

Original Article



Identification of Key N^7 -Methylguanosine-Associated Factors in NASH-Related HCC

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Abstract:

Background: Non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) have been the common causes of hepatocellular carcinoma (HCC). Methyltransferase-like 1 (METTL1) is a key factor regulating N^7 -methylguanosine (m7G) methylation. This study aimed to determine the specific roles of METTL1 and m7G in NASH-related HCC, and identify potential miRNA/mRNA axis regulated by METTL1 and m7G.

Methods: The expression of METTL1 was determined in microarray GSE83596, the Cancer Genome Atlas (TCGA) database, and Human Protein Atlas (HPA) database. Microarray GSE52821 was downloaded for identifying key m7G-regulated miRNAs. TargetScan was introduced to predict target genes of miR-760. The database for annotation, visualization, and integrated discovery database (DAVID) was used for Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis. Protein-protein interaction (PPI) analysis was used to find hub genes. Using R, we conducted a prognosis model for HCC. Drug Pair Seeker (DPS) was employed to identify potential drug combinations against NASH-related HCC.

Results: METTL1 was upregulated in NASH-related HCC, it was also related with stemness. miR-760 was identified as the key downstream miRNA of METTL1, it may be regulated by m7G methylation. 52 downregulated genes were predicted as potential target genes of METTL1/miR-760 axis. GO and KEGG analysis showed that these genes were enriched in multiple key pathways such as mitogen-activated protein kinase (MAPK) pathway and linked to the development of HCC. A prognostic signature was conducted based on METTL1 and FGF1. Finally, drug combinations were predicted, a histone deacetylase (HDAC) inhibitor, trichostatin is a potential therapeutic strategy against NASH-related HCC.

Conclusions: In the study, METTL1/miR-760 was identified as a key m7G-related axis in NASH-related HCC, targeting this axis may be a novel strategy against NASH-related HCC.

Keywords: Bioinformatic analysis; Hepatocellular carcinoma; microRNA; Nonalcoholic steatohepatitis; N^7 -methylguanosine;

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become an increasingly serious clinical burden all

over the world. The global prevalence of NAFLD has exceeded 20% ^[1]. About 20%-27% NAFLD patients would develop to the advanced stage, nonalcoholic steatohepatitis (NASH) ^[2]. NASH is characterized by inflammation, steatosis, and fibrosis ^[3]. NAFLD and NASH are the causes of multiple irrevocable liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). Lipid metabolism disorders are closely related with HCC ^[4], mounting evidence suggested that NAFLD and NASH can induce HCC. A study showed that NAFLD and NASH are the primary etiology of over 30% HCC patients ^[5, 6]. Compared with other pathogens, NASH showed the fastest growing death age-standardized rates of liver cancer ^[7]. Regular surveillance is necessary for NAFLD/NASH patients to prevent HCC ^[8]. However, the specific mechanism of how NASH develops into HCC has not been clearly clarified.

microRNAs (miRNAs) are small non-coding RNAs with ~22 nucleotides. Studies have shown that miRNAs were closely related with many diseases including metabolic diseases, cancers, and developmental diseases ^[9-12]. miRNAs can be modified by multiple biological progress such as reactive oxygen species (ROS) and methylation ^[13, 14]. *N*⁶-methyladenosine (m6A) is a type of common methylation in multiple miRNAs. m6A modification is closely related with many important progresses such as cancer progression, metastasis, and drug resistance ^[15].

*N*⁷-methylguanosine (m7G) is another common prevalent RNA methylation modification, the most well-studied regulator of m7G is methyltransferase-like 1 (METTL1). METTL1 and m7G are related with the modifications of multiple RNAs such as messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) ^[16, 17]. They are also reported closely related with multiple cancers including HCC. Upregulation of METTL1 is correlated with high alpha fetoprotein (AFP) levels and poor prognosis ^[18]. However, most studies showed that METTL1 promotes HCC mainly through m7G tRNA modification ^[19, 20]. In 2019, Pandolfini *et al.* reported that miRNAs can be modified by METTL1 and m7G. m7G can regulate miRNA structure and biogenesis, and finally change the expression level ^[21]. There is no research showing that a relationship between m7G miRNA modification and HCC.

In this study, we aimed to find key m7G-related factors regulating NASH to HCC through bioinformatic analysis, and provide potential treatment strategies against NASH-related HCC. We determined the roles of METTL1 in NASH-related by analyzing data from the Gene Expression Omnibus (GEO) database, Human Protein Atlas (HPA) database, and the Cancer Genome Atlas (TCGA) database. Key m7G-related miRNAs were obtained through GEO database. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis were used to reveal the processes that key genes enriched. A model was conducted to predict the prognosis of HCC patients. Potential drug combinations targeting NASH-related HCC were predicted using Drug Pair Seeker (DPS).

2 Methods

2.1 Analysis of miRNA/gene expression in HCC using TCGA and HPA databases

UALCAN (<http://ualcan.path.uab.edu/>) was used to analyze the expression of key miRNAs and genes in HCC patients in TCGA database ^[22]. We also explored the relationships between key factor expression and tumor grades. GEPIA (<http://gepia.cancer-pku.cn/>) was used to analyze the prognosis of HCC patients in TCGA database ^[23]. Besides, we obtained immunohistochemical data using HPA database (<https://www.proteinatlas.org/>).

2.2 Analysis using GEO database

We used GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) to filter mRNA databases and miRNA databases about NASH-related HCC. Finally, we collected two databases: GSE83596 and GSE52821. GSE83596 was a dataset based on platform GPL21810, it had 3 NASH mouse models and 4 NASH-related HCC mouse models. GSE52821 was a database about miRNAs which has 2 NASH mouse models and 3 NASH-related HCC mouse models, it was based on platform GPL16362. Data from the two databases were normalized firstly using R, and then we used the R package 'LIMMA' for differential analysis ^[24]. Screening criteria of key miRNAs/DE-genes were: $|\log_2FC| > 0.5$ in GSE52821 and $|\log_2FC| > 1.0$ in GSE83596. DE-genes were shown in volcano plots and heatmaps were drawn using R.

2.3 Prediction of target genes

TargetScan (<https://www.targetscan.org/>) is a database which can predict the target genes of miRNAs. In this study, we used this database to obtain target genes of miR-760^[25].

2.4 GO and KEGG pathway analysis

We used the database for annotation, visualization, and integrated discovery (DAVID 6.8, <https://david.ncifcrf.gov>) to perform GO and KEGG pathway analysis. GO functional analysis includes biological process (BP), cellular component (CC), and molecular function (MF)^[26].

2.5 PPI network construction and identification of hub genes

STRING database (<http://string-db.org>) was used to conduct a protein-protein interaction (PPI) network. And then we introduced the CytoHubba application in Cytoscape software (Version 3.6.1) to identify hub genes based on the calculated degree. The top 11 genes were determined as hub genes for further research.

2.6 Construction of prognosis model for HCC

We analyzed the correlation between key genes and the prognosis of HCC patients in TCGA database and filtered out genes related with prognosis. And then we used the R package “glmnet” to conduct the Lasso regression algorithm. The receiver operating characteristic (ROC) curve was introduced to test the predictive accuracy of the prognosis model. p-values and hazard ratio (HR) with 95% confidence interval (CI) of Kaplan-Meier curves were generated by log-rank tests and univariate cox proportional hazards regression.

2.7 Correlation analysis using TCGA database

We used Linked Omics (<http://www.linkedomics.org/>) to analyze the correlation between the two factors of HCC from TCGA data^[27]. In this study, we analyzed the correlation between miR-760 and METTL1, positive correlation indicated that METTL1 may induce the expression of miR-760 through m7G. We also explored the correlation between key genes and METTL1/miR-760, negative correlation indicated

that the genes may be regulated by METTL1/miR-760 axis *in vivo*.

2.8 Prediction of potential drug combinations

We used Drug Pair Seeker (DPS, Version 1.4.0, <http://www.maayanlab.net/DPS/>) to predict potential drug combinations against NASH-related HCC. And Cytoscape software (Version 3.6.1) was used to construct a drug-gene network.

2.9 Effect of METTL1 on stemness

We used one-class logistic regression machine learning algorithm (OCLR) by Malta et al. to calculate mRNAsi, which is a stemness indice. And then we compared the mRNAsi scores in different groups. The analysis was conducted through R^[28].

2.10 Statistical analysis

In this study, data were analyzed using GraphPad Prism Software (Version 7) and represented as mean ± standard deviation (SD). A *p* value < 0.05 was considered statistically significant.

3 Results

3.1 METTL1 was upregulated in NASH-related HCC

Previous studies have shown that METTL1 was upregulated in HCC, it may play important roles in the occurrence and development of HCC^[18, 19]. The expression and roles in NASH-related HCC have not been clearly clarified. We determined the expression of METTL1 using GEO database (GSE83596), results showed the upregulation of METTL1 in NASH-related HCC (Figure 1A, Figure S1A, B). Besides, we also analyzed the expression of METTL1 in HCC patients through TCGA database, we found METTL1 was also upregulated in HCC and was closely related with tumor grades. Data from HPA database were consistent with the results above. (Figure 1B, C, F). METTL1 overexpression was closely correlated with poor prognosis, including overall survival (OS) and disease free survival (DFS) (Figure 1D, E). In conclusion, METTL1 was upregulated in NASH-related HCC, and expression of METTL1 was closely related with the prognosis of HCC patients. METTL1 may be a novel factor for HCC diagnosis and treatment.

Figure 1

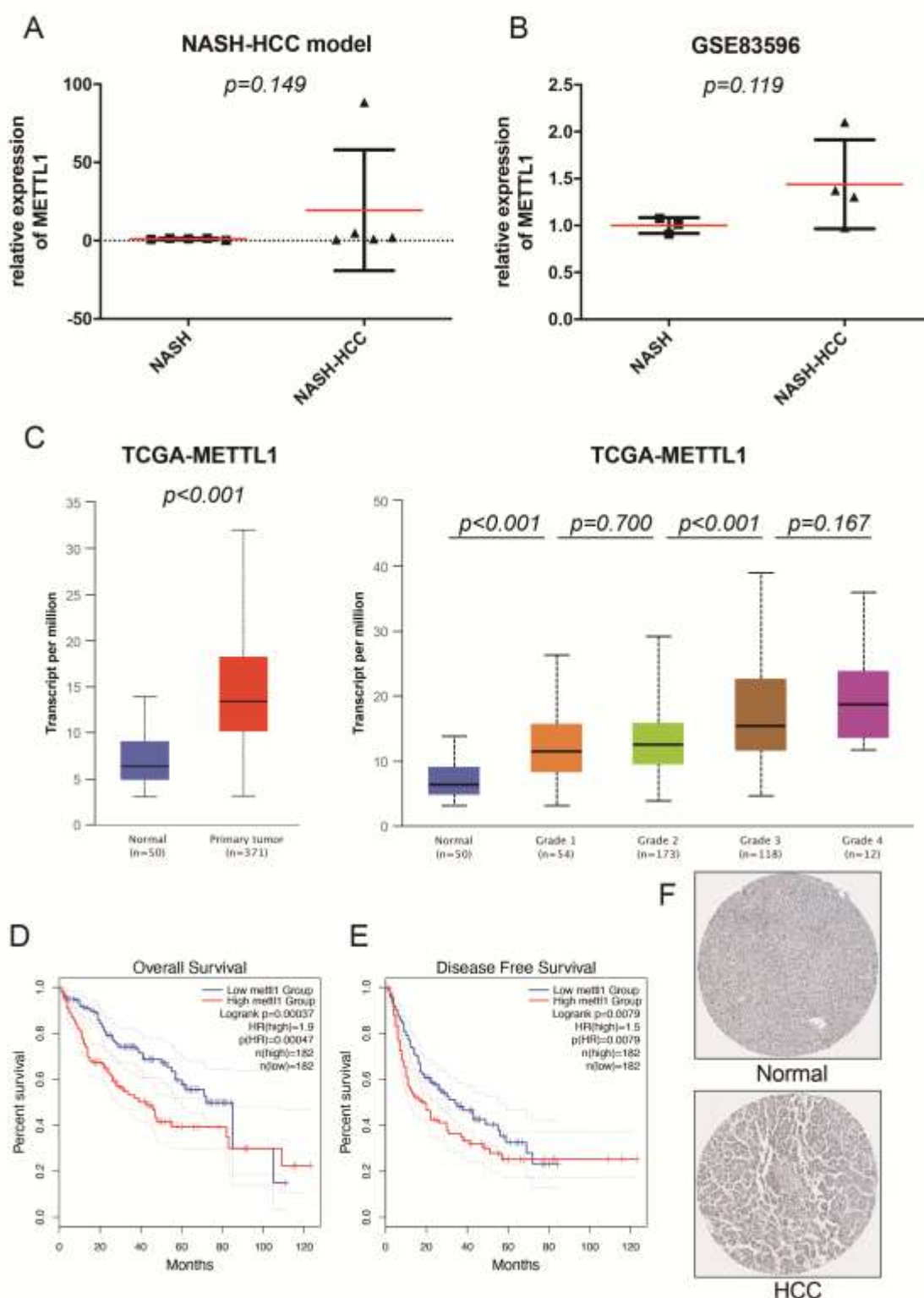


Figure 1. METTL1 was upregulated in NASH-related HCC. A: Expression of METTL1 in NASH-related HCC in GSE83596; **B:** Expression of METTL1 in HCC in TCGA database; **C:** Correlation between METTL1 expression and tumor grade; **D:** Overall Survival of METTL1 in HCC in TCGA database; **E:** Disease Free Survival of METTL1 in HCC in TCGA database; **F:** Immunohistochemical analysis of METTL1 in HCC in HPA (original magnification $\times 80$). The experiments were independently repeated at least three times. NASH: nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma; TCGA: the Cancer Genome Atlas; HPA: Human Protein Atlas.

Figure S1

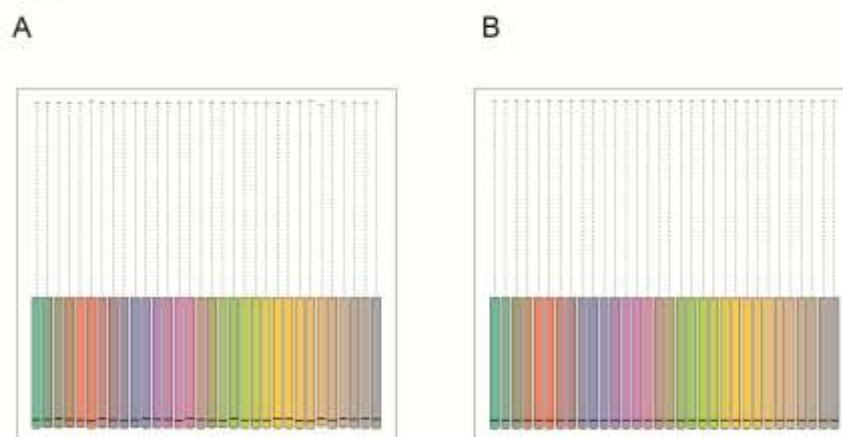


Figure S1. Normalization of data of GSE83596. A: Data before normalization; B: Data after normalization.

3.2 Identification of key m7G-modified miRNAs

METTL1 can induce the upregulation of miRNA levels through m7G miRNA precursor (pri-miRNA) modification. Pandolfini *et al.* have identified 20 miRNAs whose precursors were

modified by METTL1-dependent m7G, these miRNAs were downregulated after knocking down METTL1^[21]. To determine m7G-modified miRNAs which may play key roles in NASH-related HCC, we analyzed data from GEO database (GSE52821). Finally, we identified 5 potential miRNAs (Figure 2A, B, Figure S2A, B).

Figure 2

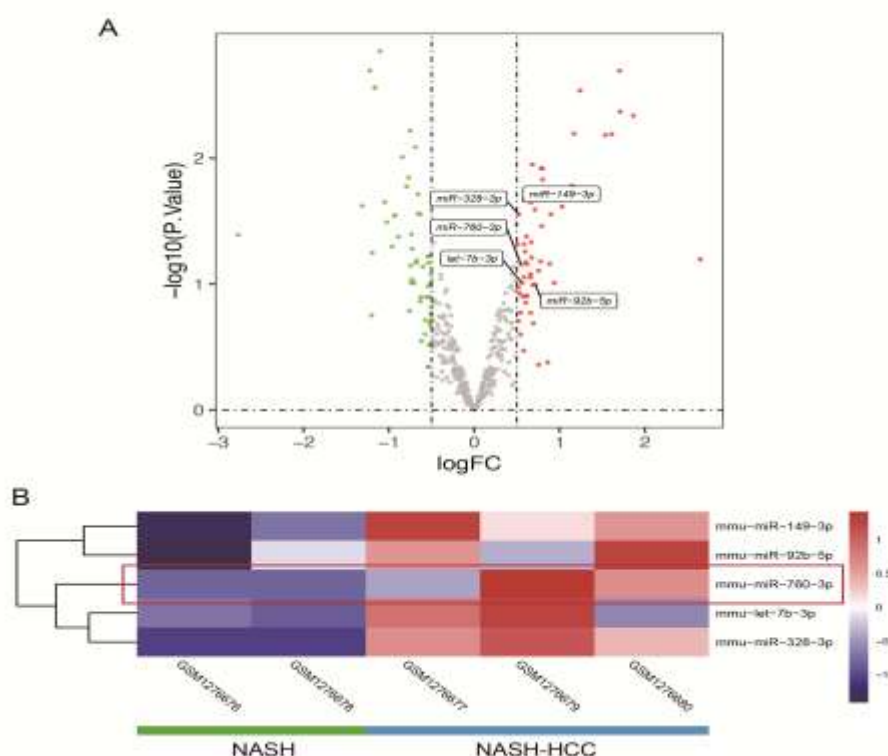


Figure 2. Volcano plot and heatmap of the m7G-modified miRNAs in GSE52821. A: Volcano plot of miRNAs. Grey dots represent miRNAs which are not differentially expressed in the NASH group and NASH-HCC group; red dots and green dots represent miRNAs that are upregulated and

downregulated significantly in NASH-HCC group; B: Heatmap of miRNAs. Red rectangles mean that miRNAs are upregulated in these samples, and blue ones mean downregulated. NASH: nonalcoholic steatohepatitis; m7G: *N*⁷-methylguanosine; HCC: Hepatocellular carcinoma; miRNA: microRNA.

Then we compared the sequences of miRNAs in *Homo sapiens* and *Mus musculus*, miR-92b-5p and miR-149-3p were excluded. We also identified the expression of let-7b-3p, miR-760-3p

(miR-760 in *Homo sapiens*) and miR-328-3p. We found let-7b-3p and miR-328-3p were downregulated in HCC patients (Figure S2C, D), miR-760 was upregulated (Figure 3A).

Figure S2

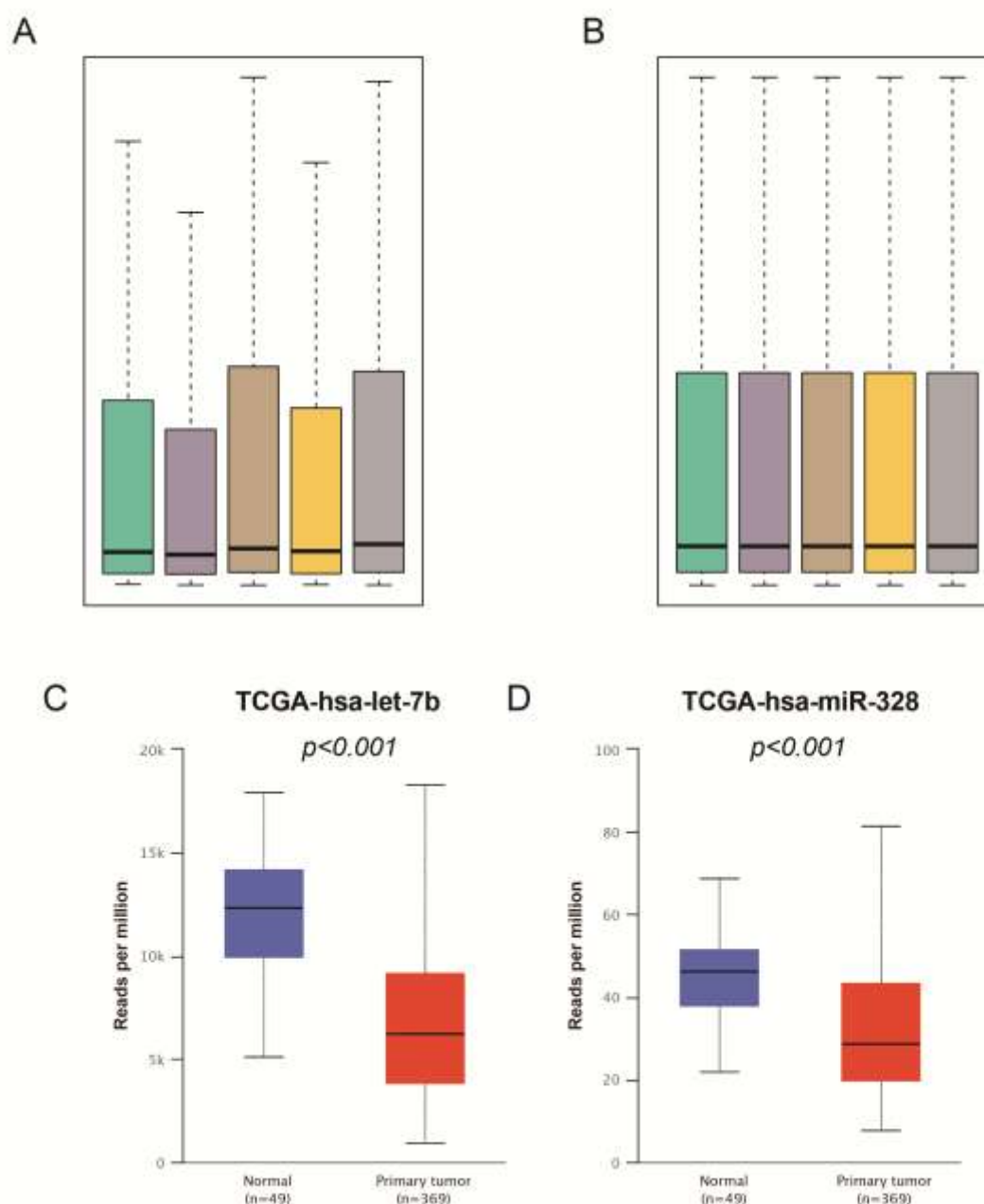


Figure S2. Normalization of data of GSE52821 and the expression of miRNAs in HCC in TCGA database. A: Data before normalization; B: Data after normalization; C: Expression of let-7b in HCC in TCGA database; D: Expression of miR-328 in HCC in TCGA database. HCC: Hepatocellular carcinoma; TCGA: the Cancer Genome Atlas.

Finally, we chose miR-760 for further research. miR-760 was not only upregulated in HCC, but was closely related with tumor grades (Figure 3B). We also analyzed the correlation between miR-760 and METTL1 in TCGA database, they

had a significantly positive correlation (Figure 3D). In summary, miR-760 was regulated by METTL1 and may play an important role in NASH-related HCC.

Figure 3

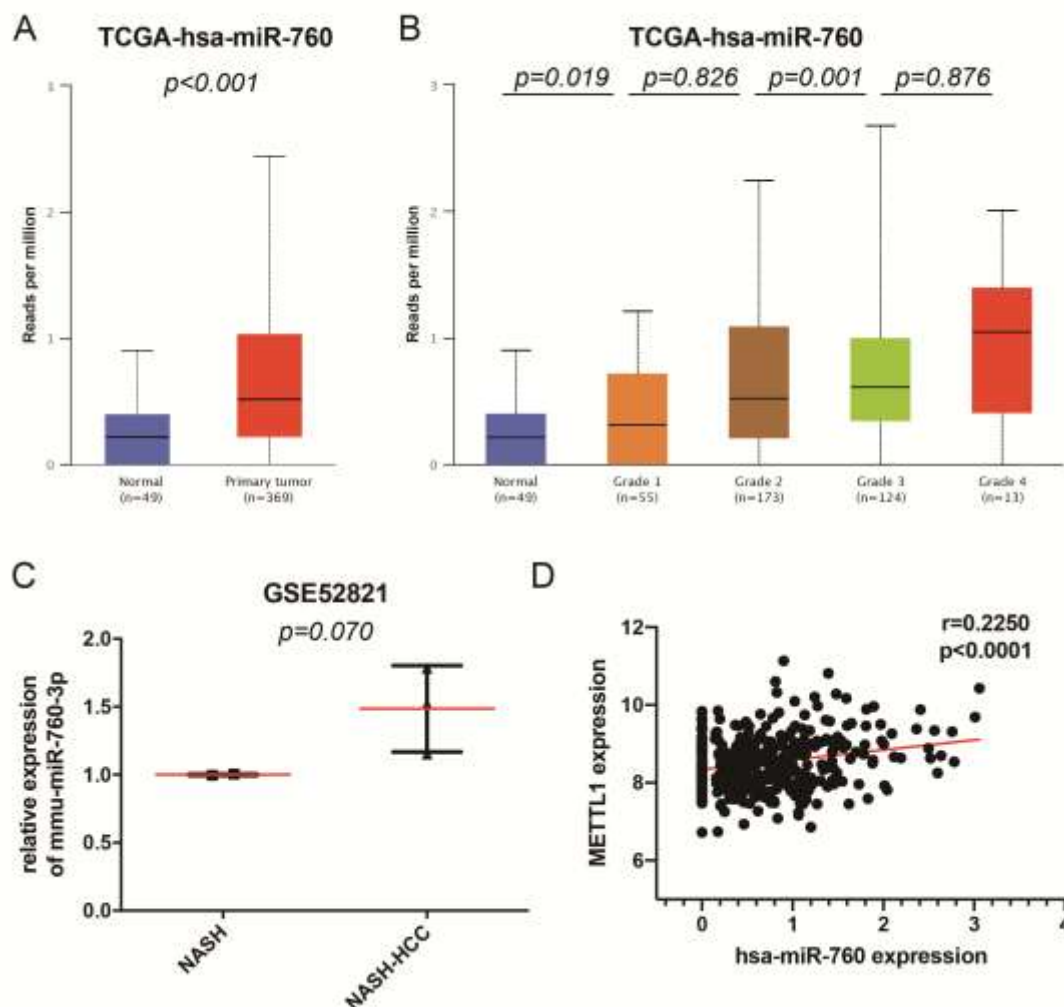


Figure 3. miR-760 was upregulated in NASH-related HCC. A: Expression of miR-760 in HCC in TCGA database; **B:** Correction between miR-760 expression and tumor grade; **C:** Expression of miR-760 in NASH-related HCC in GSE52821; **D:** Correction between METTL1 and miR-760 in HCC in TCGA database. NASH: nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma; TCGA: the Cancer Genome Atlas.

3.3 Identification of target genes of miR-760

miRNAs play important roles mainly through inhibiting the expression of target genes. So, we predicted target genes of miR-760 using TargetScan database. And we obtained 568

potential target genes. Next, we analyzed DE-genes in GSE83596. Finally, 52 downregulated genes which were both DE-gene and target gene were filtered for further research (Figure 4A-C). Volcano plot showed the top 10 most downregulated key DE-genes.

Figure 4

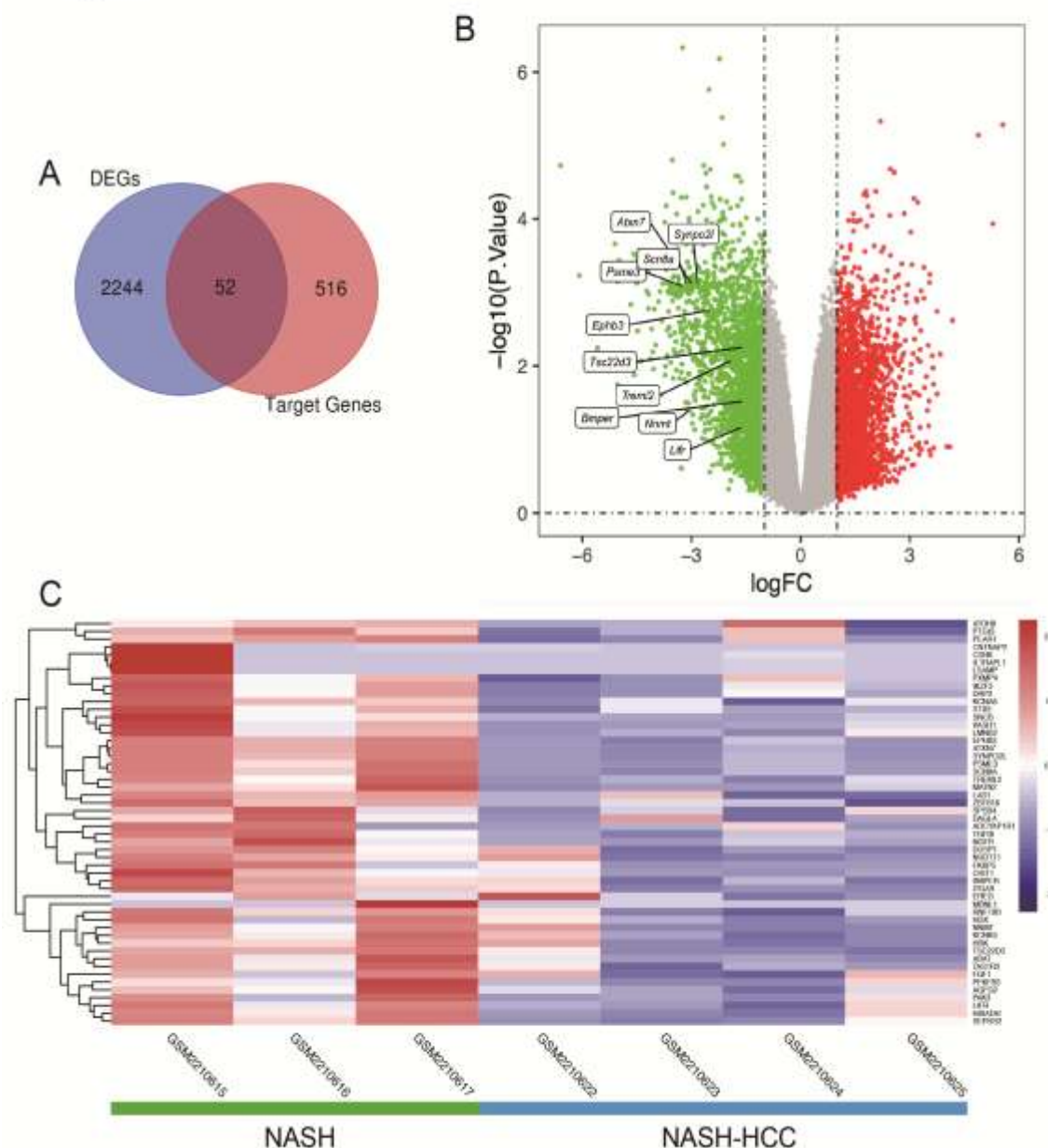


Figure 4. Target genes of miR-760. A: The intersection of differentially expressed genes (DE-genes) and target genes predicted by TargetScan; **B:** Volcano plot of DE-genes in GSE83596. Grey dots represent genes which are not differentially expressed in the NASH group and NASH-HCC group; red dots and green dots represent genes that are upregulated and downregulated significantly in NASH-HCC group; **C:** Heatmap of 52 key DE-genes. Red rectangles mean that genes are upregulated in these samples, and blue ones mean downregulated. NASH: nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

3.4 GO functional and KEGG pathway enrichment analysis

Next, GO functional and KEGG pathway enrichment analyses were performed on the 52 target genes (Table 1, Figure 5).

Figure 5

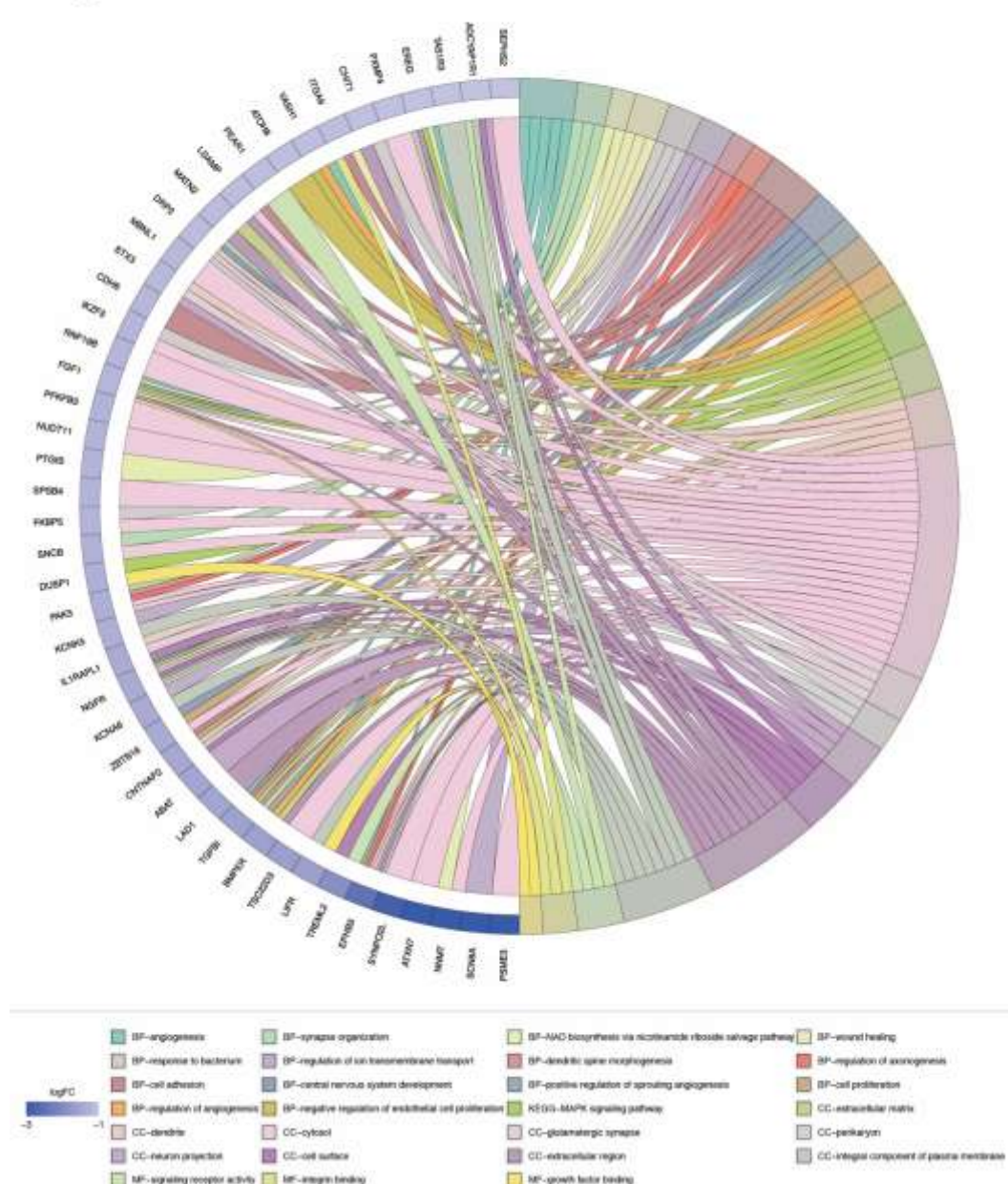


Figure 5. GO functional and KEGG pathway enrichment analysis for 52 target genes. The left side of the circle includes all related genes, and the right side is GO and KEGG terms. BP: biological process; CC: cellular component; MF: molecular function; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

The enriched GO functions for these genes include angiogenesis, synapse organization, and NAD biosynthesis via nicotinamide riboside salvage pathway in the BP category; extracellular matrix, dendrite, and cytosol in the CC category; signaling receptor activity, integrin binding, and growth factor binding in the MF category. As for

KEGG pathway enrichment analysis, these genes are mainly enriched in mitogen-activated protein kinase (MAPK) signaling pathway. In conclusion, results of enrichment analysis showed that these target genes were closely related with NASH and HCC.

Table 1. GO and KEGG terms enriched by DAVID.

ID	Term	Count	p value
GO:0001525	BP-angiogenesis	5	0.003824

GO:0050808	BP-synapse organization	3	0.00926
GO:0034356	BP-NAD biosynthesis via nicotinamide riboside salvage pathway	2	0.025163
GO:0042060	BP-wound healing	3	0.028307
GO:0009617	BP-response to bacterium	3	0.038013
GO:0034765	BP-regulation of ion transmembrane transport	3	0.045701
GO:0060997	BP-dendritic spine morphogenesis	2	0.047279
GO:0050770	BP-regulation of axonogenesis	2	0.049705
GO:0007155	BP-cell adhesion	5	0.052794
GO:0007417	BP-central nervous system development	3	0.055872
GO:1903672	BP-positive regulation of sprouting angiogenesis	2	0.071275
GO:0008283	BP-cell proliferation	3	0.081259
GO:0045765	BP-regulation of angiogenesis	2	0.08772
GO:0001937	BP-negative regulation of endothelial cell proliferation	2	0.09468
GO:0031012	CC-extracellular matrix	4	0.026694
GO:0030425	CC-dendrite	5	0.027417
GO:0005829	CC-cytosol	21	0.035305
GO:0098978	CC-glutamatergic synapse	4	0.065835
GO:0043204	CC-perikaryon	3	0.067779
GO:0043005	CC-neuron projection	4	0.068371
GO:0009986	CC-cell surface	5	0.074046
GO:0005576	CC-extracellular region	10	0.077656
GO:0005887	CC-integral component of plasma membrane	8	0.078773
GO:0038023	MF-signaling receptor activity	4	0.023463
GO:0005178	MF-integrin binding	3	0.068291
GO:0019838	MF-growth factor binding	2	0.09387
hsa04010	KEGG-MAPK signaling pathway	4	0.065428

BP: biological process; CC: cellular component; MF: molecular function; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

3.5 Construction and analysis of PPI network

Then, we used STRING database to conduct PPI network of 52 DE-genes (Figure 6A). These genes showed strong connections and then we used Cytoscape software to calculate and obtain hub

genes. Figure 6B showed the networks between the top 11 genes and the node degrees were shown in Figure 6C. The top 11 genes were FGF1, DUSP1, TGFBI, DRP2, PAK3, NGFR, SNCB, CNTNAP2, FKBP5, SCN8A, and ADCYAP1R1.

Figure 6

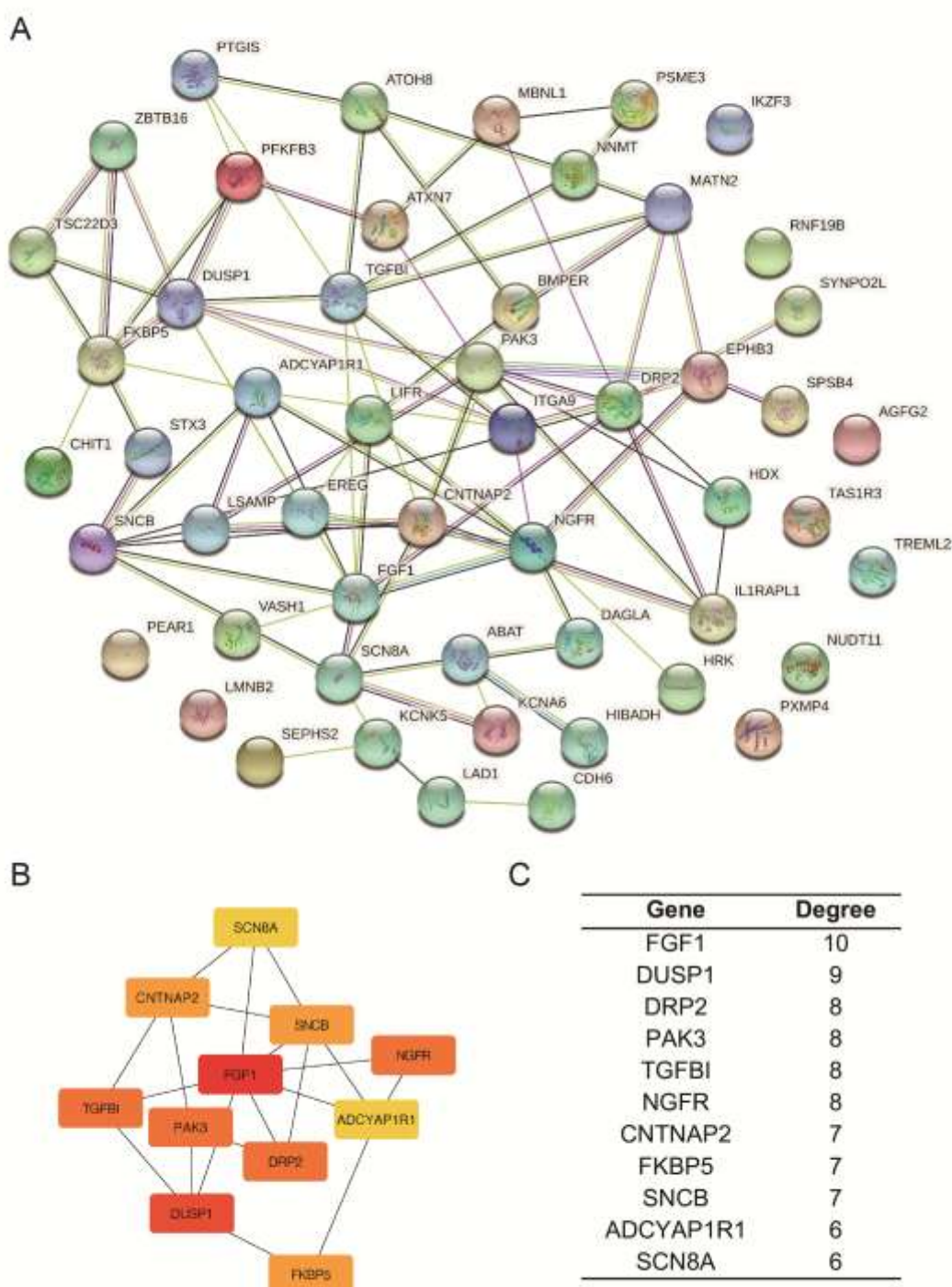


Figure 6. Identification of hub genes. A: PPI network of 52 target genes; B: Links between the top 11 hub genes; C: Node degree of hub genes. PPI: protein-protein interaction; DE-genes: differentially expressed genes.

3.6 Construction of prognosis model for HCC

Results above showed that the 11 hub genes were potential factors which may participate in the development of HCC. So, we analyzed the expression of these genes in TCGA database. And

we identified 5 genes which were downregulated in HCC for further research: FGF1, DUSP1, PAK3, NGFR, CNTNAP2 (Figure 7A, Figure 8). Other 6 genes were excluded (Table 2). And then we analyzed the relationship between the 5 genes and the prognosis of HCC patients. We found that

only the expression of FGF1 was related with the

DFS of HCC patients (Figure 7B,

Figure S3

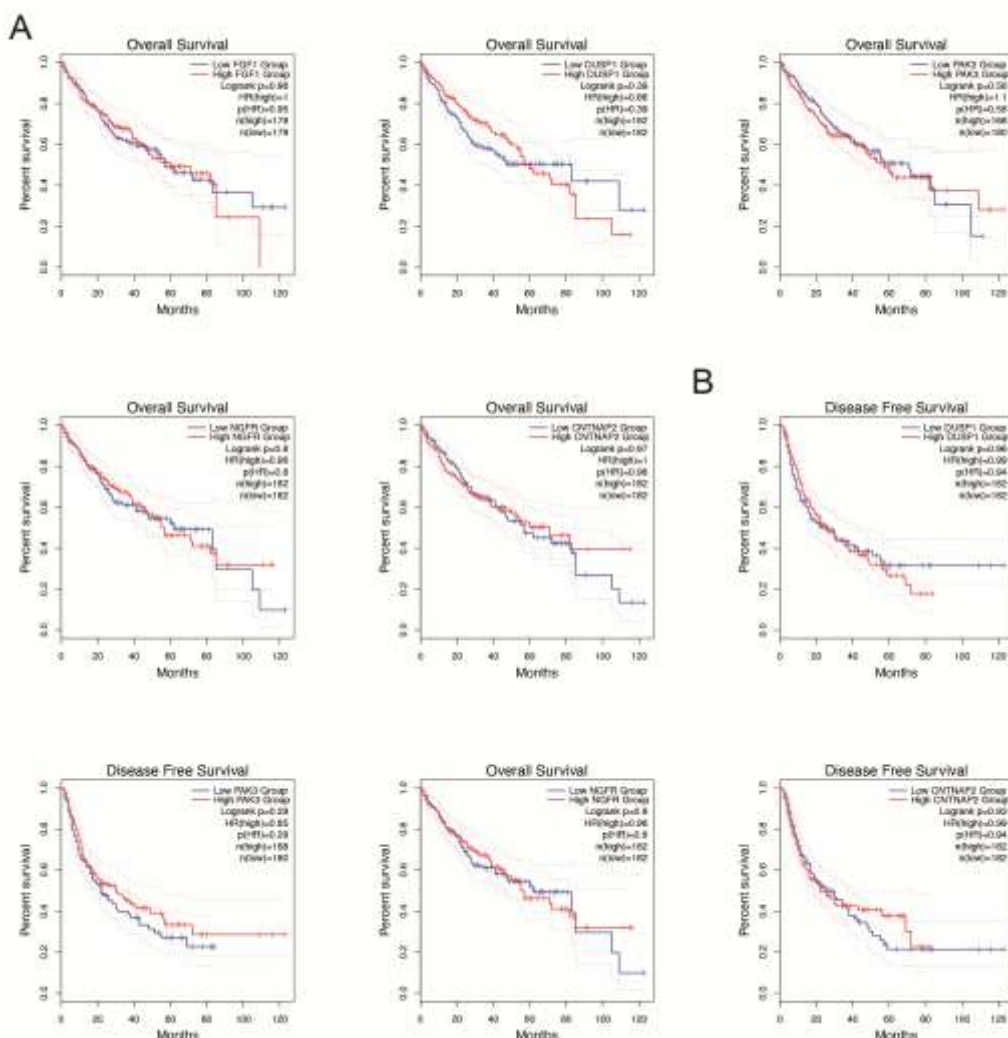


Figure S3. Correction between key genes and the prognosis of HCC patients in TCGA database. A: Overall Survival of FGF1, DUSP1, PAK3, NGFR, and CNTNAP2 in HCC in TCGA database; B: Disease Free Survival of DUSP1, PAK3, NGFR, and CNTNAP2 in HCC in TCGA database. HCC: Hepatocellular carcinoma; TCGA: the Cancer Genome Atlas. 3).

We can get the conclusion that METTL1 is closely related with the OS and DFS of HCC patients. So, we constructed a model using the expression of METTL1 and FGF1 to predict the DFS of HCC patients. The riskscore was $(0.4453) \times \text{METTL1} + (-0.3924) \times \text{FGF1}$, then we divided patients into high-risk group and low-risk group (Figure 7C). We found the DFS of high-risk

group was significantly shorter than low-risk group (Figure 7D). The ROC curve showed that the area under curve (AUC) value for 1-year, 3-year, and 5-year were 0.661, 0.612, and 0.717 (Figure 7E). In a word, we created a prognosis model with high specificity and sensitivity to predict the DFS of HCC patients.

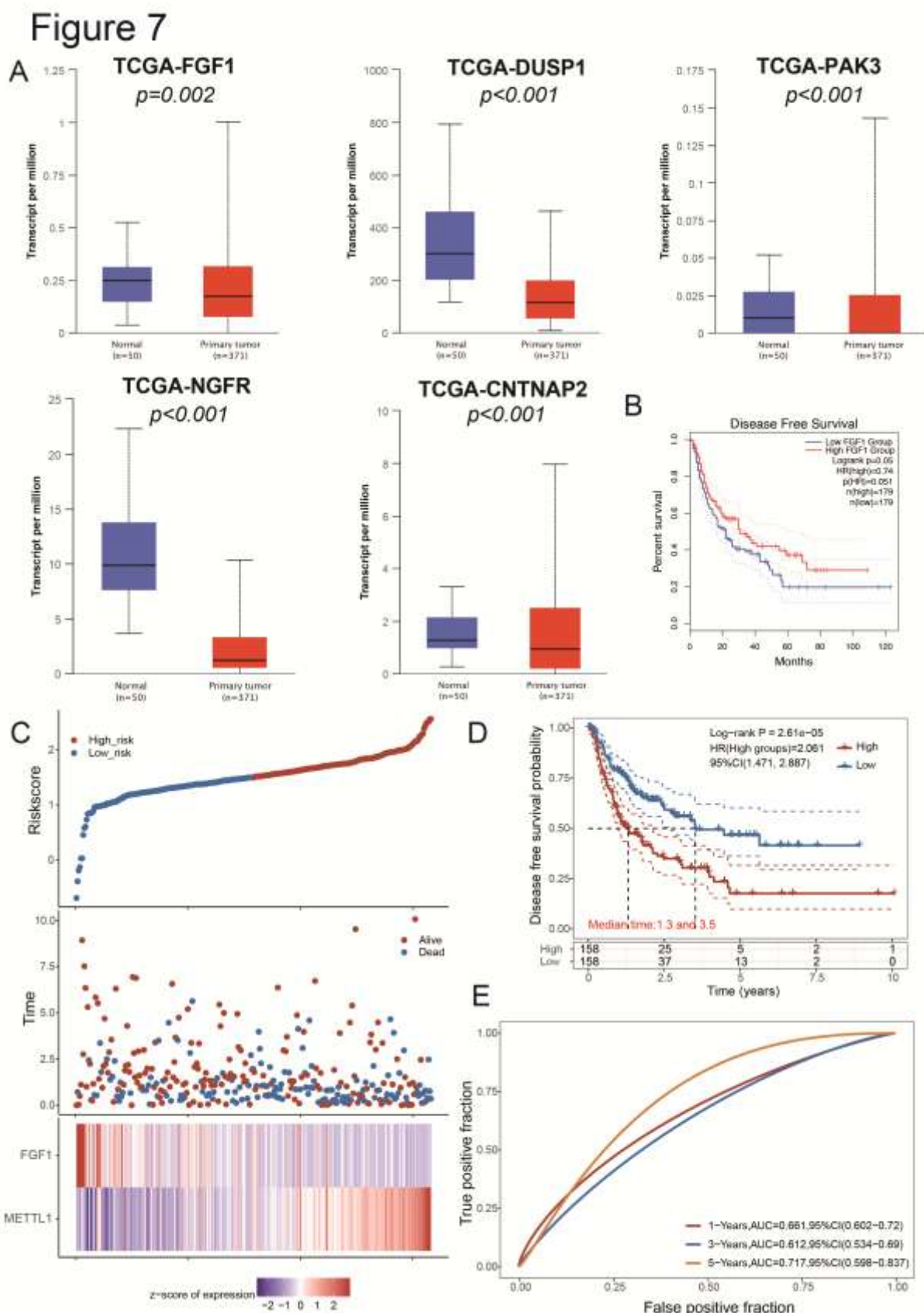


Figure 7. Further identification of key genes and construction of prognosis model for HCC. A: Expression of FGF1, DUSP1, PAK3, NGFR, and CNTNAP2 in HCC in TCGA; **B:** Disease Free Survival of FGF1 in HCC in TCGA database; **C:** Riskscores of different samples in HCC in TCGA database; **D:** Disease Free Survival of high-risk group and low-risk group; **E:** ROC curve of the prognosis model. HCC: Hepatocellular carcinoma; TCGA: the Cancer Genome Atlas; ROC: receiver

operating characteristic; AUC: area under curve.

Figure 8

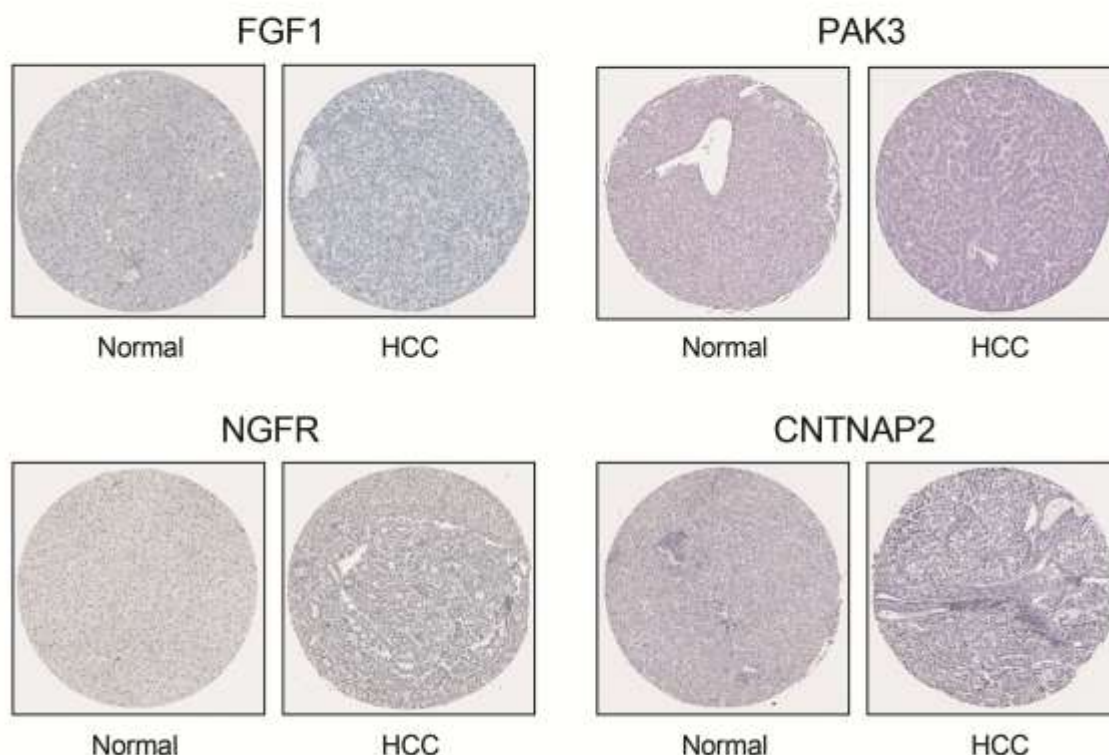


Figure 8. Immunohistochemical analysis of key genes. The expression of FGF1, PAK3, NGFR, and CNTNAP2 in HCC in HPA (original magnification $\times 80$). Data of DUSP1 were unavailable. HCC: hepatocellular carcinoma; HPA: Human Protein Atlas.

Table 2. Expression of genes in TCGA database

Genes	Expression versus normal	<i>p</i> value
TGFBI	Upregulated	<0.001
DRP2	Upregulated	<0.001
SNCB	not express	
FKBP5	Upregulated	<0.001
SCN8A	Upregulated	<0.001
ADCYAP1R1	not express	

TCGA: the Cancer Genome Atlas.

3.7 Verification of the correlation between target genes and METTL1/miR-760

Next, we analyzed the relationships between the five genes and METTL1/miR-760. We found that apart from PAK3, other 4 genes all had negative

correlations with METTL1 and miR-760 (Figure 9A, B). This result indicated that these 4 genes may play important roles in NASH-related HCC and they were regulated by METTL1/miR-760 axis.

Figure 9

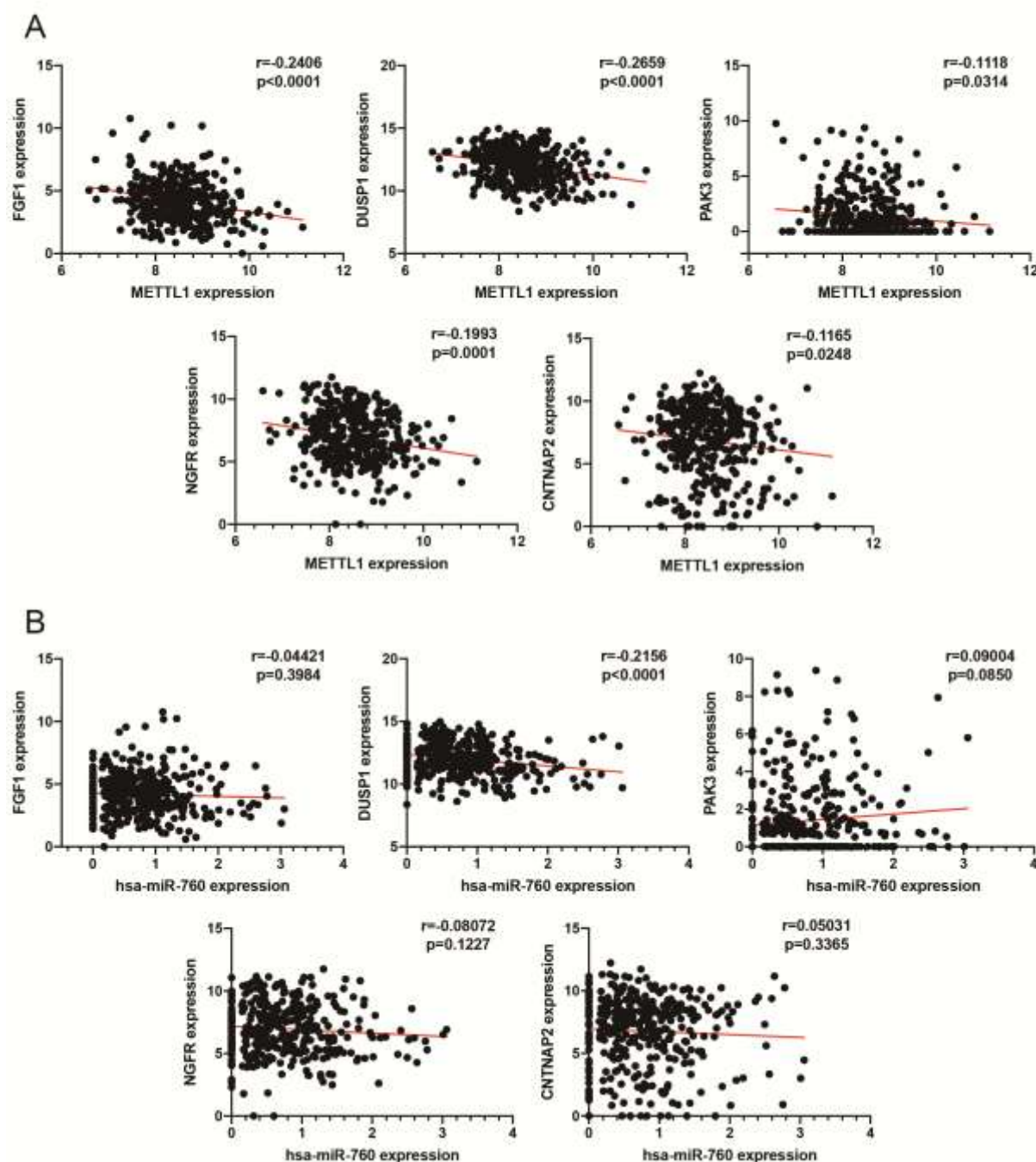


Figure 9. Correction between key genes and METTL1/miR-760. A: Correction between METTL1 and key genes in HCC in TCGA database; B: Correction between miR-760 and key genes in HCC in TCGA database. HCC: hepatocellular carcinoma; TCGA: the Cancer Genome Atlas.

3.8 Prediction of the potential drug combinations for NASH-related HCC

Then we predicted the potential drug combinations through uploading METTL1 and the four genes (Figure 10A). We noticed trichostatin

A-4710 had the highest frequency and may have an ideal treatment effect. It can upregulate the expression of NGFR, DUSP1, CNTNAP2, and inhibit METTL1. We also created the network between genes and drugs (Figure 10B).

Figure 10

A

Drug 1	Drug 2	Total Coverage	Total Conflicts	Drug 1 Coverage	Drug 1 Conflicts	Drug 2 Coverage	Drug 2 Conflicts
trichostatin A-5231	tamoxifen-6768	5	0	3	0	2	0
trichostatin A-4710	pimozide-7132	5	0	4	0	3	0
trichostatin A-4710	trichostatin A-4237	5	0	4	0	4	0
3-acetylcoumarin-3044	lisuride-1962	5	0	3	0	2	0
trichostatin A-4710	trichostatin A-3993	5	0	4	0	4	0
trichostatin A-4710	drofenine-3455	5	0	4	0	3	0
trichostatin A-4710	bupropion-5782	5	0	4	0	3	0
trichostatin A-4710	tolazamide-4003	5	0	4	0	3	0
trichostatin A-4710	picotamide-2070	5	0	4	0	3	0
pimozide-7132	3-acetylcoumarin-3044	5	0	3	0	3	0

B

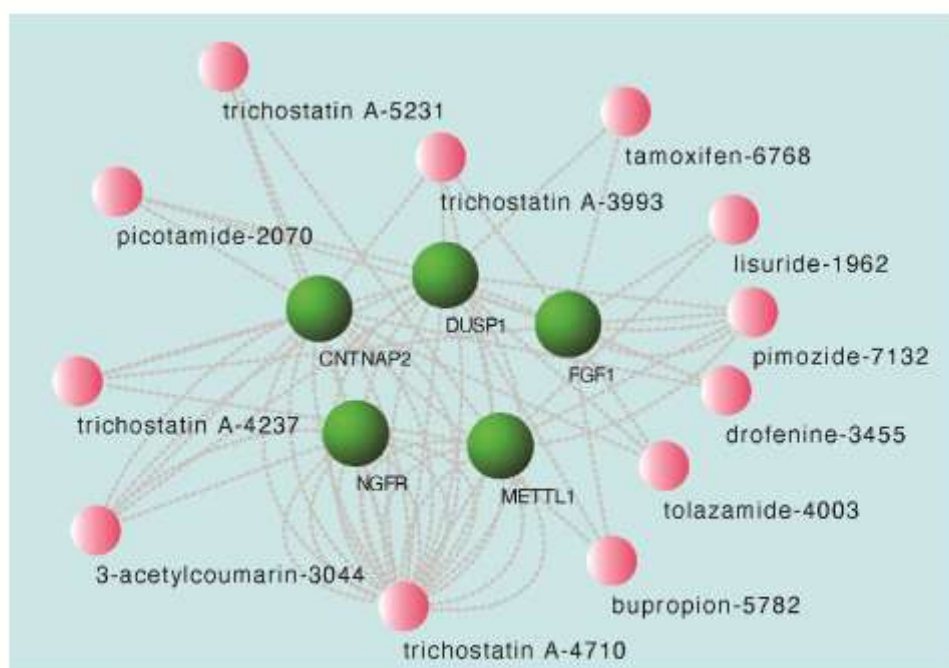


Figure 10. Prediction of drug combinations for NASH-related HCC. A: Drug combinations that may play reversal roles predicted by DPS. Coverage means the number of the drug's favorable targets, conflict is the number of genes that the drug may cause an unfavorable outcome. B: Drug-gene network. NASH: nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma; DPS: Drug Pair Seeker.

3.9 Correlation analysis between stemness and METTL1

Finally, we also explored the effect of METTL1 on HCC stemness. We found that the mRNAsi score in HCC group was higher than normal

group. Meanwhile, the score in METTL1-High group was higher than METTL1-Low group (Figure 11). These results showed that METTL1 is closely related with stemness, it may play important roles in the development of HCC.

Figure 11

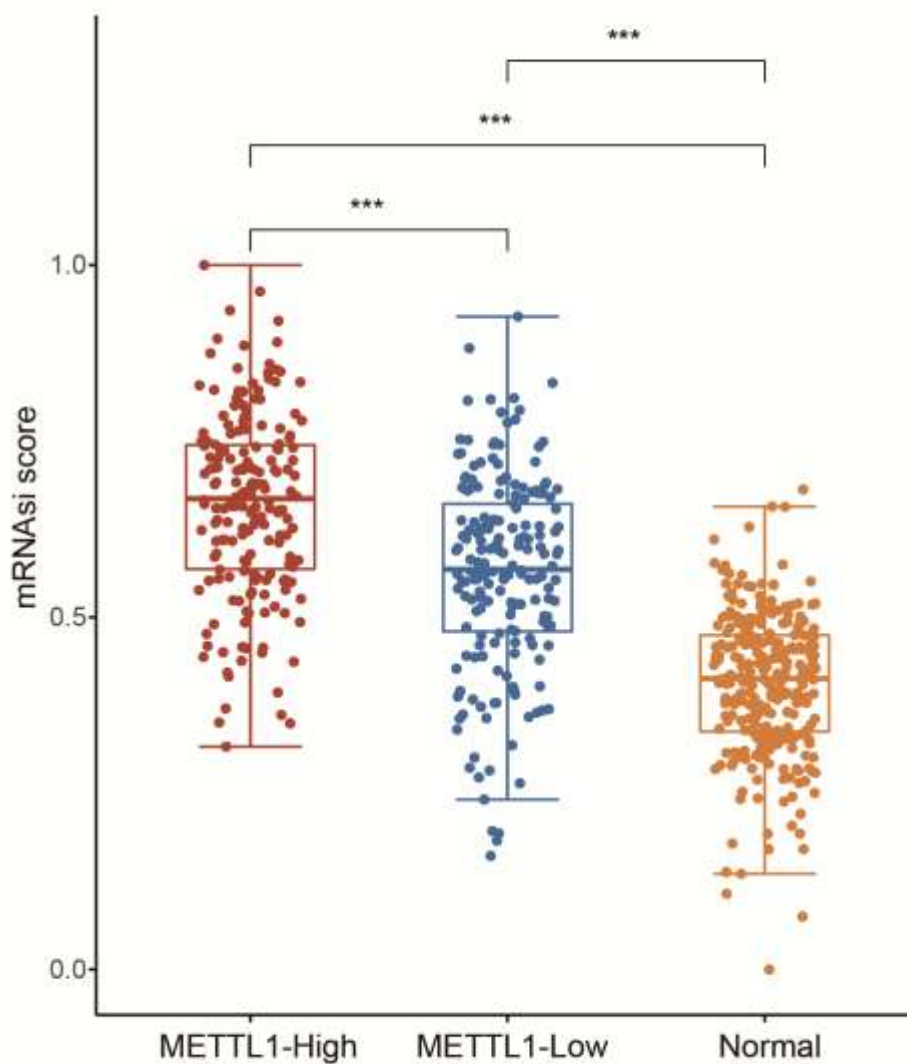


Figure 11. Effect of METTL1 in stemness in HCC. The correlation between METTL1 expression level and mRNAi score. *** $p < 0.001$. HCC: hepatocellular carcinoma.

Figure S4

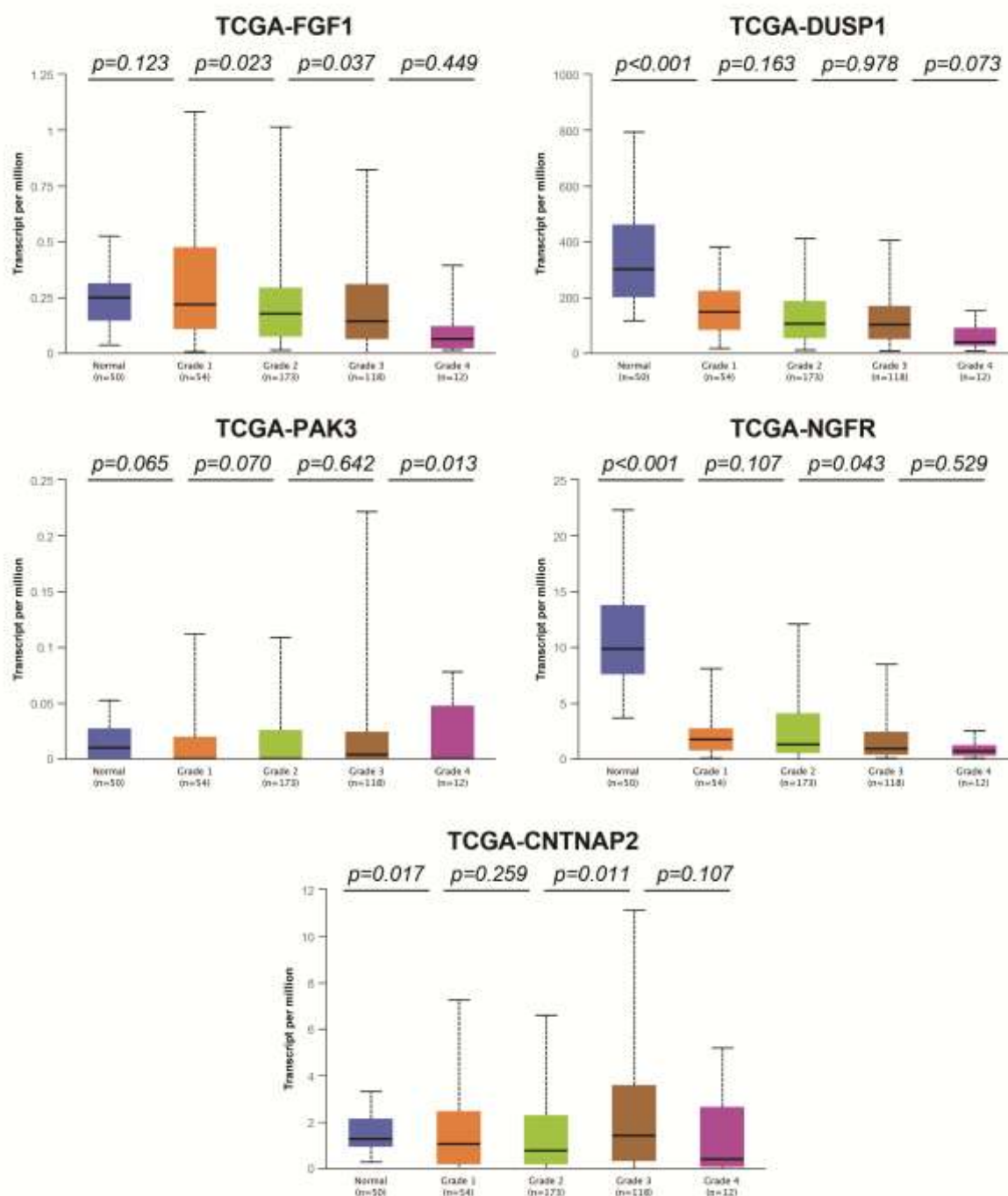


Figure S4. Correlation between key genes and tumor grade. The effect of the expression of FGF1, DUSP1, PAK3, NGFR, and CNTNAP2 on tumor grade of HCC in TCGA database. HCC: Hepatocellular carcinoma; TCGA: the Cancer Genome Atlas.

4 Discussion

In current study, we showed that METTL1 is upregulated in NASH-related HCC through bioinformatic analysis and experimental validation. METTL1 has been reported to regulate the occurrence, metastasis, resistance to

radiotherapy, and recurrence of HCC [19, 20, 29, 30]. One study showed that METTL1 may function through inhibiting phosphatase and tensin homolog (PTEN), which is mutated in multiple cancers [18]. Zeng et al. found that inhibiting the expression of METTL1 can prevent HCC recurrence through affecting the level of

polymorphonuclear-myeloid-derived suppressor cells^[29]. Apart from this mechanism, most studies reported METTL1 regulates HCC development mainly through m7G methylation on RNAs, especially tRNA. As a common methylation modification, m7G has been confirmed to participate in oncogenic cell transformation and cancer^[31]. Our study showed that METTL1 and m7G are closely related with NASH-related HCC, further research needs more experimental validation.

METTL1 and m7G methylation mainly regulate biological progresses through tRNA. They can also affect the synthesis of miRNAs. However, there is no study about m7G-related miRNA in NASH-related HCC. Our research found that miR-760 was upregulated in NASH-related HCC, and it may be regulated by METTL1 and m7G methylation. miR-760 has been reported as an oncogene for HCC, it can induce the proliferation and metastasis of HCC, upregulated miR-760 is significantly associated with poor prognosis in HCC^[32, 33]. These results were consistent with our study. However, there is no experimental research about the correction between miR-760 and NASH-related HCC, and the role of miR-760 in NASH has not been clarified yet. Our future study would focus on the expression of miR-760 in NASH-related HCC patients. Cell and mouse models would be employed to further clarify the function of miR-760, and transgenic mouse models should be used to clarify the role of miR-760 in the development from NASH to HCC.

We also finally filtered four key genes which may be regulated by miR-760, and they were differentially expressed genes (DE-genes) in NASH-related HCC database. We constructed a prediction model for HCC which contained two genes, METTL1 and FGF1. Our study found FGF1 is closely related with NASH-related HCC, upregulated FGF1 is associated with poor prognosis. However, most previous research showed that the upregulation of FGF1 can induce the development of HCC and promote sorafenib resistance. Targeting FGF1 and its upstream factors may be potential treatment strategies against HCC^[34-36]. These results seem to be contradictory with our study, we speculate the reasons may include the sample size and differences between models. Further research should clarify the expression and function of

FGF1 in NASH-related HCC using more cell and mouse models. Moreover, Xia et al. showed the activation of FGF1 can alleviate NAFLD, another research found a FGF1 variant carrying 3 substitutions of heparin-binding sites is effective for preventing and reversing NAFLD by activating adenosine monophosphate-activated protein kinase (AMPK)^[37, 38]. These results indicate the therapeutic roles of FGF1 in NAFLD, and we guess FGF1 could also treat NASH and NASH-related HCC. More studies should be conducted.

Apart from FGF1, we also identified 3 key DE-genes, DUSP1, NGFR, and CNTNAP2. DUSP1 has been reported that it has tumor suppressive role, downregulation of DUSP1 was closely related with poor differentiation and advanced HCC stage^[39]. Meanwhile, DUSP1 was also downregulated in NAFLD patients^[40]. These results showed DUSP1 plays important roles in NAFLD and HCC, which are partly consistent with our studies. There is no research clarifying the role of DUSP1 in NASH-related HCC. Our next study would focus on the function of DUSP1. Qin et al. showed that NGFR was downregulated in HCC, and it was regulated by LINC01554/miR-3681-3p axis^[41]. The conclusion of our study is similar to this. However, no study showed the correction between NGFR and NASH/NASH-related HCC. The product of CNTNAP2 is a transmembrane cell-adhesion protein, low expression of CNTNAP2 is associated with poor overall survival of oligodendrogliomas patients. There is no study about CNTNAP2 and NASH-related HCC^[42]. Our study showed these genes may be potential biomarkers and therapeutic targets of HCC, and the conclusions still need more experimental validation.

There are also several limitations in our study. Results are based on bioinformatic analysis, and further research needs more experimental models to determine the roles of key factors in NASH-related HCC. NASH-HCC mouse models need to be used in next research.

In current study, we showed trichostatin A-4710 is the most frequent drug against NASH-related HCC. Trichostatin is an inhibitor targeting histone deacetylase (HDAC). Numerous studies have demonstrated the aberrant expression of HDAC in cancers including HCC, and HDAC inhibitor have been used in clinic^[43]. Trichostatin has also been

confirmed that it can inhibit cell growth in HCC cells, and it can also prevent HCC in some cirrhosis patients^[44, 45]. Inhibiting HDAC can also attenuate NASH^[46]. The correlations between key genes and HDAC have also been clarified in previous studies. Gao et al. showed inhibiting HDAC can activate FGF1^[47]. DUSP1 can be upregulated by inhibiting HDAC, DUSP1 can overcome gefitinib-acquired resistance in non-small cell lung cancer^[48]. NGFR can also be upregulated by inhibiting HDAC^[49]. Besides, our study showed key genes were enriched in MAPK pathway, MAPK pathway is activated in more than 50% HCC and has become an important therapeutic strategy targeting HCC^[50]. So, genes identified in our study were related with AMPK and HDAC, METTL1/miR-760 may function through these signal pathways. Interestingly, the combination of the inhibitors targeting AMPK and HDAC can enhance the anti-cancer activity^[51]. And trichostatin can inhibit the two pathways simultaneously. Results have shown trichostatin can also inhibit the expression of METTL1 directly. So, trichostatin may be a potential novel strategy against NASH-related HCC by inhibiting HDAC and AMPK directly and indirectly.

5 Conclusion

In conclusion, our study determined the roles of METTL1 and m7G in NASH-related HCC. We also identified key miRNA and genes which may be downstream factors of METTL1. As such targeting these key factors through potential drug combinations may be novel strategies against NASH-related HCC.

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Conflicts of Interest

The authors have declared no conflicts of interest.

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Abbreviations

AFP	alpha fetoprotein
AMPK	adenosine monophosphate-activated protein kinase
AUC	area under curve
BP	biological process

CC	cellular component
CI	confidence interval
DAVID	the database for annotation, visualization, and integrated discovery database
DE-gene	differentially expressed gene
DFS	disease free survival
DPS	Drug Pair Seeker
GEO	the Gene Expression Omnibus
GO	Gene Ontology
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HPA	Human Protein Atlas
HR	hazard ratio
KEGG	Kyoto Encyclopedia of Genes and Genomes
m6A	N^6 -methyladenosine
m7G	N^7 -methylguanosine
MAPK	mitogen-activated protein kinase
METTL1	methyltransferase-like 1
MF	molecular function
miRNA	microRNA
mRNA	messenger RNA
NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
OCLR	one-class logistic regression machine learning algorithm
OS	overall survival
PPI	protein-protein interaction
pri-miRNA	miRNA precursor
PTEN	phosphatase and tensin homolog
ROC	receiver operating characteristic
ROS	reactive oxygen species
rRNA	ribosomal RNA
SD	standard deviation
TCGA	the Cancer Genome Atlas
tRNA	transfer RNA