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# Five lncRNAs associated with the survival of hepatocellular carcinoma: a comprehensive study based on WGCNA and competing endogenous RNA network

D.-H. LIU, S.-L. WANG, Y. HUA, G.-D. SHI, J.-H. QIAO, H. WEI

Department of Medical Imaging, Cancer Hospital of China Medical University, Liaoning Cancer Hospital & Institute, Shenyang, China

**Abstract. – OBJECTIVE: The competing endogenous RNA (ceRNA) presents a comprehensive regulatory network among lncRNAs, miRNAs and mRNA. The ceRNA provides significant information in understanding the pathology of cancer. This study aimed to explore a lncRNA-associated ceRNA network for predicting the overall survival of patients with hepatocellular carcinoma (HCC).**

**MATERIALS AND METHODS: In this study, RNA-sequencing data of HCC were downloaded from The Cancer Genomes Atlas (TCGA) database. The module-trait relationship was analyzed with Weighted gene co-expression network analysis (WGCNA). The key module associated with tumor was identified, as well as the involved lncRNAs, mRNAs and miRNAs. The preliminary ceRNA network was constructed with Cytoscape. The survival analysis was further performed to screen survival-relevant lncRNAs, mRNAs and miRNAs, and then the survival-associated ceRNA network was reconstructed.**

**RESULTS: Eventually, 5 lncRNAs, 10 miRNAs, and 25 mRNAs were included in the reconstructed ceRNA network.**

**CONCLUSIONS: The identified lncRNAs were promising candidate biomarkers in HCC diagnosis and therapeutics. This analysis process was effective to construct ceRNA network. The result will be conductive to explore the significant lncRNAs and regulatory mechanism.**

*Key Words:*
LncRNA, WGCNA, CeRNA, HCC, Survival.

## Introduction

Hepatocellular carcinoma (HCC) has been a public health issue for both developed and developing countries, leading to heavy economic and social burden. World Health Organization reported that the HCC had resulted in approximately 6% of cancer incidence and 9% of cancer mortality in the worldwide[1]. In the Western countries, there were more than 1,000,000 HCC associated death each year. HCC has also been a health crisis in China, which was 4th most common and the 3rd most lethal cancer[2-4].

The etiology of HCC has been explored and the risk factors for developing HCC have been similar, including chronic infections of hepatitis B and hepatitis C viruses, alcohol uptake, drug abuse, and aflatoxins exposure[5]. The viral hepatitis has been a known cause of HCC, which has been mitigated by hepatitis B vaccination and hepatitis C treatment[6]. However, Dimitroulis et al[2] have revealed that, the non-alcoholic fatty liver diseases and nonalcoholic steatohepatitis may become the new leading cause of HCC instead of virus infection[2]. The metabolic disorders due to the obesity and diabetes may be the next leading risk factors for HCC[6,7]. Raoul et al[8] have summarized the management strategies of HCC: prevention, screening, and treatment. Developing novel strategies for diagnosis, intervention and treatment of HCC will be important. However, the lack of HCC-specific biomarkers hindered the early diagnosis and timely monitor of treatment outcomes[9,10].

LncRNA has been considered as a linkage between RNA and cancer, since its specific roles in biological processes. LncRNA has been found as critical regulator in various fields of biology. It was involved in comprehensive interactions with miRNA, mRNA and protein. LncRNAs dysregulation may be associated with tumorigenesis and metastasis; thus, lncRNAs serve as promising targets as cancer-related biomarkers[11].

With the advances of RNA microarrays and sequencing technology, the expression profile of lncRNAs has been described. The roles of

*Corresponding Author:* Hong Wei, MD; e-mail: weihong@cancerhosp-ln-cmu.com

lncRNAs in various cancers have also been revealed. Many studies have reported the theoretical and experimental verification of lncRNAs in HCC[12-14]. The roles of lncRNAs in HCC have been summarized in a critical review, and their potential clinical applications as biomarkers for the diagnosis, prognosis, monitor and therapy of HCC have been discussed[12].

Bioinformatic analysis has been a good tool to preliminarily screen the candidate biomarkers in the massive RNA-sequencing data. In a study on recurrently deregulated lncRNAs of HCC14, 8,603 candidate lncRNAs were identified from the RNA-sequencing data from 10 HCC patients. The expression profile of 917 recurrently deregulated lncRNAs were associated with clinical traits. Then, based on the array data corresponding to 60 samples, copy number variations and DNA methylation alterations were analyzed. The recurrent deregulation of 235 lncRNAs were obtained. Recurrently deregulated lncRNAs enrichment analysis showed its co-expressed clusters of genes related to cell adhesion, immune response and metabolic processes. These identified lncRNAs may be valuable resource for exploring cancer associated biomarkers.

The competing endogenous RNA (ceRNA) exhibits comprehensive regulatory network among lncRNAs, miRNAs and mRNA, providing significant information in understanding pathology of cancer[15,16]. In this study, the HCC associated data was downloaded from The Cancer Genomes Atlas (TCGA) database. The Weighted gene co-expression network analysis (WGCNA) was performed to explore the module-trait relationships of lncRNAs and mRNAs, as well as identifying the key modules and involved lncRNAs, mRNAs and miRNAs. Then, the primary ceRNA network was constructed with Cytoscape software based on above candidates. The survival analysis was further performed to screen survival related lncRNAs, mRNAs, and miRNAs for reconstructing survival-associated ceRNA network. Finally, five HCC survival associated lncRNAs were obtained. They may be promising candidates for further verification as survival associated biomarkers in HCC.

## Material and Methods

### Data Resource

The dataset of HCC was downloaded from TCGA database (https://portal.gdc.cancer.gov/), including the expression profile of lncRNA and mRNA, clinical information of 50 normal cases and 374 HCC cases. The Fragments Per Kilobase Million (FPKM) data were utilized and the genes with mean FPKM of "0" was excluded. A total of 13585 lncRNAs and 19474 mRNAs were included in the analysis. The miRNAs of involved lncRNAs were predicted in miRcode (http://www.mircode.org/).

### WGCNA Construction

The 4528 lncRNAs with the top variance of 1/3 and 4869 mRNA with top variance of 1/4 were identified with function in R. These lncRNAs and mRNAs were utilized to construct a gene co-expression network with the package WGCNA conducted in R. Firstly, scale-free network was constructed and the soft-thresholding power (β) was determined as 7 in the lncRNAs/mRNAs WGCNA. The successful construction of scale-free network was verified with k histogram and scale-free topology. Secondly, the clustering dendrogram of lncRNAs and mRNAs was drawn by function hclust for module determination. The Dynamic Tree Cut method was applied to obtain modules. The height cut-off was set as 0.25, modules were merged together if their similarity was > 0.75. The correlations between module eigengenes and clinical traits were analyzed and compared in both tumor and non-tumor cases. Pearson's correlation coefficient (PCC) was calculated for each pair of mRNAs and lncRNAs. The $p$-values < 0.05 represent statistical significance. Key module was selected. The lncRNAs and mRNAs involved in the key modules were considered to be highly interconnected. A total of 315 lncRNAs and 1103 mRNAs were identified from the key module.

### Construction of CeRNA Network

For the lncRNA, the 315 lncRNAs included in the key module were included for constructing ceRNA network. For the miRNA, the target miRNAs were predicted based on the target lncRNA on the miRcode database (http://www.mircode.org/). The 1098 lncRNA-miRNA pairs were identified. For the mRNA, the target mRNAs were predicted on miRDB (http://www.mirdb.org/miRDB/), miRTarBase (http://mirta rbase.mbc.nctu.edu.tw), TargetScan (http://www.targe tscan.org/) databases for finding the shared target mRNAs. Finally, the intersected mRNA with those included in key module were included for the construction of ceRNA network. Finally, the construction of lncRNA-miRNA-mRNA ceRNA network was performed with Cytoscape 3.7.0 software.

### Survival Analysis

The survival analysis of lncRNA was performed with R survival package. The survival curve of all lncRNAs included in the primary ceRNA network was plotted. The survival analysis of mRNA and miRNA was performed with KM plotter (http://www.kmplot.com/). The *p*-values $< 0.05$ represented statistical significance. The significant survival-associated lncRNAs, mRNAs and miRNAs were screened and applied for reconstructing survival-associated lncRNA-miRNA-mRNA ceRNA network.

### Gene-Set Enrichment Analysis

The gene-set enrichment analysis of screened lncRNAs was implemented with GSEA 4.0.1, with the gene expression profile of 374 HCC cases. The h.all.v7.0.symbols.gmt was adopted as background gene-set. The top three results with the highest NES scores were presented.

## Results

### Data Process (Figure 1)

Expression data (FPKM) of lncRNAs and mRNAs of 374 HCC cases and 50 normal cases were downloaded from TCGA database. A total of 13585 lncRNAs and 19474 mRNAs were identified. The mRNA and lncRNA were ranked from largest to smallest based on their sum of expression quantity. The 4528 lncRNAs with the top 1/3 variance and 4869 mRNA with top 1/4 variance were identified and included for subsequent analysis.

### WGCNA and Key Module

The expression profiles of 4528 lncRNAs and 4869 mRNAs were included for the construction of co-expression network via the package WGCNA in R. The scale-free network was optimized and the soft-threshold power (β) value was determined as 7 (Figure 2A). The scale-free topology was plotted, with $R^2$=0.92 and slope=-1.56 (Figure 2B).

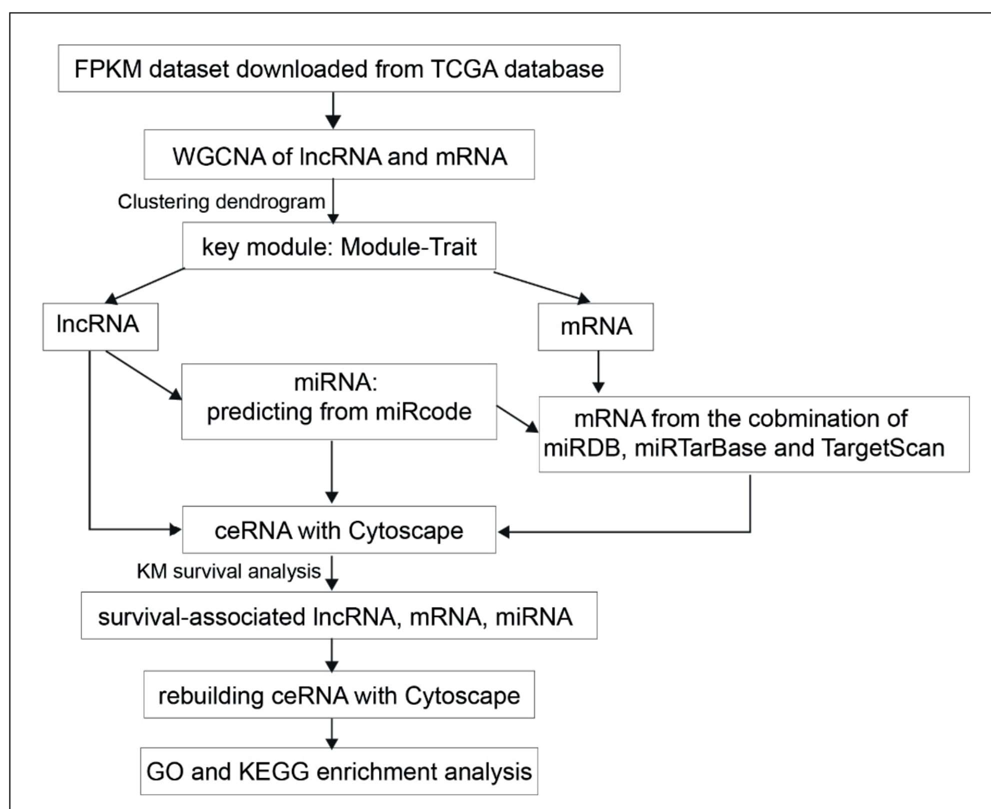


**Figure 1.** These figures are related to the female patient with uterine leiomyosarcoma pre-sacrum metastasis. **A**, and **B**, show an example of a patient-specific plan prepared before ECT procedure based on cross-sectional 2DCT images orthogonal to electrodes access route. **C**, shows the patient in interventional operatory room with needle electrodes percutaneous inserted into the pelvis, site of the lesion. **D**, represents two needle electrodes inserted into the target. Figure 1. The analysis process.

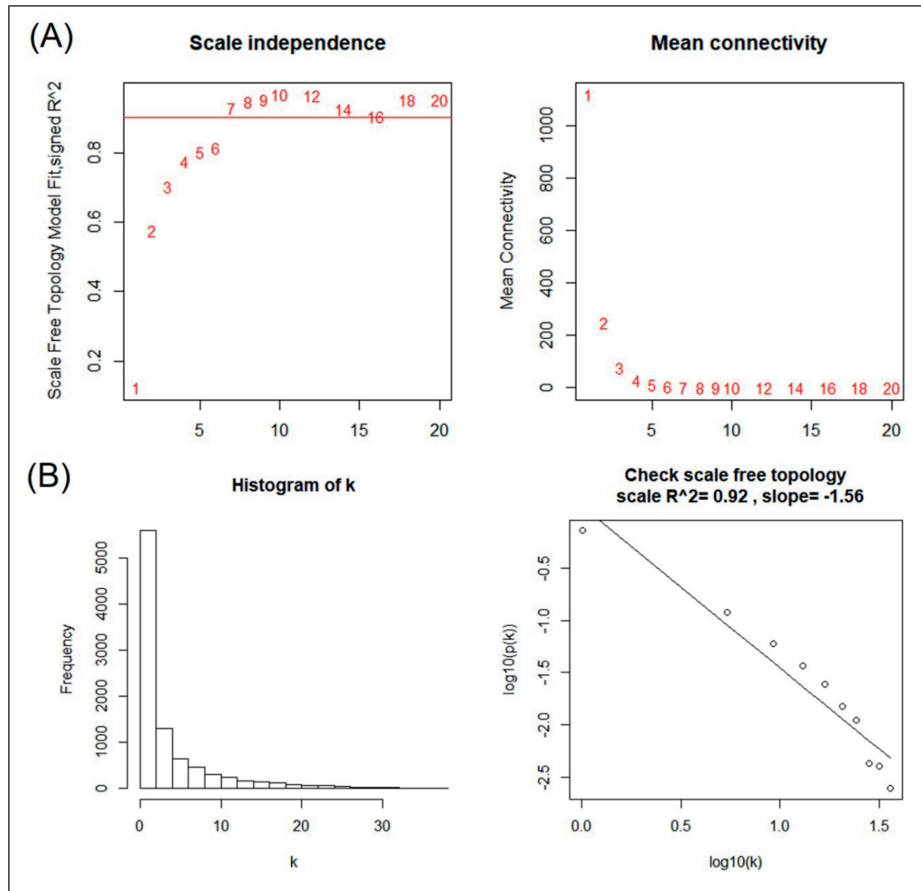


**Figure 2.** ***A***, Soft-thresholding powers (β) in the lncRNAs/mRNAs WGCNA. Right: Analysis of the mean connectivity for different β. ***B***, The verification of constructed scale-free network. R2=0.92. slope=-1.56.

The co-expression modules were then generated with Dynamic tree cutting. The parameter was set as 0.25 to merge closely associated modules into one. A total of 25 modules were produced in the lncRNAs/mRNAs co-expression network (Figure 3A). The relationship between each module and clinical trait was presented and PCC was calculated (Figure 3B). It observed that the brown module showed the highest PCC of 0.48, which was the most significant tumor-associated module ($p < 0.05$). In the brown module, 315 lncRNAs and 1103 mRNAs were included.

### CeRNA

Firstly, the miRNA corresponding to the 315 lncRNAs was predicted with miRcode database. A total of 1098 lncRNA-miRNA pairs contained 315 lncRNAs and 201 miRNAs were included. The mRNAs were further screened with above identified miRNAs from three databases, which were TargetScan, miRDB and miRTarBase. It obtained 1935 miRNA-mRNA pairs contained 43 miRNAs and 1270 mRNAs. Then, the predicted mRNA was matched with the mRNAs in the brown module, and 42 intersected mRNAs were finally included. According to above results, lncRNA-miRNA-mRNA ceRNA network was constructed using Cytoscape software, including 18 lncRNAs, 21 miRNAs and 42 mRNAs (Figure 4).

### Survival Analysis

The survival analysis of the 18 lncRNAs, 21 miRNAs and 42 mRNAs involved in the lncRNA-miRNA-mRNA ceRNA network was performed. The survival analysis of lncRNA was conducted with R survival package. The survival analysis of mRNA and miRNA was performed with KM plotter (http://www.kmplot.com/). The $p < 0.05$ represented statistical significance. Finally, there were 6 lncRNAs, 10 miRNAs and 25 mRNAs showed significance in survival analysis, indicating that they were survival-associated targets (Figure 5).

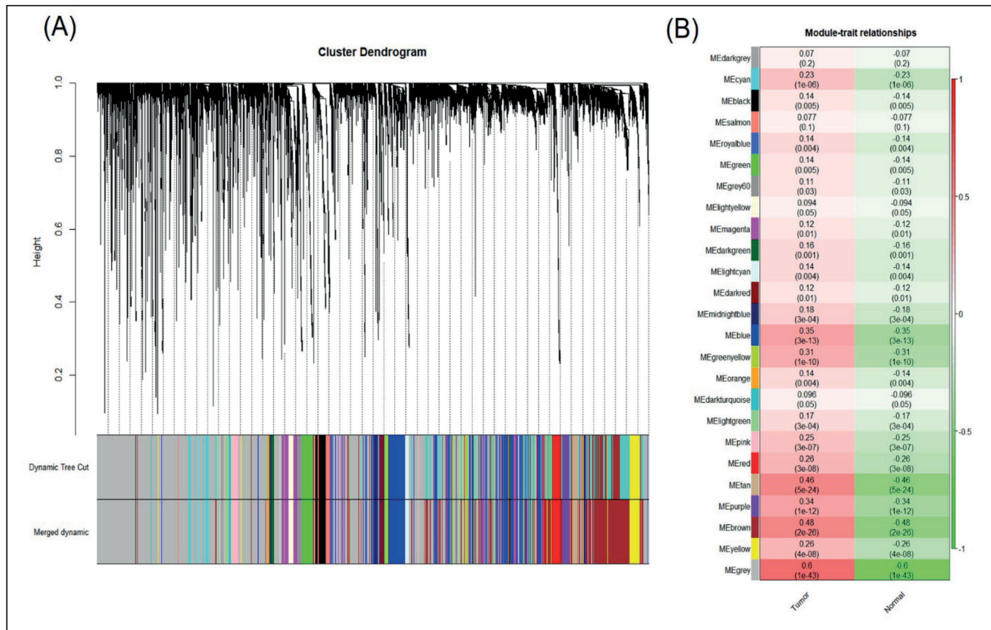


**Figure 3.** ***A***, Clustering dendrogram of lncRNAs and mRNAs. Both the original and merged modules were presented. ***B***, Module-trait relationship of lncRNAs and mRNAs were evaluated by correlations between module eigengenes (ME) and clinical traits. Note: Each row indicated to one ME and each column indicated one trait. Each cell contained corresponding correlation and $p$-value (in parentheses). The $p<0.05$ indicated statistical significance.

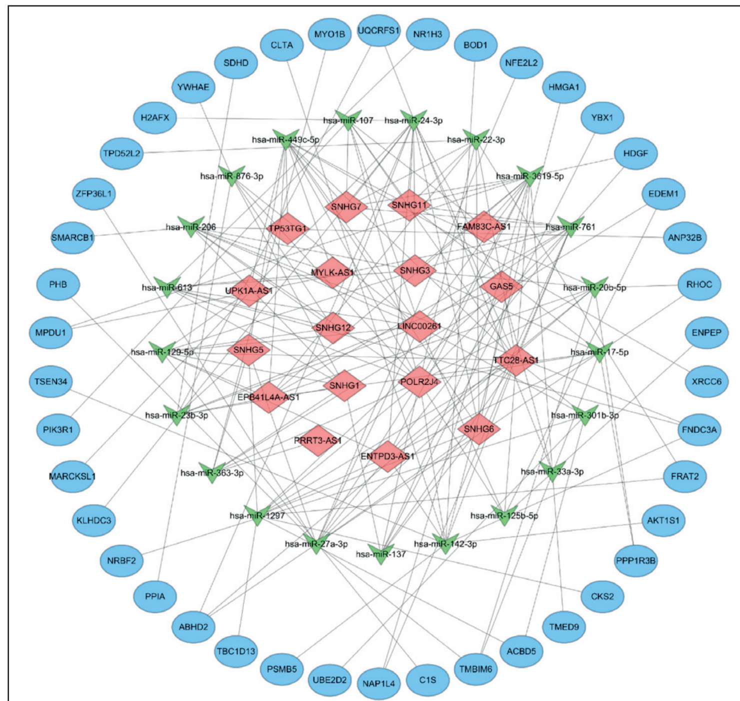


**Figure 4.** Primary ceRNA constructed with included lncRNA, mRNA and miRNA. Notes: Red diamond denoted lncRNA, green triangle represented miRNA, and blue cycle represents mRNA.

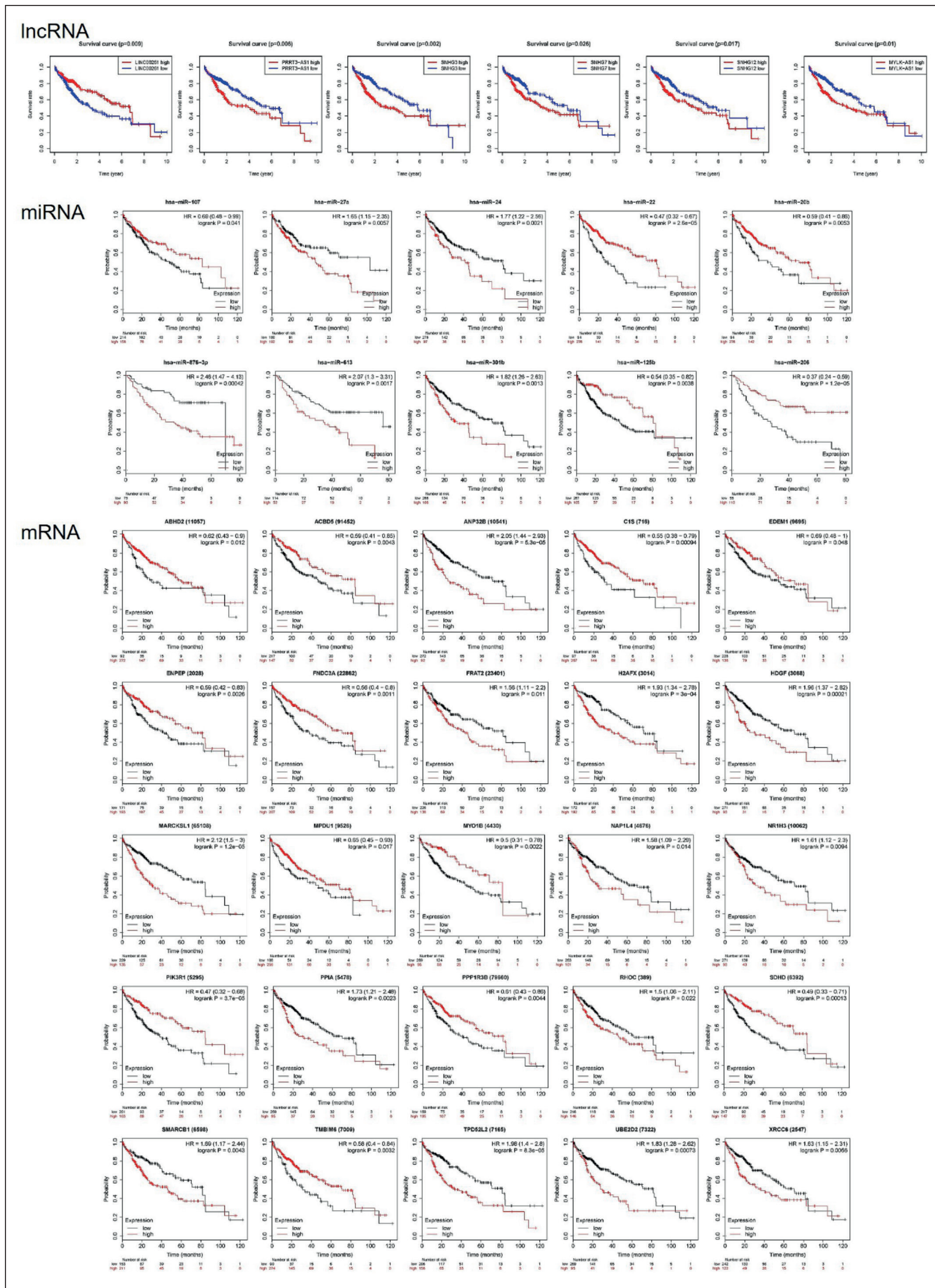


**Figure 5.** Kaplan-Meier survival analysis of lncRNA, miRNA and mRNA. The $p<0.05$ indicated statistical significance.

### *Survival-Associated CeRNA Network*

With the 6 lncRNAs, 10 miRNAs and 25 mRNAs associated with survival, the ceRNA network was reconstructed with Cytoscape software. In the reconstructed ceRNA network, 5 lncRNAs, 10 miRNAs and 18 mRNAs were included (Figure 6).

### *The Screened LncRNA and Gene-Set Enrichment Analysis*

The expression levels of 5 screened lncRNAs were analyzed, which were LINC00261, MYLK-AS1, SNHG12, SNHG3 and SNHG7. The results indicated that lower expression level of LINC00261, higher expression levels of MYLK-AS1, SNHG12, SNHG3 and SNHG7 were observed in HCC cases, compared to that of normal cases (Figure 7). It may suggest that LINC00261 was negatively associated with survival of tumor, while MYLK-AS1, SNHG12, SNHG3 and SNHG7 was positively associated with survival of HCC. To explore the potential biological functions of lncRNAs, functional enrichment analysis was performed with GSEA. For each lncRNA, three most enriched terms were presented (Figure 8). For LINC00261, the enriched hallmarks were bile acid metabolism, xenobiotic metabolism and peroxisome. For MYLK-AS1, SNHG12, SNHG7 and SNHG3, the enriched hallmarks were DNA repair, Myc targets V1 and Myc targets V2. The metabolite associated pathway dominated.

## Discussion

The ceRNA hypothesis proposed that there was a comprehensive ceRNA regulatory network across the transcriptome, consist of miRNAs, mRNAs and lncRNAs. The ceRNA was able to regulate other RNA transcripts at post-transcription level by competing for shared miRNA. Thus, the genetic functions can be expanded to make critical effects in various biological processes[16]. The roles of ceRNA network have been intensively explored. The dysregulated ceRNA network was proved to link with various diseases, including cancer. It may be applied as diagnostic biomarkers or therapeutic targets[16,17]. Several studies have been performed on the regulatory effects of ceRNA in normal and pathological conditions of patients with cancers. However, the understanding of ceRNA regulatory network remained limited[18].

Bioinformatic analysis of RNA-sequencing data has been powerful and effective tool for constructing ceRNA, exploring significant biomarkers, and understanding the potential regulatory mechanism.

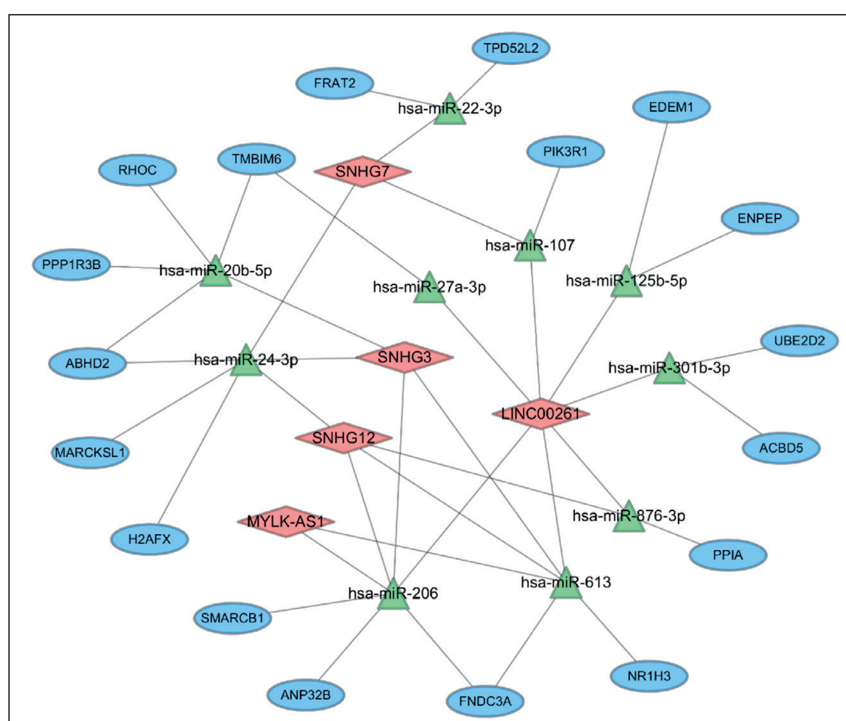


**Figure 6.** Reconstructed ceRNA regulatory network with survival-associated lncRNAs, miRNAs and mRNAs.

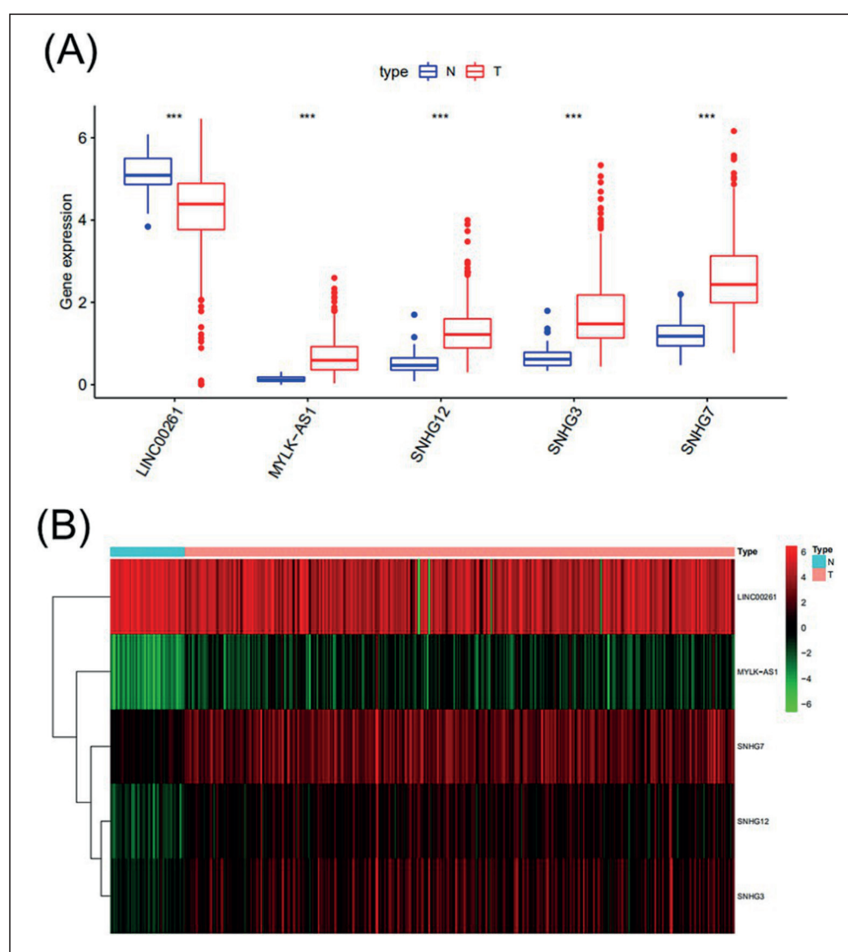


**Figure 7. A**, The differential expression of screened lncRNAs and (**B**) heatmap.

In this study, RNA-sequencing data were downloaded from TCGA database, and WGCNA was applied to identify the key module significantly related to HCC. Then, based on the lncRNA, miRNA and mRNA involved in the key module, the ceRNA network was constructed. The survival analysis was subsequently performed on all lncRNA, miRNA and mRNA identified with the constructed ceRNA network. The survival-associated indicators were identified and then applied for reconstructing survival-associated ceRNA network. Finally, potential prognostic lncRNAs were screened, as well as the underlining regulatory mechanism.

LncRNA has been considered as promising biomarker for HCC, either diagnosis or prognostic prediction[18]. Several lncRNAs were determined resulted from the advance of sequencing technology. Their functions in the tumorigenesis and development of human HCC were also explored. The identified HCC-related lncRNAs included HULC, HOTAIR, MALAT1 and H19[12]. The lncRNA HULC has been proved to trigger autophagy *via* interacting with Sirt1 and attenuated the chemosensitivity of HCC cells[13]. The upregulation of lncRNA-UCA1 was proved to promote HCC progression *via* suppressing miR-216b and activating FGFR1/ERK signaling pathway[19]. Similarly, lncRNA CRNDE inhibited miR-384 thus promoting proliferation, migration and invasion of HCC cells[20]. Based on the RNA-sequencing data from 20 HCC patients, 917 recurrently deregulated lncRNAs of 8,603 candidate lncRNAs were observed to correlate with clinical data. There were 235 recurrent deregulation lncRNAs with copy number variations and DNA methylation alterations. Above identified lncRNAs may be the resource for further theoretical and experimental verification[14]. A better understanding of the function mechanism in lncRNAs will assist in lncRNA-targeted therapies. In our analysis, we determined five lncRNAs, which were LINC00261, MYLK-AS1, SNHG12, SNHG3 and

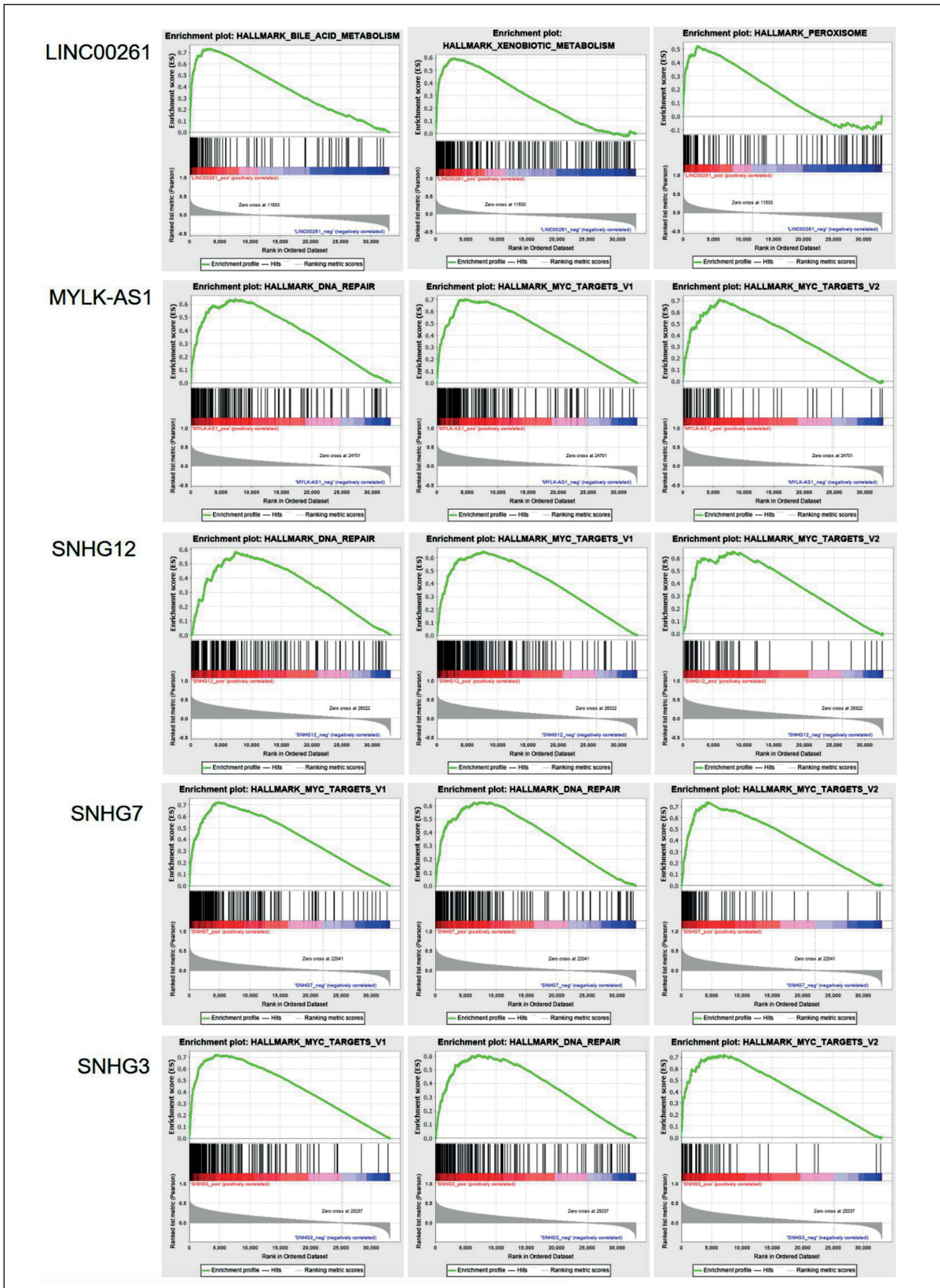


**Figure 8.** The gene-set enrichment analysis of screened lncRNAs.

SNHG7. The results indicated that lower expression level of LINC00261, higher expression levels of MYLK-AS1, SNHG12, SNHG3 and SNHG7 were observed in tumor cases, compared to those of in normal cases.

LINC00261 was a lncRNA with tumor inhibitory effects, which was downregulated in various cancers. Proliferation, migration, and invasion of cancer cells would be suppressed with overexpressed LINC00261. In several bioinfor-

matic analysis, LINC00261 has been identified as survival-associated lncRNA included in the constructed ceRNA network[21]. The association between survival outcome and lncRNA expression was analyzed with univariate and multivariate Cox proportional hazards regression analyses in HCC, in which LINC00261 was identified to be significantly correlated with overall survival[22]. The roles of LINC00261 in HCC were only reported in a few studies, and upregulation of LINC00261 significantly inhibited Notch signaling by downregulating Notch1 and Hes-1 expression in HCC cells[23]. Consistent with above result, our study suggested that the lower expression of LINC00261 was associated with HCC compared to that of in normal cases.

Besides of bioinformatic analysis, the effects of LINC00261 in cancers were also proved with experimental data. The downregulation of LINC00261 was observed in endometrial carcinoma. LINC00261 upregulated the levels of FOXO1 protein *via* inhibiting FOXO1-targeted miRNAs. Then, the proliferation, migration, and invasion of endometrial carcinoma cells could be suppressed[21]. High level of LINC00261 in cholangiocarcinoma indicated a poor prognosis, and promoted a metastasis via EMT process[24]. In addition, LINC00261 was reported to improve chemosensitivity of human colon cancer cells[25]. LINC00261 suppressed non-small cell lung cancer (NSCLC) cells progression via sponging miR-522-3p and inhibiting Wnt signaling[26]. The effects of LINC00261 could be further verified both *in vitro* and *in vivo*.

MYLK-AS1 (short for MYLK antisense RNA 1) was reported to be involved in the ceRNA regulatory network of HCC, which was associated with the survival rate of HCC patients[21,27]. In another bioinformatic analysis, the MYLK-AS1 was screened and included in the lncRNA expression-based risk score system for overall survival, which can effectively predict the survival of HCC patients[28]. MYLK-AS1 was reported to make effects in other cancers, including colon cancer and gastric cancer. In colon cancer, the lncRNA-lncRNA pairs were identified to make synergistic effects, including BVES-AS1/MYLK-AS1, ADAMTS9-AS1/MYLK-AS1 and FENDRR/MYLK-AS1. These lncRNAs were included in the signature for predicting prognosis[29]. The lncRNA and mRNA expression profiles correlated to gastric cancer with or without lymph node-metastasis was determined and MYLK-AS1 was validated to be downregulated compared to those without lymph node metastasis and normal samples[30]. It is worth noting that MYLK-AS1 may play various roles in different cancers. Different from the results obtained in gastric cancer, MYLK-AS1 was up-regulated in HCC relative to that of normal cases. The upregulation was also observed in previous studies on HCC[27,28].

LncRNAs encoded small nucleolar RNAs was named small nucleolar RNA host genes (SNHGs), which made comprehensive effects on cellular processes. In a bioinformatic analysis for exploring the prognostic value of the SNHGs in HCC, SNHG1, GAS5, SNHG3-7 and SNHG10-12 were identified as significantly upregulated SNHGs in HCC specimens relative to normal controls[31]. Similarly, our results observed that the higher expression of SNHG12, SNHG3 and SNHG7 was correlated with the HCC. These SNHGs were intensively explored as star molecules, both in HCC and other cancers, which would be demonstrated in detail.

SNHG12 has been reported as a promising therapeutic target and biomarker for human cancers[32]. The experimental results indicated that SNHG12 was significantly upregulated in the HCC tissues than that of in normal control. SNHG12 would directly bind to miR-199a/b-5p as an endogenous sponge. The expression of MLK3 can be regulated thus affecting the NF-κB pathway[33]. SNHG12 also participated in tumorigenesis, progression and metastasis of other cancers, including glioma, osteosarcoma[34], nasopharyngeal carcinoma[35], NSCLC[36] and bladder cancer[37]. Several miRNAs and signaling pathways were involved, supporting that SNHG12 may be a multi-functional lncRNAs playing various roles in various biological processes. So, SNHG12 also made effects on chemoresistance. LncRNA SNHG12 contributed to multi-drug resistance by activating the MAPK/Slug pathway by sponging miR-181a in NSCLC. It may be a therapeutic target[38]. All these results proved SNHG12 a promising prognostic biomarker and therapeutic target worthy of further exploration.

Similar to SNHG12, SNHG3 was also frequently reported in HCC[21]. The expression of SNHG3 and its clinical significance in HCC has been verified. The results derived from 51 HCC clinical specimens indicated that the expression level of SNHG3 was significantly upregulated relative to normal control[39]. It observed significant correlations between SNHG3 expression and some clinical features, including tumor size, portal vein tumor thrombus and relapse. In short,

high SNHG3 level was markedly associated with overall survival, recurrence-free survival and disease-free survival. Increased SNHG3 expression could be an independent prognostic indicator for malignant status and poor prognosis in HCC patients[39]. The regulatory mechanism of SNHG3 was also investigated. HCC progression could be promoted by SNHG3 *via* the miR-326/SMAD3/ZEB1 signaling pathway[40]. Cell proliferation, migration, and invasion of HCC could be stimulated *via* SNHG3/miR-139-5p/BMI1 axis[41]. In addition, SNHG3 overexpression induced HCC cells EMT *via* miR-128/CD151 cascade activation, which was associated with poor chemotherapy response and HCC survival[42]. The roles of SNHG3 in other cancers were also reported. Similar to HCC, SNHG3 promoted malignant development of colorectal cancer and proliferation of lung adenocarcinoma[43,44].

SNHG7 was also intensively studied as one of the most promising SNHGs with prognostic significance in HCC. The expression of SNHG7 was significantly upregulated in HCC specimens relative to normal control[31], and the upregulation of SNHG7 was correlated with higher grade of HCC[45]. SNHG7 was reported to participate in HCC metastasis. The result of loss-of-function assays confirmed that HCC cells invasion would be impaired with the knockdown of SNHG7[46]. In short, the elevated expression of SNHG7 indicated poor prognosis of HCC. From the cellular level, SNHG7 promoted HCC cell proliferation, migration and invasion *via* regulating the levels of miR-122-5p and RPL4[47]. SNHG7 could promote proliferation and metastasis of HCC cell *in vitro* and *in vivo*, as miR-425 sponge *via* Wnt/β-catenin/EMT pathway[48]. Similar to other SNHGs, SNHG7 also stimulated the tumor progression in other cancers, such as gastric cancer, osteosarcoma, glioblastoma, colorectal cancer[49] and pancreatic cancer[47].

The bioinformatic analysis of ceRNA regulatory network provided new candidate markers and useful information to understand the molecular mechanism in HCC. It also facilitated innovative applications in HCC diagnosis and treatment.

Based on the consequences of GSEA, the regulatory mechanism of involved lncRNAs in HCC development and progression can be further revealed. Some liver function related hallmarks were enriched, including bile acid metabolism, xenobiotic metabolism and peroxisome. For the snoRNAs, the most enriched hallmarks were DNA repair, Myc targets V1 and Myc targets V2. From these cues obtained from enrichment, we could further explore the clinical significance of these lncRNAs in the future.

## Conclusions

WGCNA was performed to explore the module-trait relationship of lncRNAs and mRNAs, as well as identifying the key module and involved lncRNAs, mRNAs and miRNAs. Two rounds of ceRNA network were successfully constructed for further screening the survival associated lncRNAs, mRNAs and miRNAs included in regulatory network. Five HCC survival associated lncRNAs were obtained, which have been proved to be promising biomarkers in HCC diagnosis. The analysis process was effective to find significant lncRNAs and the constructed ceRNA network was assistive for exploring the regulatory mechanism.

### Conflict of Interests

The authors declare that they have no conflicts of interest in this work.

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