ceRNA network development and tumor-infiltrating immune cell analysis in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma is among the primary causes of cancer deaths globally. Despite efforts to understand liver cancer, its high morbidity and mortality remain high. Herein, we constructed two nomograms based on ceRNA networks and invading immune cells to describe the molecular mechanisms along with the clinical prognosis of HCC patients.

Methods: RNA maps of tumors and normal samples were downloaded from TCGA. HTseq counts and fragments per megapons per thousand bases were read from 421 samples, including 371 tumor samples and 50 normal samples. We established a ceRNA network based on differential gene expression in normal versus tumor subjects. CIBERSORT was employed to differentiate 22 immune cell types according to tumor transcriptomes. Kaplan-Meier along with Cox proportional hazard analyses were employed to determine the prognosis-linked factors. Nomograms were constructed based on prognostic immune cells and ceRNAs. We employed ROC (Receiver operating characteristic) and calibration curve analyses to estimate these nomogram.

Results: The difference analysis found 2028 mRNAs, 128 miRNAs, and 136 lncRNAs to be significantly differentially expressed in tumor samples relative to normal samples. We set up a ceRNA network containing 21 protein-coding mRNAs, 12 miRNAs, and 3 lncRNAs. In kaplan-Meier analysis, 21 of the 36 ceRNAs were considered significant. Of the 22 cell types, resting dendritic cell levels were markedly different in tumor samples versus normal controls. Calibration and ROC curve analysis of the ceRNA network, as well as immune-infiltration of tumor showed resultful accuracy (three-year survival AUC: 0.691, five-year survival AUC: 0.700; three-years survival AUC: 0.674, five-year survival AUC: 0.694). Our data suggest that Tregs, CD4 T-cells, mast cells, SNHG1, HMMR and hsa-miR-421 are associated with HCC based on ceRNA-immune cells co-expression patterns.

Conclusion: On the basis of ceRNA network modeling and immune cell infiltration analysis, our study offers an effective bioinformatics strategy for studying HCC molecular mechanisms and prognosis.

Declarations

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Conflicts of interest/Competing interests (include appropriate disclosures)

The authors declare no conflict of interest.

Availability of data and material (data transparency)

The data was downloaded from The Cancer Genome Atlas (TCGA) database.

Code availability (software application or custom code)

Not applicable.

Authors' contributions (optional: please review the submission guidelines from the journal whether statements are mandatory)

L.C. and C.N. conceived and designed the experiments; W.Z. performed the experiments; L.C., H.S. and L.Z. analyzed the data; Z.L. and L.C. contributed reagents and materials.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Not applicable.

Ethics approval (include appropriate approvals or waivers)

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Consent to participate (include appropriate statements)

All authors agree to submit articles for publication.

Consent for publication (include appropriate statements)

All authors agree with publication.

Introduction

Hepatocellular carcinoma (HCC) is the 6th most frequent cancer globally and is linked to rising morbidity and mortality [1]. Despite advances in HCC treatment, including chemotherapy, targeted therapy, radiotherapy, Iodine-125 seed implantation, transcatheter arterial chemoembolization (TACE), radiofrequency ablation, and immunotherapy, its overall 5-year survival has increased by 1-3% only and HCC relapse rate may reach 70% in the 5 years. Remarkably, a median survival of 7.1 months has been reported in advanced liver cancer patients without treatment [2]. Liver cancer is a major clinical challenge globally. Thus, effective personalized HCC biomarker and therapeutic strategies, as well as prognostic factors are urgently needed.

CeRNA networks, correlations between mRNA, miRNA, and lncRNA have been identified in many diseases [3–6]. However, HCC ceRNA networks remain unknown. Understanding the molecular mechanisms underlying HCC tumorigenesis is critical for early detection, diagnosis, successful treatment, and prognosis determination.

Here, we established a HCC-associated ceRNA network on the basis of gene expression datasets from TCGA. Using CIBERSORT, we evaluated HCC tumor sample infiltration by various immune cells. We then established 2 nomograms for predicting HCC prognosis on the basis of important immune cells and the ceRNA network. The association of HCC-linked ceRNA with immune cells networks was assessed to determine potential molecular mechanisms.

Materials And Methods

3.1. Data abstraction and differential gene expression analysis

HCC RNA profiles along with the matching patient clinical data were abstracted from the TCGA data resource (<https://tcga-data.nci.nih.gov/tcga/>). HTseq counts and fragment mapping per million exons per thousand bases for 421 samples (371 tumors and 50 normal samples) were assembled. edgeR was employed to uncover mRNA, lncRNA, and miRNA that were differentially expressed. False discovery rate (FDR), $|\log(\text{fold change})| > 1.0$ coupled with $p < 0.05$ were set as cutoff thresholds for differentially expressed genes.

3.2. Construction of the ceRNA network

lncRNA-miRNA-mRNA ceRNA networks are hinged on the theory that lncRNAs can directly or indirectly serve as miRNA sponges to modulate mRNA activity. The ceRNA network was built using the package

“GDCRNATools” R (<http://bioconductor.org/packages/devel/bioc/html/GDCRNATools.html>). Cytoscape web resource (<http://www.cytoscape.org/>) was employed to visualize the expression locations of the ceRNA network [7].

3.3. Survival analyses and nomograms of essential members in the ceRNA network

Kaplan-Meier (K-M) survival assessment was done to evaluate correlation between Biomarker expression and HCC survival. Next, important variables in the initial Cox model were analyzed to identify those with prognostic value and important biomarkers integrated into the reduced Cox proportional hazard model. LASSO (minimal equivalent contraction and recovery of selective operators) utilizes contraction to lower the data value to a specific point to verify the validity of the constructed multifactorial model. Finally, a nomogram was created on the basis of the multivariate model to predict HCC prognosis. The nomogram may enable clinicians determine a prognostic score for each biomarker based on its expression. The sum of individual scores may indicate prognosis and overall three- and five-year survival. Calibration and recipient behavioral characteristic (ROC) curve analyses were used to assess the nomograms accuracy.

3.4. CIBERSORT estimation

CIBERSORT uses gene expression data to determine the abundance, as well as the proportion of various types of immune cells in a mixed cell population [8]. Here, we employed CIBERSORT to evaluate the fractions of 22 immune cell types in HCC samples. Only cases with CIBERSORT outputs of $p \leq 0.05$ were eligible for subsequent analysis. Wilcoxon rank-sum was used identify important immune cells in tumor vs non-malignant samples. Next, K-M survival assessment was employed to assess the association of the proportion of specific immune cells with HCC overall survival. Upon LASSO regression analysis, distinct immune cells were integrated into a Cox proportional hazard model and a nomogram developed for HCC prognosis prediction. The concordance index of the Cox model was employed to assess the nomogram’s bias and precision. Pearson’s correlation explored correlation between HCC biomarkers and immune cells.

3.5. Statistical analysis

Statistical analyses were done on R version 4.0.2 software using edgeR, ggplot2, GDCRNATools [9], rms, glmnet, preprocessCore, survminer, and timeROC packages. Two-sided $p \leq 0.05$ indicates statistical significance.

Results

4.1. Criterion and expression analysis of genes with essential differences

A schematic of our analysis process is shown in Figure 1. Genes with $FDR \leq 0.05$ as well as a log fold change >1.0 or <-1.0 were regarded as differentially expressed. Differential expression of mRNAs, miRNAs, and lncRNAs in tumor vs normal samples was calculated using DESeq2 (Figure 2A-C). Our analysis identified 104 lncRNAs, 107 miRNAs, and 1222 mRNAs as upregulated and 32 lncRNAs, 21 miRNAs, and 806 mRNAs as downregulated (Figure 2D).

4.2. CeRNA network construction and survival assessment

The ceRNA network was developed on the basis of interactions between 14 pairs of lncRNA–miRNA and 42 pairs of miRNA–mRNA (Figure 3A, Table 1). K-M survival assessment evaluated the relationship of the prognosis with biomarkers in the HCC-linked ceRNA network. These analysis revealed that hsa-miR-421 ($p < 0.001$) and hyaluronan mediated motility receptor (HMMR) ($p < 0.001$) signified significance. These analysis revealed that hsa-miR-421 ($p < 0.001$) and hyaluronan mediated motility receptor (HMMR) ($p < 0.001$) signified significance (Figures 3B-C).

4.3. Establishment of the prediction model on the basis of the ceRNA network

LASSO regression analysis found HMMR, ARL2, RNF24, has-miR-326, RAP2A, S100A10, and hsa-miR-421 as essential for modeling, which were then integrated into Cox regression analysis and a nomogram built for prognosis prediction. Area under curve (AUC) values for three- and five-year survival were 0.691 and 0.700, respectively, indicating good efficiency and *accuracy*. Additionally, the identification of the nomogram can be seen through the calibration curves (Figure 4A-G).

4.4 Development of the prediction model on the basis of the immune cells

We integrated five of twenty-two immune cells with remarkable prognostic potential in the Cox regression into the final multivariable model with satisfactory estimation power (concordance index = 0.660) and used to establish the nomogram. LASSO regression analysis revealed that all 6 genes were important for modeling. ROC and calibration curve analysis revealed acceptable accuracy (three-year survival AUC: 0.674; five-year survival AUC: 0.694), indicating good concordance (Figure 6A-G).

4.4 Comprehensive assessment of genes and immune cells

Pearson's correlation analyses of co-expression patterns between various immune cells and the correlation between biomarkers and immune cells (Figure 7A-B) found that HMMR and M0 macrophages ($p = 0.00046$, $R = 0.18$), RAP2A and M0 macrophages ($p = 0.000017$, $R = -0.22$), RNF24 and M0 macrophages ($p = 0.000057$, $R = 0.21$) and RNF24 and resting mast cells ($p = 0.000000009$, $R = -0.3$) had good correlation (Figure 7C-F).

Discussion

Mounting evidence shows that genetic alterations in signaling pathways drive HCC tumorigenesis, highlighting the importance of molecular biomarkers in liver cancer detection. Although the mechanisms underlying HCC tumorigenesis are unclear, cellular and molecular changes are influential factors. Here, we first evaluated differentially expressed ceRNA and immune cells infiltration in normal vs tumor HCC tissues. We then developed nomograms for predicting HCC prognosis. The excellent concordance index and AUC value in the 2 nomograms may guide HCC survival prediction. K-M survival along with correlation analysis indicated that the ceRNA modulatory network of SNHG1 (lncRNA), HMMR (mRNA), hsa-miR-421 (miRNA), and Tregs may influence HCC progression.

It is estimated <2% of the human genome is protein-coding, suggesting that most human transcripts are non-coding [10]. mRNAs, miRNAs, and lncRNAs are linked in an intricate crosstalk network by competing endogenous RNA [11]. Interaction between miRNA, lncRNA, and mRNA, in ceRNA networks is extensively studied in various disorders, including lung, gastric, and gallbladder cancers [12,13]. However, the mechanisms of ceRNA networks in HCC oncogenesis are largely unclear. Here, we find that in a ceRNA network, SNHG1 (a lncRNA), may downregulate hsa-miR-421 and upregulate HMMR to promote HCC tumorigenesis. Hsa-miR-421 is reported to play a core role in malignant transformation, which is consistent with our findings [14,15]. HMMR is an important component of polo-like kinase 1 (PLK1)-dependent mitotic spindle localization cascade, which is essential for neurodevelopment, neonatal survival, and tumorigenesis [16]. HMMR is upregulated in many cancers, such as lung cancer, glioblastoma, prostatic adenocarcinoma, and leukemia [17–20]. HMMR has been documented to enhance EMT (epithelial-mesenchymal transition) and carcinogenesis by activating TGF- β /Smad2 signaling in gastric cancer [21]. Assessment of 1420 colorectal cancer tissues indicated that HMMR may be a better prognostic factor relative to tumor grade as well as vascular infiltration [22]. How HMMR modulates cell cycle-linked gene expression has not been studied. HMMR antisense lncRNA HMMR-AS1 stabilizes HMMR mRNA and enhances the progress of lung adenocarcinoma, epithelial ovarian cancer, as well as glioblastoma [23–25]. HMMR negatively correlates with poor HCC prognosis, highlighting it as a potential indicator of HCC prognosis [26].

We also uncovered different proportions of immune cells in liver cancer. Naïve CD4 T-cells, resting mast cells, gamma delta T-cells, Tregs, and activated dendritic cells have been shown to be associated with HCC. A nomogram based on 5 immune cell types, built for overall survival prediction (consistency index = 0.66) may have high clinical value.

Tregs, which are CD25⁺, account for 5-10% of CD4⁺ T-cells and are one of the most widely studied immune cell types due to their inhibitory effects on tumor pathogenesis. Tregs repress the activation as well as the proliferation of CD4⁺ and CD8⁺ T-cells *in vitro* and *in vivo* using various mechanisms consisting of cell-cell contact along with secretion of immunosuppressive cytokines like TGF- β , IL-10, and IL-35 [27]. Tregs are believed to be the most important determinant of hepatitis B prognosis [28]. Infection with hepatitis B virus (HBV) is linked to >60% of liver cancer cases in developing countries and <25% in developed countries. Our data show that Tregs are upregulated in HCC. It is suggested that Tregs depletion may slow HCC progression[29].

Levels of tumor infiltrating dendritic cells (DCs) correlate with clinical outcomes. CD14⁺CTLA4⁺ regulatory DCs have the same inhibitory effect as Tregs in HCC. These DCs inhibit T-cell response and upregulate PD-1 via IL-10 and CTLA4 [30].

High CD4⁺ T-cell levels also hinder liver cancer development. Anti-programmed death 1 antibody on the surface of CD4⁺ cells can demonstrate the clinical outcomes of HCC patients.

In the innate immune cells, total mast cells, monocytes, DCs, as well as neutrophils are considerably changed. Mast cells have key immune regulation functions, and the mechanism of cell inactivation in HCC remains unclear. IgE, a mast cell activator has been reported in HBV-linked HCC [31]. Our data show a higher proportion of active mast cells in healthy livers and a higher proportion of inactive subtypes in advanced HCC. COX evaluation also established that the proportion of resting mast cells remarkably correlated with prognosis. Mast cells constitute the fastest immune cell responders that are particularly enriched in barrier sites. Their rapid release is mediated by factors like vasoactive amines, proteases, cytokines, and proteoglycans, from intracellular storage upon FCER1-bound IgE crosslinking [32]. However, mast cell function in abnormal livers is still unclear and warrants further investigation.

Correlation analysis found that CD4 T-cells were linked to HMMR ($R=-0.164$, $p=0.0016$). CeRNA network correlation analysis and hypergeometry tests found that SNHG1 (a lncRNA), HMMR (a protein-coding RNA), and hsa-miR-421 (a miRNA) were remarkably associated ($p=0.007847$), suggesting that the interaction between hsa-miR-421, SNHG1, HMMR, and CD4 T-cells is associated with HCC development.

This study has several limitations. Firstly, multivariable survival assessment included only basic prognostic factors from TCGA and could not suggest other potential clinical factors like metastatic lesions status and patients' performance status. Secondly, preponderant evidence suggests that HCC is a very heterogeneous tumor at genetic and molecular levels. Due to study limitations, gene expression data from GEO, SYMH, and ICGA cohorts was determined from one piece of HCC tissue from a single patient. Future studies should evaluate gene expression in several HCC specimens from a patient via single-cell whole-genome sequencing or RT-qPCR analysis to determine if the line map is a reliable and viable predictor of HCC overall survival markers. In addition, cooperation with other hospitals should be considered to recruit more patients and samples for gene model validation.

Conclusions

Based on a ceRNA network along with tumor-invading immune cells, we established 2 nomograms with high concordance index as well as AUC values for predicting HCC prognosis. On the basis of comprehensive clinical data from predictive nomograms, individualized management of HCC patients can be significantly enhanced and has huge potential to be a candidate biomarker for the diagnosis, prognosis as well as therapeutic targets of HCC patients.

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Table 1. Hypergeometric testing and correlation analysis results of ceRNAs network

LncRNA	Protein-coding RNA	MiRNAs	Hypergeometric test P	Correlation P
SNHG1	E2F8	hsa-miR-421	0.00784745	8.58865E-31
SNHG1	HMMR	hsa-miR-421	0.042928897	2.93371E-27
SNHG1	NMB	hsa-miR-421	0.042928897	1.9713E-25
SNHG1	MSH2	hsa-miR-21-5p	0.042928897	1.54818E-24
SNHG1	CRYBG2	hsa-miR-21-5p	0.042928897	9.00723E-19
SNHG1	NETO2	hsa-miR-21-5p	0.041029046	1.71971E-08
SNHG1	S100A10	hsa-miR-21-5p	0.042928897	5.98165E-05
SNHG1	LMNB2	hsa-miR-326,hsa-miR-330-5p	0.002308944	1.56875E-38
SNHG1	FANCE	hsa-miR-326,hsa-miR-330-5p	0.000392403	6.37741E-30
SNHG1	ANKRD13B	hsa-miR-326,hsa-miR-330-5p	0.041029046	3.34609E-20
PVT1	ABCC5	hsa-miR-17-5p,hsa-miR-93-5p	0.026876318	1.97873E-13
PVT1	RNF24	hsa-miR-17-5p,hsa-miR-20a-5p,hsa-miR-93-5p,hsa-miR-20b-5p	0.000133481	3.03984E-09
PVT1	ANKRD52	hsa-miR-17-5p,hsa-miR-20a-5p,hsa-miR-93-5p,hsa-miR-106b-5p,hsa-miR-20b-5p	0.007044169	1.04891E-08
PVT1	PLAG1	hsa-miR-17-5p,hsa-miR-20a-5p,hsa-miR-93-5p,hsa-miR-106b-5p,hsa-miR-20b-5p	0.000138113	0.000120269
KCNQ1OT1	NDOR1	hsa-miR-214-3p	0.006618532	8.40638E-11
KCNQ1OT1	CDC7	hsa-miR-335-5p	0.008147588	1.13552E-09
KCNQ1OT1	KLC2	hsa-miR-214-3p	0.000409319	8.95697E-05
KCNQ1OT1	ARL2	hsa-miR-214-3p	0.040952018	0.000207007
KCNQ1OT1	CDC25B	hsa-miR-214-3p	0.020800997	0.001761624
KCNQ1OT1	LMNB2	hsa-miR-326,hsa-miR-330-5p	0.001910986	6.64665E-15
KCNQ1OT1	ANKRD13B	hsa-miR-326,hsa-miR-330-5p	0.00598274	4.41844E-06
KCNQ1OT1	FANCE	hsa-miR-326,hsa-miR-330-5p	0.006618532	6.25071E-06
KCNQ1OT1	RAP2A	hsa-miR-326,hsa-miR-330-5p	0.020888159	6.77642E-06
KCNQ1OT1	MECOM	hsa-miR-214-3p,hsa-miR-338-3p	0.015398832	0.034334548
KCNQ1OT1	ANKRD52	hsa-miR-214-3p,hsa-miR-326,hsa-miR-330-5p	0.038275504	2.38934E-13

ceRNAs, competing endogenous RNAs; lncRNA, long noncoding RNA; miRNA, microRNA.

Figures

Figure 1

The flowchart of the analysis process.

Figure 2

The heat maps of differentially expressed (A) RNAs, (C) miRNAs, (E) lncRNAs between the tumor and normal tissues of HCC. (B) Bar plot showing differentially expressed protein coding genes, long noncoding genes, pseudogenes, and other RNAs. Red and blue represent up-regulated and down-regulated RNAs, respectively. It shows that 806 of 2028 differentially expressed protein-coding genes are down-regulated and 1222 are up-regulated. Besides, among 136 differentially expressed lncRNAs, 32 lncRNAs are down-

regulated, and 104 are up-regulated. Volcano plots of differentially expressed miRNAs (D) and lncRNAs (F). We defined the log (fold change) >1.0 or <-1.0 and FDR <0.05 as the critical point. Thus, the red and green dots in the plots represent high and low expression RNAs with statistical significance, respectively. Meanwhile, black dots represent mRNAs and lncRNAs without statistical significance.

Figure 3

(A) Overview of the lncRNA-miRNA-mRNA ceRNA network of HCC with 14 pairs of lncRNA-miRNA and 42 pairs of miRNA-mRNA. Kaplan-Meier survival curves based on the expression of biomarkers involved in ceRNA network related to the bone metastasis in melanoma shows that (B) HMMR ($P<0.001$), (C) hsa-miR-421 ($P<0.001$), (D) E2F8($P<0.001$) and (E) FANCE($P< 0.001$) had significantly prognostic values. The flowchart of the analysis process.

Figure 4

The Cox proportional hazards model based on RNAs selected by (B) (C) Lasso regression. HMMR, RNF24, RAP2A, S100A10, ARL2, has-miR-326 and hsa-miR-421 are incorporated into the Cox proportional hazards model. (E) Nomogram for predicting patients' outcome based on RNAs (HMMR, RNF24, RAP2A, S100A10, ARL2, has-miR-326 and hsa-miR-421) in Panel (A). (D) ROC curves and (F) calibration curves for assessing the discrimination and accuracy of the nomogram. Besides, AUCs of the 3- and 5-year survival were 0.691 and 0.700, respectively. AUC, area under curve; ROC, receiver operating characteristic.

Figure 5

(A) Bar plot showing cell types and relative percent in HCC. Different colors represent different cell types, which are listed in the right as y axis, while x axis represents different samples. (B) Heat map of tumor-infiltrating cells in tumor tissues in HCC patients. Annotations on top show clustering of samples. While the blue represents the normal, the red symbolizes the HCC. (C) Violin plot for comparing cells' proportion between the tumor and normal. It illustrates that the proportion of the T cells CD4 naïve in tumor tissues was relatively less than that in the normal tissues ($P = 0.004$), and Tregs, T cells gamma delta dendritic cell resting and mast cell activated were relatively greater in the tumor than the normal tissues ($P = 0.016$, 0.005 , 0.031 and 0.005).

Figure 6

(A) Cox proportional hazards model integrated by 4 different types of immune cells. (B) Nomogram for predicting patients' outcome based on 4 cells in Panel (A). (C) Calibration curves for evaluating the accuracy of the nomogram. *P < 0.05; **P < 0.001.

Figure 7

(A) Correlation analysis (Pearson analysis) of different tumor-infiltrating cells and (B) the relationships between different tumor-infiltrating cells and differentially expressed genes in HCC. Scatterplots further illustrate the exact relationship between HMMR and Macrophages M0 ($P = 0.00046$, $R = 0.18$) (C), RAP2A and Macrophages M0 ($P = 0.000017$, $R = -0.22$) (D), RNF24 and Macrophages M0 ($P = 0.000057$, $R = 0.21$) (E) and RNF24 represented good correlation with Mast cells resting ($P = 0.000000009$, $R = -0.3$) (F).

Besides, gray-shaded areas in two graphs represent the standard errors of the blue regression lines. R, correlation coefficient.