Co-expression analysis and ceRNA network reveal eight novel potential lncRNA biomarkers in hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) is the most common primary liver cancer in the world, with a high degree of malignancy and recurrence. The influence of ceRNA network in tumor on the biological function of liver cancer is very important, It has been reported that many lncRNA play key role in liver cancer development. In our study, integrated data analysis revealed potential eight novel lncRNA biomarkers in hepatocellular carcinoma. **Methods:** Transcriptome data and clinical data were downloaded from the The Cancer Genome Atlas (TCGA) data portal.Weighted gene co - expression network analysis was performed to identify the expression pattern of genes in liver cancer. Then ceRNA network was constructed using transcriptome data. **Results:** The integrated analysis of miRNA and RNAseq in the database show eight novel lncRNAs that may be involved in important biological pathways including TNM and disease development in liver cancer. We performed function enrichment analysis about mRNAs affected by these lncRNAs. **Conclusions:** By identifying the ceRNA network and lncRNAs that affect liver cancer, we showed that eight novel lncRNAs played an important role in the development and progress of liver cancer.

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23 **Methods:** Transcriptome data and clinical data were downloaded from the The Cancer Genome

24 Atlas (TCGA) data portal. Weighted gene co-expression network analysis was performed to

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28   lncRNAs that may be involved in important biological pathways including TNM and disease
29   development in liver cancer. We performed function enrichment analysis about mRNAs affected
30   by these lncRNAs.

31   **Conclusions:** By identifying the ceRNA network and lncRNAs that affect liver cancer, we showed
32   that eight novel lncRNAs played an important role in the development and progress of liver cancer.

33   **1. Introduction**

34   Liver cancer, most common among male patients, is the high leading cause of death (Wang et al.
35   2015). In the United States, there are as many as 42,030 new cases and 31,780 deaths related to
36   liver cancer every year, based on the latest statistics record (Siegel et al. 2019). The degree of
37   malignancy of cancer can be determined by study of the histology of the tumor, and patients can
38   be divided into three classes, low, medium, and high according to the degree of malignancy.
39   Clinically, primary liver carcinoma is considered to be one of the most common malignant tumors,
40   and about 90% of these tumors are hepatocellular carcinoma (HCC). In cases of patients diagnosed
41   with HCC, 50.7% of them achieve a 5-year survival rate, while the median survival time is 60
42   months (Lee et al. 2006). The prognosis of HCC patients is related to the patient's disease stage.
43   Currently, the tumor-node-metastasis (TNM) pathological staging standard developed by the
44   American Joint Committee on Cancer (AJCC) is the most commonly used malignant tumor staging
45   system worldwide. However, the relationship between long non-coding RNAs (lncRNAs) and
46   tumor staging has raised concern among researchers (Chen et al. 2015; Ou et al. 2018; Yao et al.
47   2018), suggesting that many lncRNAs play progressive key roles in HCC development (Chen et
48   al. 2016a). For example, the MALAT-1 gene is upregulated in HCC and also correlates with
49   prognostics and recurrence (Guerrieri 2015; Lai et al. 2012); the overexpression of the HULC gene
50   may reduce the mir-372 gene, while at the same time may promote reprogramming during
51   tumorigenesis (Du et al. 2012); and DANCR induces stemness features and could serve as a
52   potential prognostic marker and therapeutic target for HCC (Yuan et al. 2016). Previous studies
53   have mainly focused on the single biomarker use of miRNAs in HCC.

54   The weighted gene co-expression network analysis (WGCNA) is a popular bioinformatic method
55   used in the construction of gene networks and the detection of gene modules and their phenotypic
56   traits (Langfelder & Horvath 2008; Yin et al. 2018). In this study, we identified eight novel
57   lncRNAs correlated with TNM using WGCNA. Additionally, functional enrichment analysis
58   shows that these eight novel lncRNAs play an important role in the regulation of gene expression
59   that affect development and progression of liver cancer.

60   **2. Materials and Methods**

2.1. Data preprocessing and differential gene selection

The clinical data, RNAseq data and microRNA data of LIHC were downloaded from the The Cancer Genome Atlas (TCGA) database (Akbani et al. 2014) (Table S1).There were 49 pairs of microRNA samples and 50 pairs of RNAseq samples in total. The variation between the RNA and microRNAs data was calculated using EdgeR package (Reimers & Carey 2006; Varet et al. 2016). Only microRNAs recorded with first 10 and last 10 FC values were selected for subsequent analysis(Li et al. 2018; Shao & Li 2019). Because these biggest expression changed miRNA may have real major function in HCC. For RNAseq data, the EdgeR package was used to calculate the differential correlationship, and the threshold value for FDR was set at < 0.01, $|\,logFC\,| > 1$ (Table S2). Clinical data were used to calculate the correlation matrix of clinical information for integrated analysis. We used the "heatmap.2" function in the "gplots" package to create the heatmap.

2.2. Determination of ceRNA

Using microRNAs and differential expression genes as input data for target prediction, the RNA22 program was used to predict binding sites of microRNAs based on their sequence characteristics (Loher & Rigoutsos 2012). Based on these ceRNA interactions, we obtained all the mRNA-miRNA pairs with sharing the number of common miRNAs. Because the number of the ceRNA pairs is obey hypergeometric distribution. We estimated their statistical significance by a hypergeometric test. The potential top 20 different expression miRNA with hypergeometric test in ceRNA network P value less than 0.05 was obtained (Li et al. 2018; Shao & Li 2019). Specific formulas, such as the differential expression matrix of lncRNA, were used to get the Pearson correlation coefficient (PCC) (Table S3). Based on ceRNA's mechanism, the expression matrix (EM) for lncRNA to bind between themselves resulted in a PCC of EM > 0. Co-expression is one of the features of ceRNA network on account of their interactions. The final ceRNA pair was obtained by intersecting the ceRNA threshold with cutoff p-value <0.05. Cytoscape v3.0 was used to construct the ceRNA network. The overall Kaplan–Meier (KM) survival analysis in each subtype was performed using GEPIA2 database (Tang, 2017).

2.3. WGCNA model related computing

Weighted gene co-expression network analysis (WGCNA), a bioinformatic method used to find correlation patterns among genes, was utilized in this research. WGCNA assumes that gene expression networks is scale-free, it uses a 'soft' threshold to determine the weights of the edges connecting genes and merge individual genes to a module. An appropriate soft threshold will make

93   the co-expression network closer to a scale-free network. Then we constructed a signed weighted
94   co-expression network using WGCNA based on the gene expression value across the TCGA
95   samples. We obtained 60 co-expression modules according to the correlations of fpkm value
96   among samples. Each module is represented by an value belongs to the 'eigengene'. This value is
97   identified from the principal component analysis (PCA) of all the gene expression value in the
98   module. Then, we find the relationship between modules and the trait, Eventually, unsupervised
99   WGCNA identified major lncRNAs expression modules with different degrees of correlation to
100   TNM staging using WGCNA R software (Langfelder & Horvath 2008).

101   **2.4. Prediction of interrelationship between lncRNA-related mRNAs**

102   Using RNAseq data from TCGA, the correlation prediction of the lncRNA-lncRNA network and
103   lncRNA-mRNA network was constructed. The cutoff of PCC was 0.7. We retained the intersection
104   with the lncRNA using a cutoff of the top 20% degree. Ultimately, we found eight potentially
105   important lncRNAs with high degrees of connection in the ceRNA network and high correlation
106   at the expression level.

107   **2.5. Gene Ontology and KEGG Enrichment Analysis**

108   Gene ontology (GO) annotation analysis was performed using DAVID software (Huang da et al.
109   2009a). Gene functions for these important indirectly regulated mRNA genes elucidate that this
110   mRNA regulated by lncRNA may have some key biological functions (Huang da et al. 2009a;
111   Huang da et al. 2009b). String database was used for protein-protein analysis(Szklarczyk et al.
112   2017).

113   **3. Results**

114   **3.1. Analysis of differential miRNAs and differential lncRNAs**

115   The differential expression of miRNAs and lncRNAs between normal samples and cancer samples
116   was calculated separately using the EdgeR package (Chen et al. 2017; Law et al. 2016; Maza
117   2016). There were 1962 significant differential expression genes in lncRNAs, and 310
118   differentially expressed miRNA genes found between healthy and cancer-treated samples. Almost
119   half of the mRNAs are upregulated and half downregulated (Fig.1A). Most of the gene expression
120   for lncRNAs and miRNAs was upregulated in cancer-treated samples (Fig.1B-C).

121   **3.2. Weighted gene co-expression network analysis of lncRNA**

122 To determine if any of the identified coexpression modules were associated with TNM stage, we
123 calculated the PCC between the MEs and TNM stage . All lncRNAs were merged to 60 modules
124 according the degree of coexpression across the data set in WGCNA. As in the previous study, we
125 assigned each coexpression module an arbitrary color for reference(Di et al. 2019; Zhussupbekova
126 et al. 2016). The hierarchical clustering dendrogram of the eigengenes shows the module size (the
127 number of genes per module) and relationships among these modules (Fig.2A). Most modules
128 had minimal relationships with each other.

129 Comparison of module-characteristic eigengenes showed TNM was best correlated with the
130 module MElightpink4 (p=4E-04) and MElightcyan (P<0.006), composed of 125 lncRNAs (Fig.
131 2B). The PCC values ranged from −1 to +1 depending on the power of the relationship. A positive
132 value indicated that the lncRNA within a particular co-expression module increased as the TNM
133 increased, whereas the opposite occurred if the sign of the PCC was negative. We learned that the
134 correlationship between the module and TNM stage with PCC value was accompanied by the
135 corresponding P -value in brackets. These modules included genes that were co-expressed in a
136 particular TNM stage can be used to represent the TNM stage of HCC development (Fig. 2B).
137 These gene may be the risk factors and therapeutic targets in the treatment of HCC.

138 **3.3. ceRNA network of lncRNA reveals potential biomarkers in liver cancer**

139 As shown in Figure 3A , ceRNA network was constructed by high degree lncRNAs. The
140 topological characteristics of ceRNA network were analyzed with degree, betweenness, and
141 closeness (Table S4). Many genes in ceRNA network are with high degree, betweenness, or
142 closeness like AC016773.1, AC145285.2, LINC01569 and DANCR, and so on. This implied
143 ceRNA network may regulate many gene expression through these lncRNAs to have effect on
144 progression of liver cancer. To verify the function of these lncRNAs, Kaplan-Meier survival
145 analysis was perform ed for expression level of these lncRNAs. The results of the survival
146 analysis presented in Figure 3B and Figure3C show patients with high expression level of DANCR
147 or AL671710.1 have poor prognosis. It is a validation of our analysis and two of the lncRNAs
148 may affect prognosis.

149 **3.4. The interactions between lncRNAs and mRNAs**

150 Combining WGCNA with the results of ceRNA prediction analysis, lncRNA was combined with
151 correlation prediction to find eight important lncRNAs including AL671710.1, TRIM52-AS1,
152 C1orf220, AC022762.2, DANCR, LINC01569, AC084018.1 and MIR194-2HG. Among these
153 lncRNAs, downregulation of TRIM52-AS1 play key role in renal cell carcinoma(Liu et al. 2016).
154 DANCR increases stemness features of hepatocellular carcinoma (Yuan et al. 2016). DANCR is
155 also associated with various cancer(Lu et al. 2018a; Lu et al. 2018b; Mao et al. 2017; Sha et al.

156   2017; Xu et al. 2018; Yuan et al. 2016). However, there is little known about the function of other
157   six lncRNAs in cancer. There were 124 genes targeted by these eight lncRNAs (Fig.4A-H),
158   including EIF3 which functions during the initiation phase of translation. TRIM52-AS1 may
159   influence cancer behavior and function through interactions with regulator EIF3. EIF3 plays a key
160   role in human diseases (Gomes-Duarte et al. 2018; Valasek et al. 2017). Also, there were 30 genes
161   targeted by lncRNA ac084018.1, including m6A reader methyltransferase like 3 (METTL3). As
162   reported in previous research, METTL3 promotes liver cancer progression through YTHDF2
163   (Balacco & Soller 2019; Berlivet et al. 2019; Chen et al. 2018; Weng et al. 2018). DANCR and
164   AL671710.1 also have crucial roles through certain key genes (Fig.4C,E).

165   **3.5. GO and KEGG pathway enrichment analyses of lncRNA-targeted gene**

166   Additionally, we performed GO and KEGG enrichment analysis of the mRNAs in the network
167   (Fig.5A-C). We analysed target genes of the lncRNA based on their enrichment scores for
168   associated GO terms and KEGG pathways using David tools (Huang da et al. 2009a; Huang da et
169   al. 2009b) . The GO and KEGG enrichment analysis concerning the target genes of lncRNAs
170   indicated that the top regulated pathways of lncRNAs were Huntington's disease, RNA polymerase
171   and Pyrimidine metabolism, and the top regulated functions of lncRNAs were SRP-dependent co-
172   translational protein targeting to membrane, translational initiation, viral transcription, nuclear-
173   transcribed mRNA catabolic process, and nonsense-mediated decay. Further-more, those essential
174   mRNA may interact with each other and function in HCC (Fig. 6A). We can conclude that those
175   lncRNAs affect HCC through the functions and pathways listed above (the flow chart was depicted
176   in Fig. 6B).

177   **4. Discussion**

178   Hepatocellular carcinoma (HCC) is one of the primary causes of cancer-related death worldwide
179   (Balogh et al. 2016). Many genes influence HCC, TP53 tumor-suppressor gene, p16INK4A and
180   Rb-associated with various risk factors have been largely reported (Bae et al. 2016; Buendia 2000;
181   Nishida & Fukuda 2001; Peng et al. 2013; Wang et al. 2017; Zhang et al. 2014). Some even take
182   part in the cancer biography progress through lncRNA (Su et al. 2017). Long non-coding RNAs
183   (lncRNAs) which are transcribed but do not encode proteins, play key roles in HCC development
184   (Abbastabar et al. 2018). These include MALAT-1 and also NEAT-2, which regulates splicing
185   factors mostly situated in nuclear speckles. In addition, MALAT-1 is a biomarker in various
186   cancers including HCC(Lai et al. 2012; Wang et al. 2016). LncRNA GAS5 is a biomarker and
187   have potential applications in HCC therapy(Fang et al. 2019). Using WGCNA and hypergeometric
188   test analysis, we found eight lncRNAs with important functions: AL671710.1, TRIM52-AS1,
189   C1orf220, AC022762.2, DANCR, LINC01569, AC084018.1 and MIR194-2HG. TRIM52-AS1 is

190 one of the eight lncRNAs that has been reported as a function of a tumor suppressor (Liu et al.
191 2016; Zhang et al. 2017).Targeted by MYC, DANCR promotes cancer (Chen et al. 2016b;
192 Dhanasekaran et al. 2017; Huang et al. 2013; Kron et al. 2012; Lu et al. 2018b). Taken together,
193 we concluded that these lncRNAs may function as a potential tumor regulator in HCC.
194 Additionally, some lncRNAs were associated with the TNM stage in HCC tissues (Abbastabar et
195 al. 2018). The American Joint Committee on Cancer (AJCC) stratifies patients using a Tumor-
196 Node-Metastasis (TNM) classification, representing a group of models useful in the assessment of
197 tumor extension (Selcuk 2017; Tellapuri et al. 2018). Among several staging systems, the TNM
198 system was one of the most widely accepted, and had a higher prognostic competency than the
199 other systems (Prognosis Evaluation in Patients with Hepatocellular Carcinoma after
200 Hepatectomy, Comparison of BCLC and Hangzhou Criteria Staging Systems). In our work, we
201 found two lncRNAs modules associated with the TNM stage. Those lncRNAs may function as the
202 biomarker of node size and metastasis status in HCC. Systematic analysis of transcriptomics data
203 reveal those novel potential therapeutic target may be involved in cancer-related pathway in liver
204 cancer. Our study has limitations, the specific mechanism of these lncRNAs remains unexplored.

205 **5. Conclusions**

206 In summary, our results demonstrated that lncRNAs AL671710.1, TRIM52-AS1, C1orf220,
207 AC022762.2, DANCR, LINC01569, AC084018.1, and MIR194-2HG play an essential role in the
208 HCC stage, and their targeted mRNA have key functions in HCC. Those lncRNAs might be a
209 novel prognostic biomarker for HCC.

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391

Figure 1

Fig 1. Different expression of mRNA, miRNA and lncRNA levels between tumor and normal samples.

(A) Heatmap showing 2763 different expression of mRNA between tumor and normal samples in LIHC. (B) Volcano map for 310 different expression miRNA. Dots in red and green indicate high and low expression of miRNA in cancer, respectively. (C) Volcano map for 1962 different LncRNA. Dots in red and green indicate high and low expression of lncRNA in cancer, respectively.
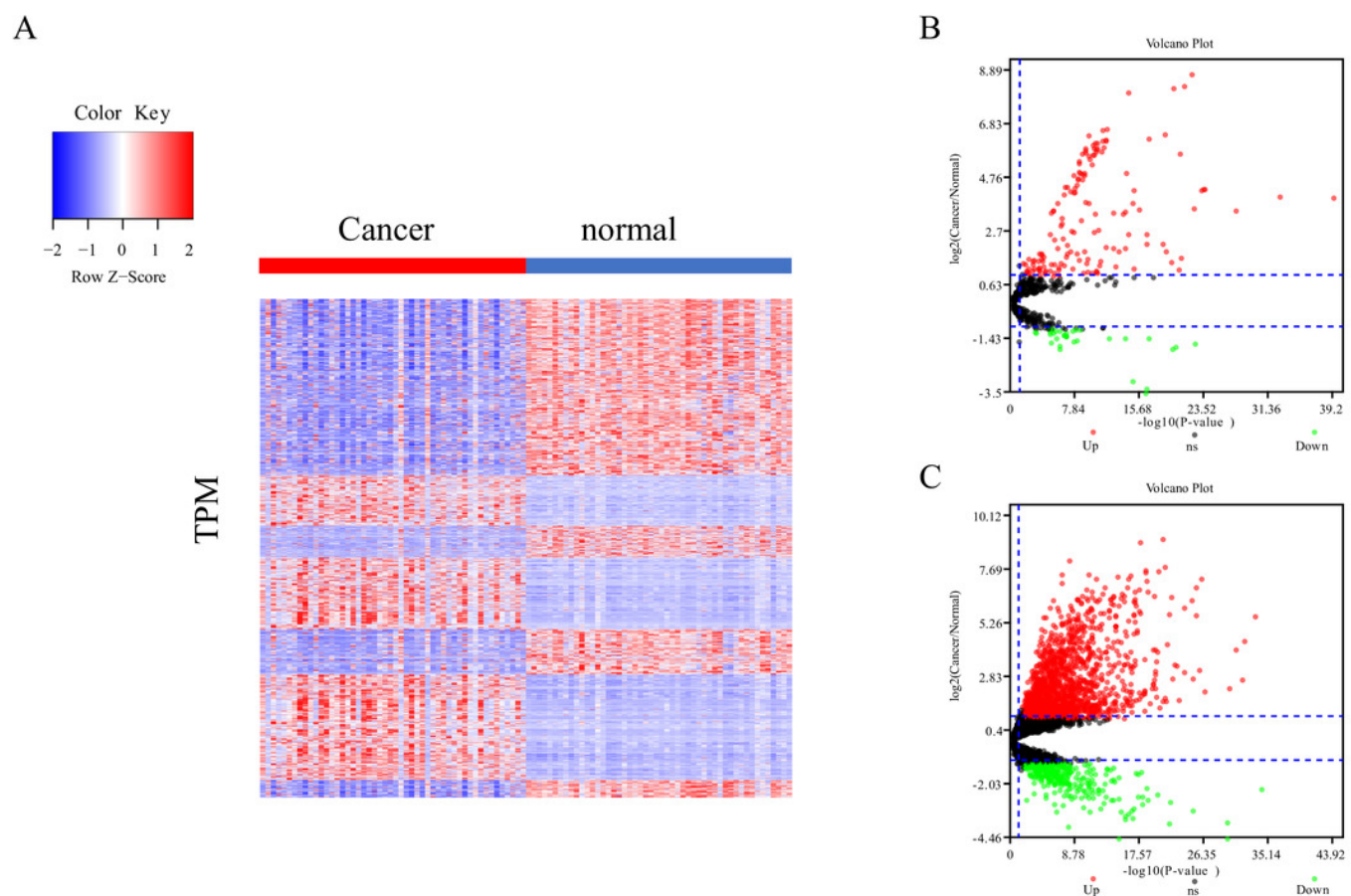
Figure 2

Fig 2. Results of Weight Gene Co-expression Network Analysis (WGCNA).

(A) shows the clustering dendrogram of co-expression lncRNA based on topological overlap.

(B) Module-TNM stage correlative analysis. Each row corresponds to a module eigengene,

each column corresponds to a TNM stage. Heatmap block with p-values and correlation

coefficient. The red box in the figure shows the module with higher correlation coefficient in

the three stages of TNM. The blue box in the figure shows the module with negative

correlation coefficient in the three stages of TNM.

A

B
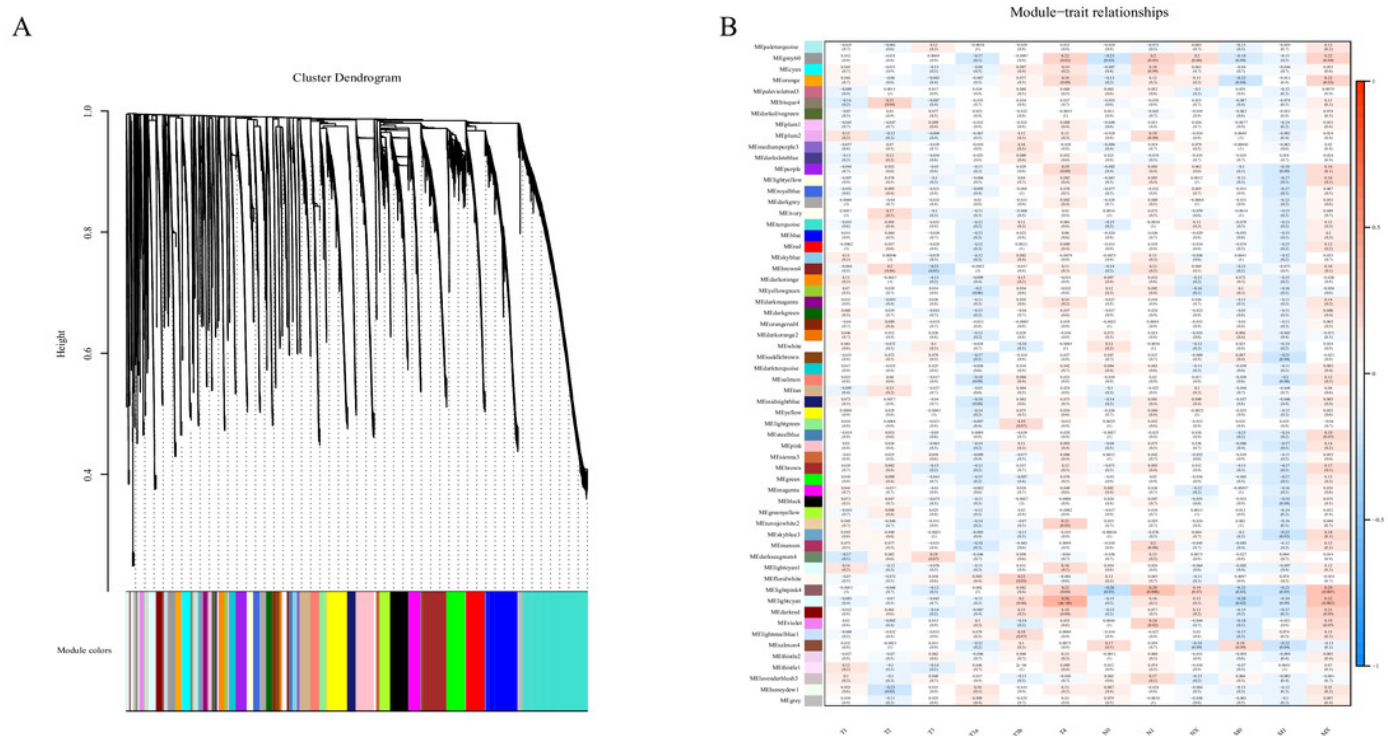
Figure 3

Fig 3. CeRNA network in LIHC and survival curves.

(A) Rosered dots represent overlaping lncRNA selected from co-expression analysis and RNA22 which can identify microRNA binding sites. The bigger the dots, the higher the degree value, the more important nodes in the network. (B) Survival curve of patients with high expression of DANCR and low expression of DANCR. Survival curve of patients with high expression of AL671710.1 and low expression of AL671710.1.
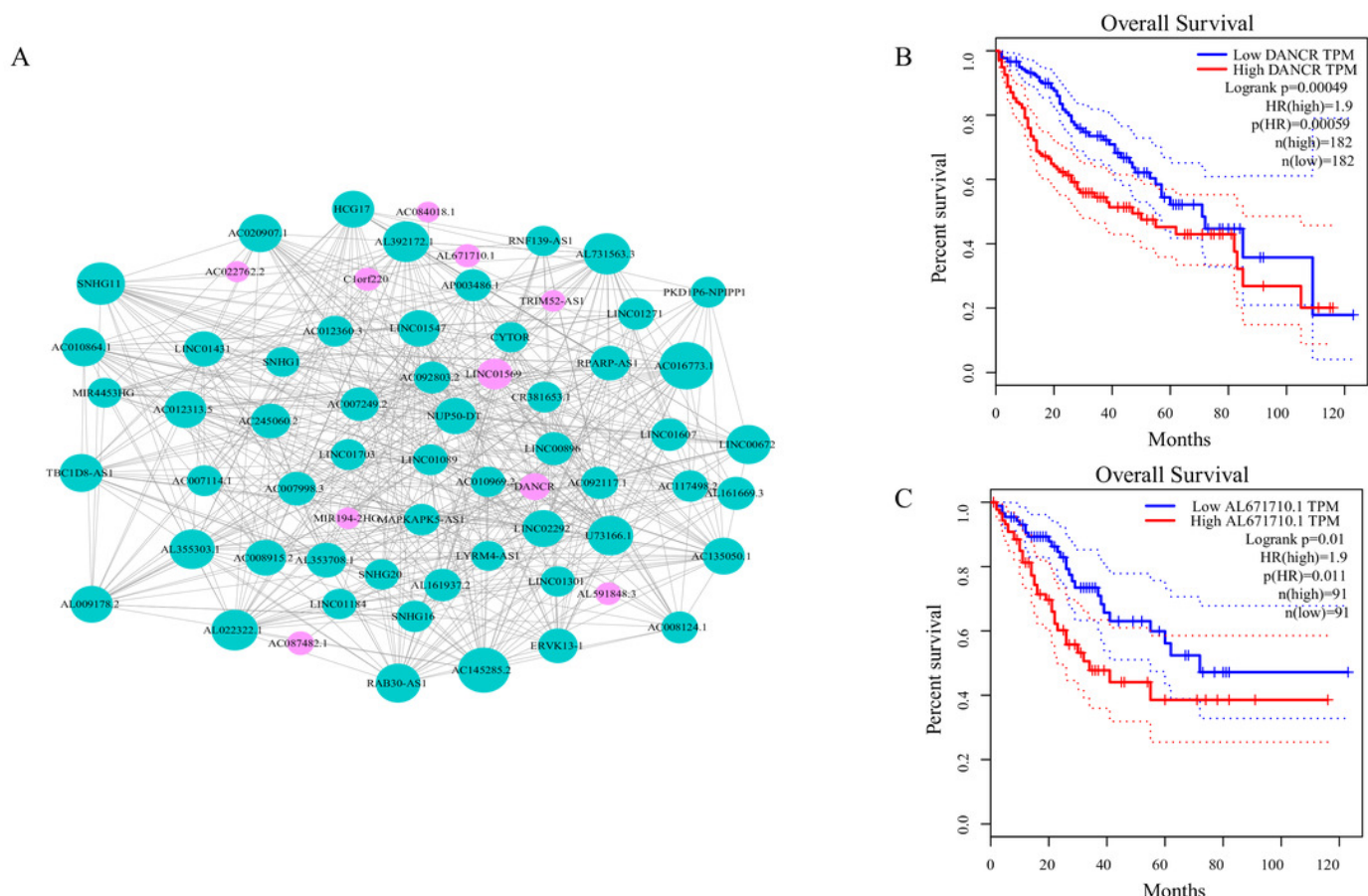
Figure 4

Fig 4. Interaction of eight lncRNA and target mRNA.

The dots in the arrow shape represent the lncRNA, and red wine color dots represent the mRNA in the figure. It can be seen that the eight important lncRNA AL6717.1, TRIM52-AS1, C1220C1, DANCR, LINC01569, AC084018.1 participated in many regulation of mRNA.

Figure 5

Fig 5. GO and KEGG pathway enrichment analysis of target gene.

(A) GO enrichment of mRNA interact with lncRNA. Points of different shapes represent BP, CC and MF of GO, and the size of points represents the number of gene enriched in the GO function, the number of different colors represents P value. (B) KEGG pathway is enriched in Ribosome and huntington disease.
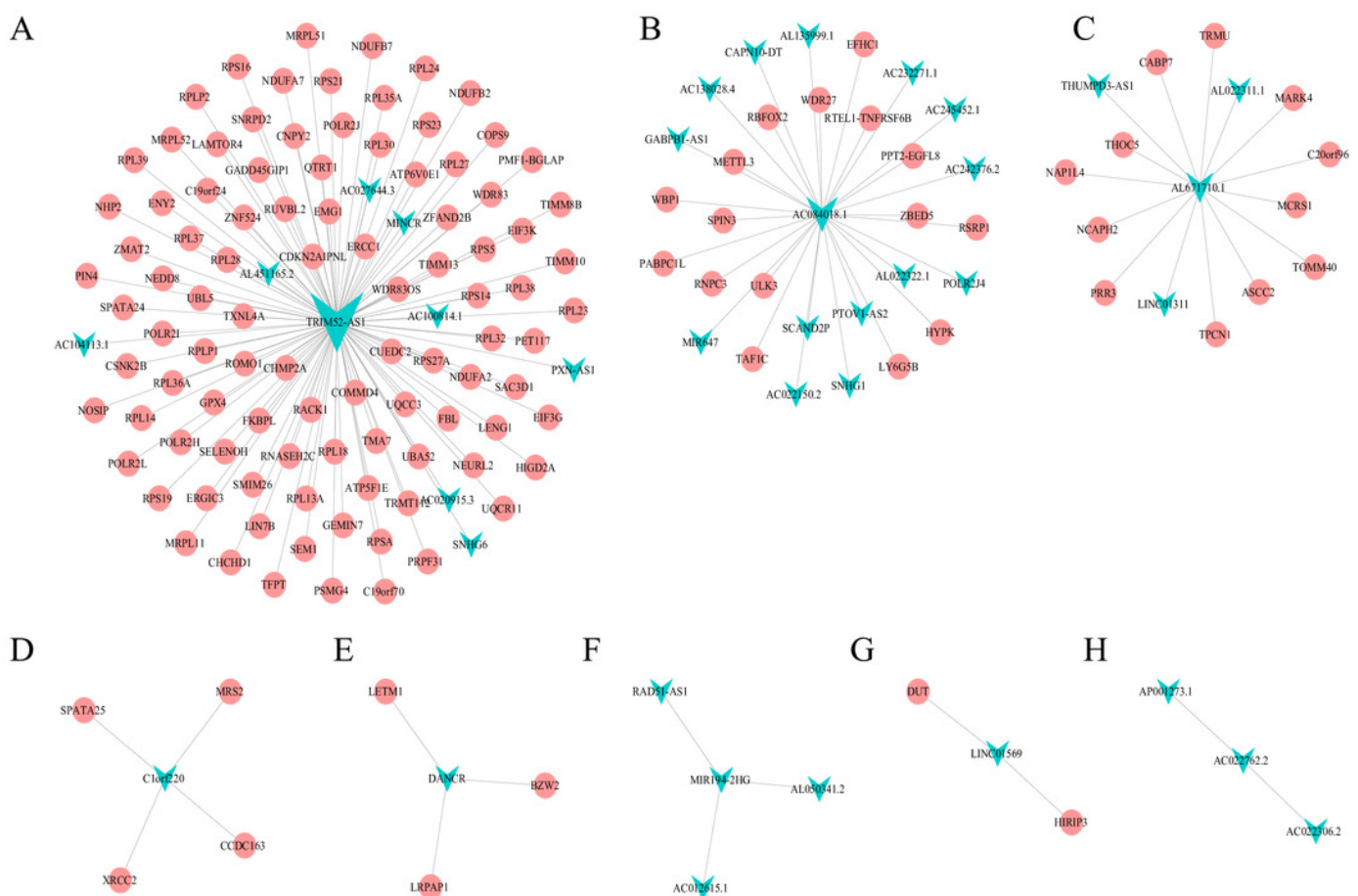
Figure 6

Fig 6. The protein–protein interactions of RNA and conclusion summary.

(A) The PPI network of RNA with degree bigger than 10 in string database. The larger the dots in the graph, the more interaction between the RNA corresponding proteins and other proteins. (B) Conclusion summary.