) in diff-mRNA

The PPIs of diff-mRNA analysis was performed using STRING (Version 10.0, <http://www.string-db.org/>) database with the score = 0.7, which was visualized by Cytoscap (version 3.2.0, <http://www.cytoscape.org/>). Furthermore, based on MCODE (Version 1.4.2, <http://apps.cytoscape.org/apps/MCODE>) with the score 10, the network modules were obtained and evaluated from the original PPIs network.

Functional enrichment analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for diff-mRNA, mRNAs in modules that were significantly clustered in PPIs, mRNAs in ceRNA network were carried out, via the R pakage clusterprofiler (Version 2.4.3, <http://bioconductor.org/packages/3.2/bioc/html/clusterProfiler.html>). In KEGG pathway enrichment analysis, p-value < 0.05, and gene count > 2 were chosen as the cuts-off criteria.

Prediction of diff-miRNA-diff-mRNA and diff-miRNA-diff-lncRNA

The miRWalk 2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) was used to presume the target miRNAs of the diff-miRNA. The selected genes must have results in six of the eight databases (miRanda, miRWalk, miRDB, Pictar2, miRMap, RNA22, PITA, Targetscan). miRanda (Version:3.3a, <https://omictools.com/miranda-tool>) was used to analyze potential diff-miRNA-diff-lncRNA binding sites with the score 150 and energy 20.

Co-expression analysis of dif-lncRNA and dif-mRNA

The Pearson correlation coefficient (PCC) was calculated between lncRNA and mRNA. And the PPC 0.95 and p-value 0.05 were considered to be meaningful correlation. Cytoscape was used to illustrate the co-expression network.

Survival analysis

The colon cancer tumor samples from which mRNA, lncRNA, miRNA and survival prognosis messages were extracted from the TCGA database, and the expression values and survival prognosis information of all node elements in the lncRNA-miRNA-mRNA network were extracted from the TCGA colon cancer samples. K-M survival curve was performed using Survival (Version: 2.42-6, <https://cran.r-project.org/web/packages/survival/index.html>). P-value 0.05 was considered as the significant threshold.

Results

Clustering of differentially expressed RNAs in CRC tissues

A total of 688, 241, and 103 differentially expressed mRNA (diff-mRNA), diff-lncRNA, and diff-miRNA were obtained, respectively, which including 266 up-regulated diff-mRNA, 462 down-regulated diff-mRNA, 85 up-regulated diff-lncRNA, 156 down-regulated diff-lncRNA, 103 up-regulated diff-miRNA and 95 down-regulated diff-miRNA. As shown in Figure 2, the two groups of samples could be separated significantly.

The top 10 expression diff-mRNA were MMP7, LEMD1, CLDN1, SLC01B3, REG1A, COL10A1, ETV4, COL11A1, APOBEC1, and LGR5. The top 10 expression of the following diff-lncRNA were CCAT1, LOC401585, AC123023.1, UCA1, EVADR, RP11-132A1.4, CTD-2008A1.3, SNAR-H, SNAR-C1, and SNAR-E. the top 10 expression diff-miRNA were hsa-miR-31-5p, hsa-miR-224-5p, hsa-miR-1244, hsa-miR-188-5p, hsa-miR-764, hsa-miR-301b, hsa-miR-19b-1-5p, hsa-miR-3648, hsa-miR-452-3p, and hsa-miR-3157-3p. The details were shown in Supplementary table 1, 2 and 3.

Screening the cell pathways with regard to differentially expressed mRNA in CRC

KEGG pathway analysis was performed to make a deep understanding between the diff-mRNA and cell pathways. The result indicated that all 266 up-regulated diff-mRNA were enriched in 10 pathways, including cell cycle, oocyte meiosis, progesterone-mediated oocyte, cytokine-cytokine receptor interaction etc. And the 462 down-regulated diff-mRNA were enriched in 21 pathway, including hypertrophic cardiomyopathy, dilated cardiomyopathy, cGMP-PKG signaling pathway, cAMP signaling pathway etc. (Table 1).

PPIs network with regard to Differentially expressed mRNAs in CRC

PPIs network was constructed, which integrates large amount of known and predicted interactions of proteins. As shown in Figure 3, 354 nodes and 1,500 edges were included in the network. Module analysis was performed using MCODE, and two modules were obtained (module-A, module-B). Module-A (score = 32.778) was constructed with 37 nodes and 590 edges, and the module-B (score = 15) was constructed with 15 nodes and 105 edges (Figure 4A, 4B). KEGG analysis was also performed. The result indicated that 37 diff-mRNA in Module-A were enriched in 4 pathways, and 15 diff-mRNA in Modules-B were enriched in 8 pathways (Table 2).

miRNA-lncRNA/mRNA prediction and lncRNA-mRNA co-expression

The top 10 up-regulated and all 8 down-regulated diff-miRNA were predicted using miRWALK 2.0 database. The edges were screened which the target genes were diff-mRNA. And a total of 308 edges of dif-miRNA- diff-mRNA were obtained finally, including 15 diff-miRNA and 177 diff-mRNA. Thirty-nine

edges of lncRNA-miRNA were obtained including 10 diff-miRNA and 33 diff-lncRNA. Furthermore, 143 target genes regulated by 10 miRNAs in the lncRNA-miRNA regulatory network were screened and co-expressed with 33 lncRNAs in the lncRNA-miRNA regulatory network. Based on the screening threshold, 77 edges of positive correlation lncRNA-mRNA were obtained.

lncRNA-miRNA-mRNA network

According to the whole relationship of edges above, the lncRNA-miRNA-mRNA edges were further collated. A total of 315 edges were obtained, of which 77 lncRNA-mRNA positive correlation co-expression edges, 199 miRNA-mRNA regulatory edges and 39 lncRNA-miRNA regulatory edges. And all the edges contained 186 nodes, 7 up-miRNAs, 3 down-miRNAs, 34 up-mRNAs, 109 down-mRNAs, 16 up-lncRNAs, and 17 down-lncRNAs.

lncRNA-miRNA-mRNA network was shown in Figure 5, and the nodes in the network was shown in Supplementary Table 4. The diff-mRNA in lncRNA-miRNA-mRNA network were analyzed using KEGG pathway enrichment analysis, of which 15 pathways (Table 3).

Differentially expressed RNAs with regard to the prognosis of CRC

All the nodes in the Figure 6 were used to do the survival analysis, of which a lncRNA RP11-108K3.2 and one mRNA ONECUT2 correlated with prognosis.

Discussion

In this study, to screen for RNAs and pathways associated with CRC, a lncRNA-miRNA-mRNA expression network was constructed using two datasets downloaded from the GEO database. A total of 688 diff-mRNA, 241 diff-lncRNA, and 103 diff-miRNA were identified at the present study. A functional enrichment analysis of these 688 diff-mRNA revealed that they were mainly enriched in 21 pathways. A PPI network analysis was also performed on these diff-mRNA. Finally, a ceRNA network was constructed and a survival analysis was performed on the nodes to obtain two prognostic-related RNAs. The expression levels of RP11-108K3.2 and ONECUT2 were both up-regulated in CRC, and low levels were associated with better prognosis, suggesting that they may play a positive role in CRC.

LncRNA can participate in the initiation and development of many diseases by directly regulating proteins or indirectly regulating the target genes of related miRNA. The result of ceRNA showed that RP11-108K3.2 was regulated by hsa-miR-224-5p. Study has shown that the great expression level of RP11-108K3.2 correlated with low overall survival of CRC, and this result was consistent with our study [15]. In a study on lung adenocarcinoma, the researchers study 346 differentially expressed lncRNAs, of which

RP11-108K3.2 is highly expressed in lung cancer tissues [16]. Furthermore, *hsa-miR-224-5p is up-regulated both in adeoma and cancer tissues* [17]. A study has been revealed that *hsa-miR-224-5p could suppress the cell growth of two oral squamous cell carcinoma cell lines (SCC4, SCC15)*. However, when *hsa-miR-224-5p and pcDNA3.1-CT-GFP-FTH1P3 are co-transfected, the growth inhibition of hsa-miR-224-5p on oral squamous cell carcinoma will be reversed* [18]. In this study, the expression of *RP11-108K3.2 and hsa-miR-224-5p were both up-regulated in CRC samples*. This result indicate that *lncRNA RP11-108K3.2 and hsa-miR-224-5p may play a same role as hsa-miR-224-5p and FTH1P3*.

ONECUT2 is a member of the onecut2 transcription factor family that interacted with hsa-miR-139-5p, hsa-miR-188-5p, hsa-miR-19b-1-5p, hsa-miR-31-5p, and hsa-miR-497-5p in ceRNA network in this study [19]. It has been reported that ONECUT2 acts a critial position on CRC gene network and is significantly associated with the development of cance. Interfernce with endogenous ONECUT2 expression inhibited the CRC cell line SW620 cell migration [20]. A series of experiments have shown that hsa-miR-139-5p acts as a crutial part in colon tumor. Compared with normal tissues, hsa-miR-139-5p expression level is significantly declined in colon tumor, and its expression level is significantly correlated with tumor stage. Subsequently, experiments have shown that the direct object of hsa-miR-139-5p is BCL2, and the expression of BCL2 is negatively correlated with the expression of hsa-miR-139-5p. The tumor metastasis and drug sensitivity of CRC could be diminished by hsa-miR-139-5p targeting the BCL2 pathway [21]. In present study, hsa-miR-139-5p was interacted with ONECUT2, and its expression level was negatively connected with the expression level of the later one. These two RNAs may have a similar mechanism with hsa-miR-139-5p and BCL2. hsa-miR-31-5p as a infrequently expressed miRNA, was associated with stage when assessing CRC cases. The up-regulated hsa-miR-31-5p makes a more advanced disease stage more likely than a lower disease stage [22]. At this research, the expression level of hsa-miR-31-5p was higher in CRC tissues than in control ($\log FC = 9.153176$). In addition to being significantly up-regulated in CRC tissues, it has been reported to be significantly up-regulated in uterine cervical cancer tissues [23].

In conclusion, at the present study, several CRC-related diff-miRNAs, diff-lncRNAs, and diff-mRNAs were obtained. A ceRNA network was also constructed to analyze the crosstalk among the identified diff-miRNAs, diff-lncRNAs, and diff-mRNA. The lncRNA RP11-108K3.2 and mRNA ONECUT2 in the network may play crutial role in CRC, with their low expression levels being correlated with better prognosis. Although the mechanism of RP11-108K3.2 and ONECUT2 remain to be reveald, but both of two may use as a novel clinical predictors of CRC. Further experimental studies are needed to reveal the mechanism of RP11-108K3.2 and ONECUT2 in CRC in the future.

Declarations

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing Interests

The authors declare that no conflicts of interest exist.

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- Authors' Contributions

All authors participated in the conception and design of the study;

- Conceived the manuscript: Han Shuwen;
- Processed the data: Wang Zhanwei and Yang Xi;

Analyzed the data: Zhao Hui and Liu Jin and Zhuang Jing;

Wrote the paper: Zhao Hui and Zhuang Jing;

All authors read and approved the paper.

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Not Applicable.

Availability of data and materials

The datasets generated during the current study are not publicly available but identified and anonymized information is potentially available on reasonable request.

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Tables

Table 1. KEGG enrichment pathway.

	KEGG Pathway	Count	PValue
up-regulated	hsa04110:Cell cycle	15	1.09E-09
	hsa04114:Oocyte meiosis	10	1.98E-05
	hsa04914:Progesterone-mediated oocyte maturation	9	2.35E-05
	hsa04060:Cytokine-cytokine receptor interaction	14	2.56E-05
	hsa05322:Systemic lupus erythematosus	8	2.42E-03
	hsa05323:Rheumatoid arthritis	6	7.18E-03
	hsa05034:Alcoholism	8	1.09E-02
	hsa04062:Chemokine signaling pathway	8	1.41E-02
down-regulated	hsa05132:Salmonella infection	5	2.74E-02
	hsa03460:Fanconi anemia pathway	4	3.64E-02
	hsa05410:Hypertrophic cardiomyopathy (HCM)	9	7.97E-04
	hsa05414:Dilated cardiomyopathy	9	1.30E-03
	hsa04022:cGMP-PKG signaling pathway	12	2.32E-03
	hsa04024:cAMP signaling pathway	13	4.68E-03
	hsa04080:Neuroactive ligand-receptor interaction	16	4.74E-03
	hsa04020:Calcium signaling pathway	12	6.04E-03
	hsa04724:Glutamatergic synapse	9	8.61E-03
	hsa04261:Adrenergic signaling in cardiomyocytes	10	8.69E-03
	hsa04713:Circadian entrainment	8	1.06E-02
	hsa00980:Metabolism of xenobiotics by cytochrome P450	7	1.13E-02
	hsa04911:Insulin secretion	7	2.13E-02
	hsa00071:Fatty acid degradation	5	2.18E-02
	hsa04725:Cholinergic synapse	8	2.33E-02
	hsa04726:Serotonergic synapse	8	2.33E-02
	hsa00830:Retinol metabolism	6	2.34E-02
	hsa04978:Mineral absorption	5	2.54E-02
	hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	6	2.79E-02
	hsa03320:PPAR signaling pathway	6	2.79E-02
	hsa04920:Adipocytokine signaling pathway	6	3.29E-02
	hsa04921:Oxytocin signaling pathway	9	3.82E-02
	hsa04728:Dopaminergic synapse	8	4.54E-02

Table 2. KEGG enrichment pathway of Modules.

	KEGG Pathway	Count	PValue
Module-A	hsa04110:Cell cycle	12	5.33E-17
	hsa04114:Oocyte meiosis	9	2.09E-11
	hsa04914:Progesterone-mediated oocyte maturation	7	1.57E-08
	hsa04115:p53 signaling pathway	3	9.04E-03

Module-B	hsa04062:Chemokine signaling pathway hsa04060:Cytokine-cytokine receptor interaction hsa05134:Legionellosis hsa04621:NOD-like receptor signaling pathway hsa05132:Salmonella infection hsa05323:Rheumatoid arthritis hsa04668:TNF signaling pathway hsa05200:Pathways in cancer	9 7 3 3 3 3 3 4	3.79E-11 7.28E-07 3.18E-03 3.42E-03 7.37E-03 8.26E-03 1.20E-02 2.17E-02
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Table 3. KEGG enrichment pathway of diff-mRNAs in ceRNA network.

KEGG Pathway	Pvalue	Count
hsa04724:Glutamatergic synapse	3.40E-03	5
hsa05410:Hypertrophic cardiomyopathy (HCM)	6.85E-03	4
hsa04725:Cholinergic synapse	1.76E-02	4
hsa04530:Tight junction	1.76E-02	5
hsa04726:Serotonergic synapse	1.92E-02	4
hsa05230:Central carbon metabolism in cancer	2.01E-02	3
hsa04360:Axon guidance	2.24E-02	5
hsa04920:Adipocytokine signaling pathway	2.35E-02	3
hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	2.62E-02	3
hsa04728:Dopaminergic synapse	2.93E-02	4
hsa00980:Metabolism of xenobiotics by cytochrome P450	3.01E-02	3
hsa04514:Cell adhesion molecules (CAMs)	3.94E-02	4
hsa05032:Morphine addiction	4.74E-02	3
hsa05414:Dilated cardiomyopathy (DCM)	4.74E-02	3
hsa05033:Nicotine addiction	4.93E-02	2

Highlights

1. A total of 688, 241, and 103 differentially expressed mRNA (diff-mRNA), diff-lncRNA, and diff-miRNA were obtained.
2. In the ceRNA network, a total of 315 edges were obtained.
3. lncRNA RP11-108K3.2 and mRNA ONECUT2 correlated with prognosis.

Figures

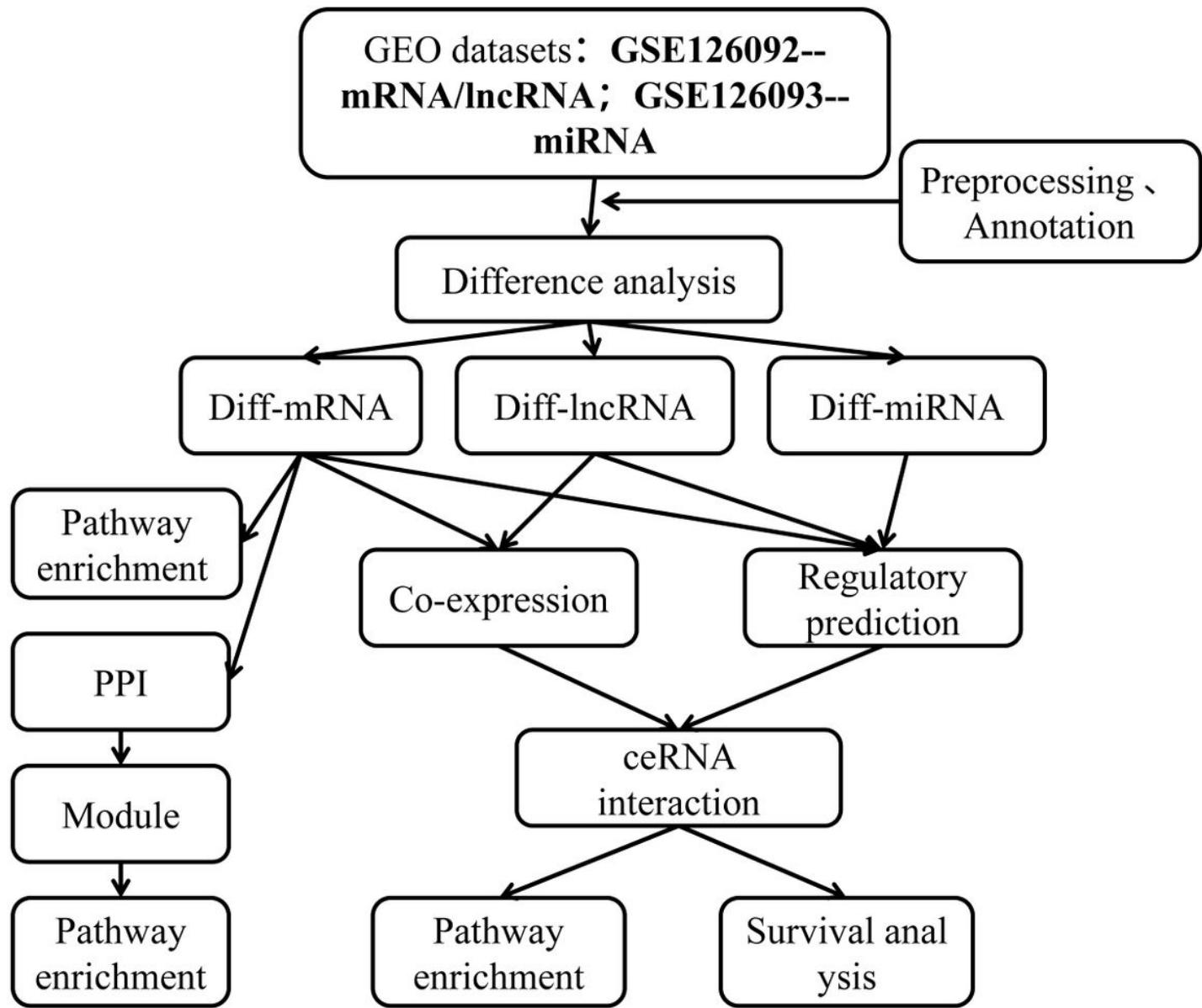


Figure 1

Diagram of bioinformatics analysis

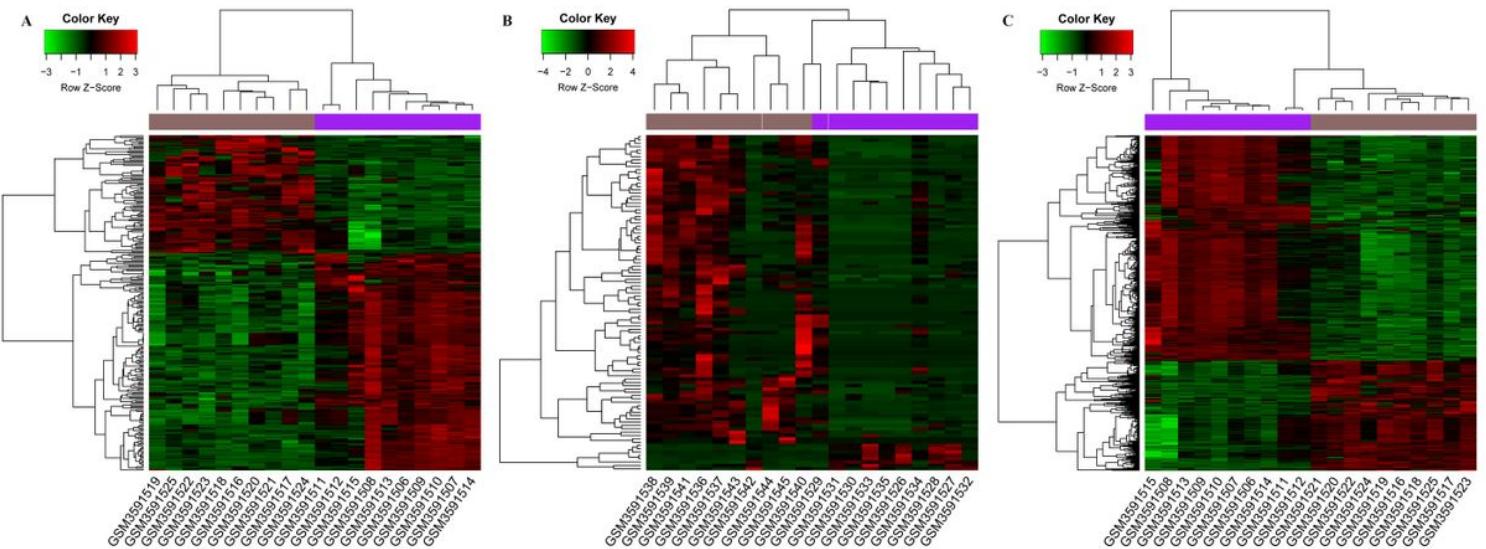


Figure 2

The heat maps of the differentially expressed lncRNAs, miRNAs, and mRNAs in CRC. The relative expression of the differentially expressed lncRNAs (A), miRNAs (B), and mRNAs (C), respectively, between the cancer tissues and normal tissues. Green indicates down-regulated RNAs, and red indicates up-regulated RNAs.

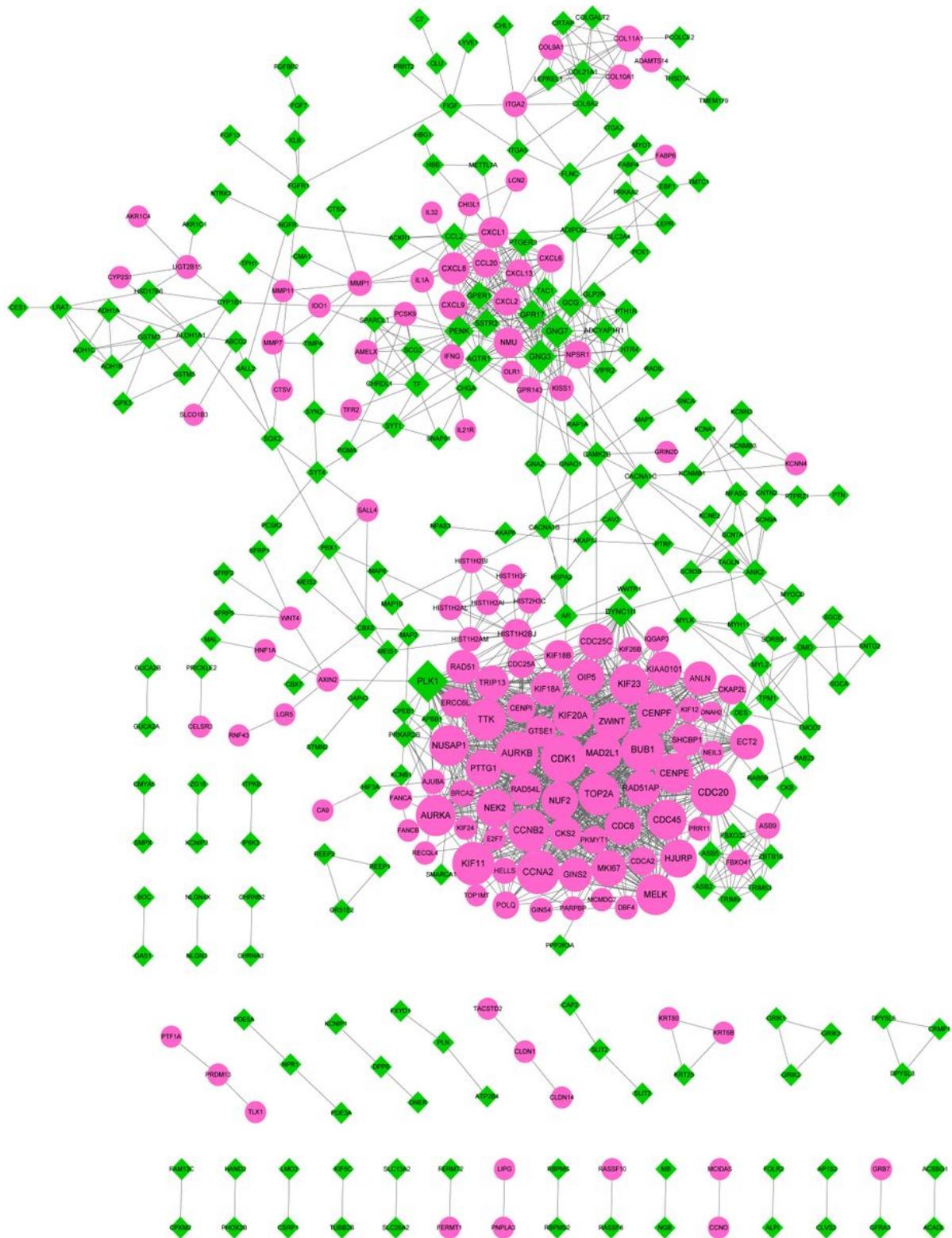


Figure 3

The protein-protein interaction network of differentially expressed mRNAs. The purple nodes represented the up-regulated mRNAs, and the green nodes represented the down-regulated mRNAs. The higher the degree value is, the larger the nodes.

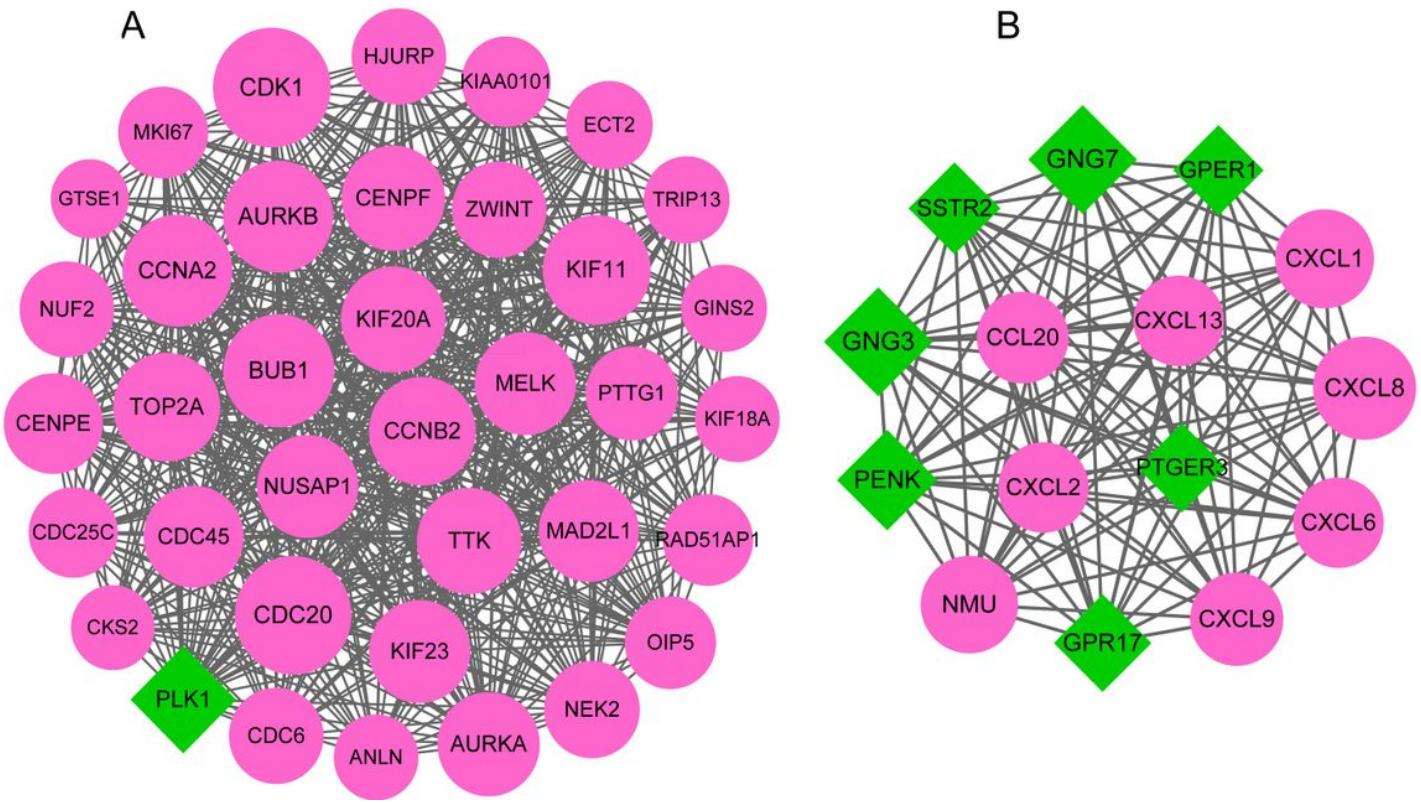


Figure 4

The protein-protein interaction network of differentially expressed mRNAs in module A (A) and module B (B). The purple nodes represented the up-regulated mRNAs, and the green nodes represented the down-regulated mRNAs. The higher the degree value is, the larger the nodes.

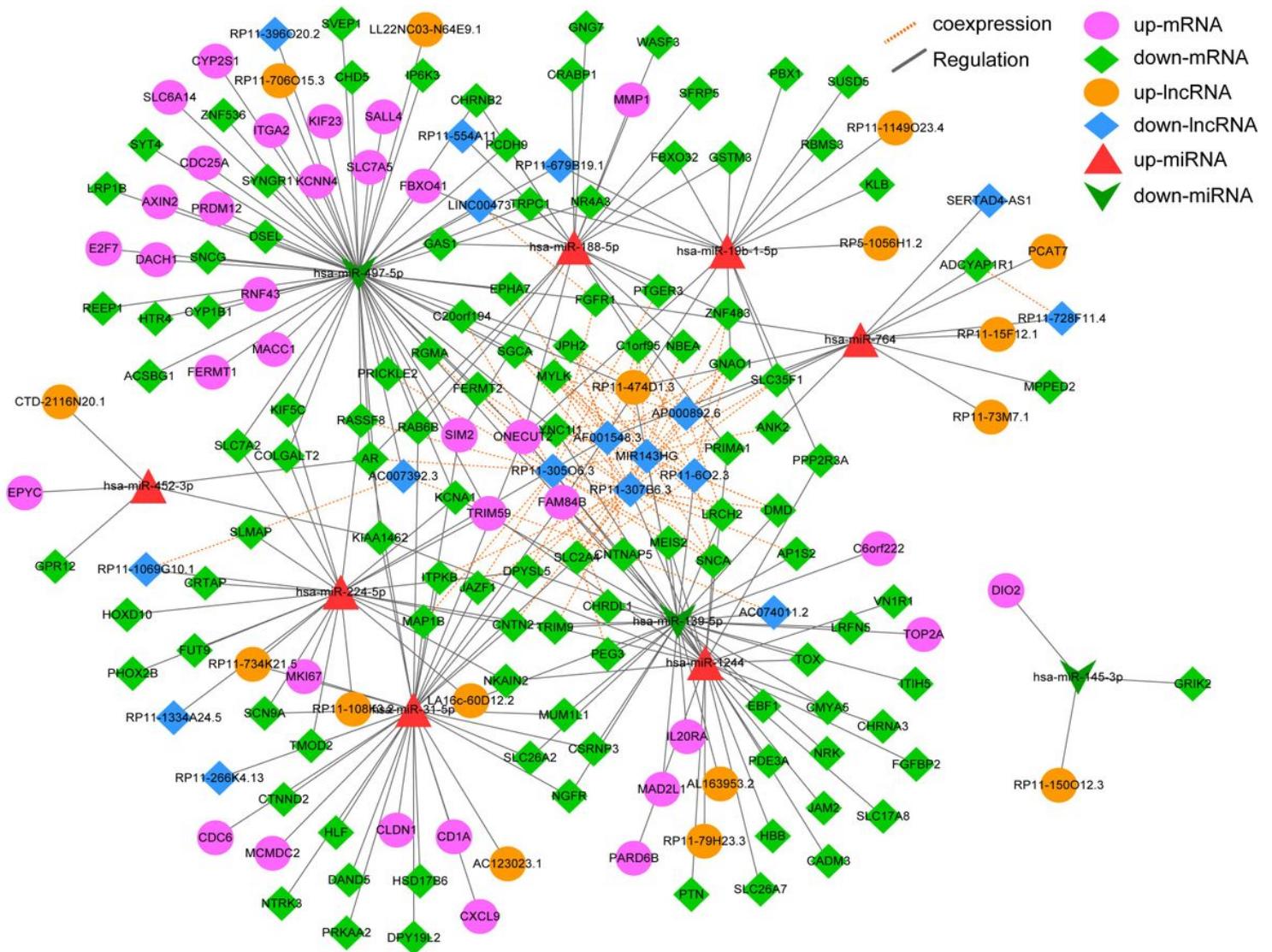


Figure 5

ceRNA network in CRC. The purple ellipse and the green diamond represented the up- and down-regulated mRNAs, respectively. The orange ellipse and the blue diamond represented the up- and down-regulated lncRNAs, respectively. The red triangle and the dark green arrow represented the up- and down-regulated miRNAs, respectively. The red dotted line represented the coexpression relationships, and the black line represented the regulation relationships.

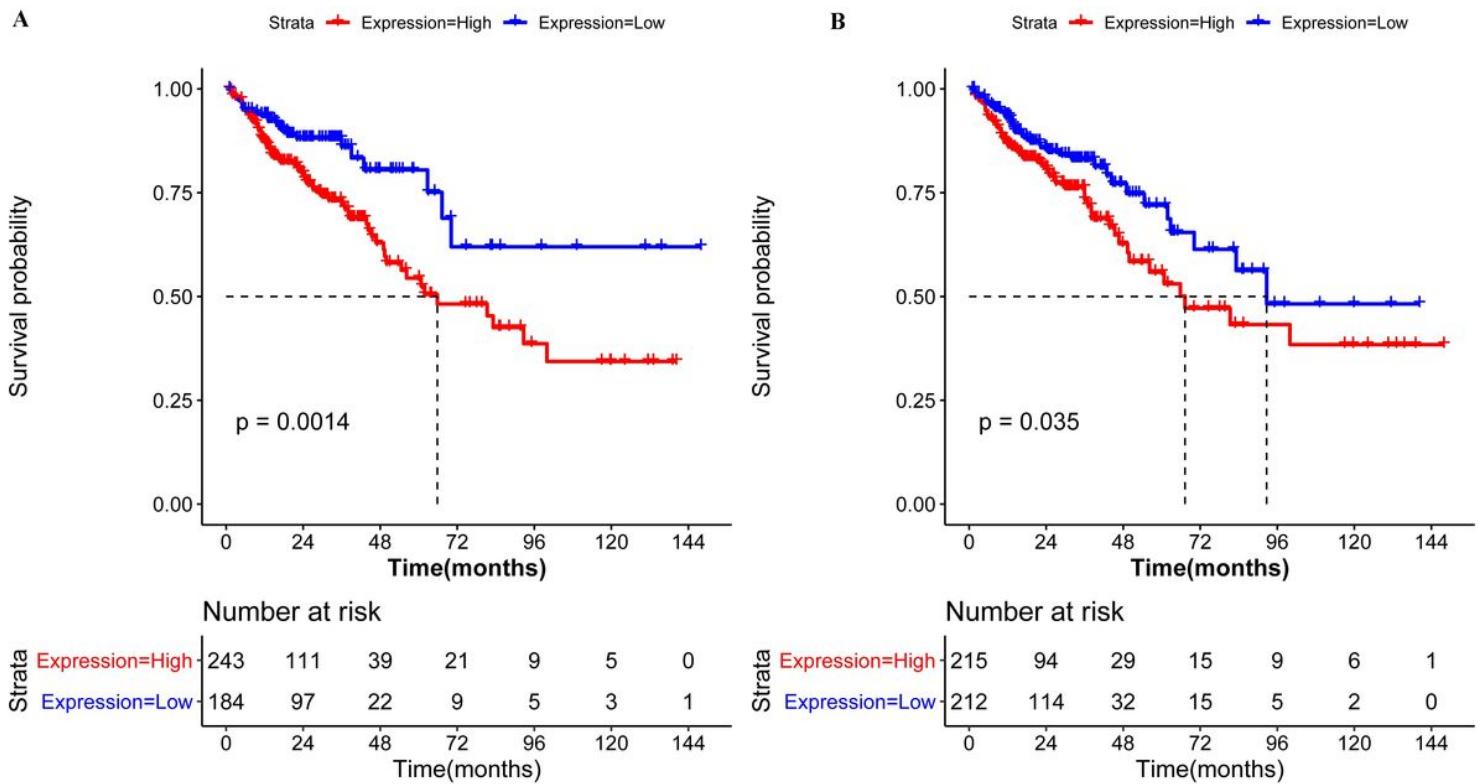


Figure 6

Survival curve with CRC containing differentially expressed RNAs. The mRNA, lncRNA, and miRNA expression levels and patient survival information from TCGA CRC data were used to plot the KM survival curve. The figure showed that the lncRNA RP11-108K3.2 (A) and mRNA ONCUT2 (B) significantly correlated with prognosis of CRC.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarytables.docx](#)