

Potential prognostic value of hsa-miR-18a in hepatocellular carcinoma and the underlying key down-stream gene *CHRM2*: a comprehensive bioinformatic analysis*

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Abstract

Background

Hepatocellular carcinoma (HCC) is still the fourth leading cause of cancer-related death. Better prognosticators are warranted for HCC. Hsa-miR-18a has been considered implicated in the pathogenesis of several tumors including HCC.

Methods

Bioinformatic analyses were conducted to predict target genes and carry out enrichment analysis. Validated downstream genes of hsa-miR-18a were obtained from PubMed database. Differential expression analysis was conducted within the “edgeR” R package based on the TCGA datasets. Survival analysis was performed by Kaplan-Meier survival analysis. All the visualizations were implemented by R.

Results

Bioinformatic analysis obtained a total of 90 target genes of hsa-miR-18a and revealed that target genes were involved in pathways essential for cancer onset and development such as cell cycle and PI3K/AKT signaling pathway. A review of literatures found target genes of miR-18a indeed participating in the biological processes of HCC. *CHRM2* was identified as a special gene after the intersection analysis of TCGA differentially expressed genes (DEGs) and predicted target genes. Survival analysis validated that hsa-miR-18a and *CHRM2* significantly affected the prognosis of HCC patients.

Conclusion

There is a strong association between hsa-miR-18a with tumors including HCC via participating essential tumor-promoting pathways including cell cycle, PI3K/AKT signaling pathway, *etc.* Furthermore, high miR-18a expression and low *CHRM2* expression could lead to a poor prognosis in HCC. In conclusion, miR-18a could serve as an expectational prognostic biomarker in HCC.

Background

As one of the most prevalent cancer in the alimentary system, hepatocellular carcinoma (HCC) ranks sixth in terms of morbidity and fourth in terms of mortality worldwide. Due to the remained difficulties in early diagnosis and the high possibility of tumor relapse and metastasis after resection, HCC has been posing a huge threat upon human health and survival over the years[1]. Hence, profound study into the mechanism of tumorigenesis and progression, discovering biomarkers with high sensitivity and specificity aiming HCC for the early diagnosis and prognosis assessment is essential.

MicroRNAs (miRNAs) are short, single-stranded non-coding RNAs of around 22 nucleotides. Expression levels of target genes are down-regulated post-transcriptionally by miRNAs through binding to the 3'untranslated region (3'UTR) of their mRNAs, by which mean miRNAs participate in the transcriptional regulation of various cellular events including cell cycle, proliferation and protein phosphorylation and play key roles in tumorigenesis and malignancy development[2, 3].

It has been revealed that aberrant expression levels of miRNAs in HCC cases are bound up with their biological behaviors and clinicopathological characteristics, making miRNAs undergoing extensive investigations[4–6]. Furthermore, precisely because of the identity of miRNAs mentioned above, their potentials of serving as in-situ or serum prognostic biomarkers has been acquiring increasing concerns from the oncology community[7–9]. However, relatively bulk bioinformatic minings and experimental validations are necessary before the prognostic value of select miRNA or miRNA panel is finally authenticated in clinical practice.

Meanwhile, hsa-miR-18a has been implicated in important biological processes of several cancer types including HCC, indicating the essentiality of comprehensively identifying downstream targets together with their functions as well as their prognostic influences upon HCC[10].

In this article, the target genes of miR-18a were predicted and analyzed for gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) to explore the functions and pathways possibly involved in HCC, and the results were demonstrated through literature researches. Next, differentially expressed genes were extracted from TCGA datasets and a test was made to identify whether an intersection with predicted genes was authentically existing. Finally, survival analysis was carried out to illustrate the diagnostic and prognostic monitoring value of miR-18a, and the underlying molecular mechanism was preliminarily explored.

Materials And Methods

Mature sequence of miR-18a in several species

Sequences of miR-18a in different species were obtained from miRBase (<http://www.mirbase.org>) [11]. Using custom R scripts for mature sequence identification.

Target gene prediction of hsa-miR-18a

TargetScan (<http://www.targetscan.org>) [12], miRWalk (<http://mirwalk.umm.uni-heidelberg.de>) [13], miRDB (<http://www.mirdb.org/cgi-bin/search.cgi>) [14], mirDIP (<http://ophid.utoronto.ca/mirDIP/index.jsp#r>) [15] databases were used to predict the target genes. For the authenticity of the prediction, intersection of the 4 gene sets was extracted for further analysis.

Expression level of target genes in normal tissue

TiGER database (<http://bioinfo.wilmer.jhu.edu/tiger/>)[16] was used to obtain the expression level of target genes in various normal tissues. Only a gross picture of the expression was observed because several target genes could not be found in TiGER database.

GO and KEGG pathway enrichment analysis

DAVID online tool (<https://david.ncifcrf.gov/tools.jsp>) [17] was used to conduct GO enrichment to identify the biological processes, cellular components and molecular functions of target genes as well as the KEGG pathway information[18]. The KEGG database (<http://www.genome.jp/kegg/>) was used for generating a certain pathway map. $p < 0.05$ was considered significant.

Protein-protein interaction (PPI) network construction

Interactions between target genes of hsa-miR-18a were obtained from the online database resource Search Tool for the Retrieval of Interacting Genes (STRING) (<https://www.string-db.org>) [19].

Data selection and statistical analysis

A total of 424 cases were extracted from TCGA database (<https://www.cancer.gov/>) with 374 HCC tissues and 50 normal tissues. The genes expressed differentially between tumor and normal tissues were analyzed and identified by “edgeR” R package. Survival curves were depicted with the Kaplan-Meier Plotter online tool (<http://kmpplot.com/analysis/>)[20, 21]. $p < 0.05$ was considered significant.

Data visualization

Visualization was conducted in R. The gene expression matrix, enrichment plots and differential expression boxplot were depicted by “ggplot2” R package[22]. The venn diagram was drawn with “VennDiagram” R package[23].

Results

Literature research on hsa-miR-18a

A research based on PubMed database was performed to obtain preliminary details of the effect of miR-18a in various tumors (Table 1). The results suggested a possible role for miR-18a as an onco-miRNA in cancers such as cervical cancer and gastric cancer because of the positive correlation between its increased expression and the tumorigenesis and progress. A study focused on nasopharyngeal cancer evidenced an increased expression of miR-18a in nasopharyngeal tissues, which can promote tumor proliferation and metastasis through down-regulating *SMG1* expression[24, 25]. However, another research demonstrated that decreased miR-18a level led to an over-expression of *SREBP1*, promoting the invasion of breast cancer through enhancing the epithelial-to-mesenchymal transition (EMT) [26]. Taken together, the results in Table 1 show that miR-18a has different characters in various tumors[27].

Table 1
Summary of literature findings on hsa-miR-18a

Disease	Level	Role	Author(Year)
Epstein–Barr virus-associated lymphomas	Increased	Oncogene(Gene damaging and EBV multiplication)	Cao PF(2018) [28]
Cervical cancer	Increased	Oncogene(Promoting PD-L1 expression)	Dong PX(2018)[29]
Gastric cancer	Increased	Oncogene(Activating Wnt/ β -catenin pathway, enhancing metastasis)	Yuan JS(2019) [30]
glioblastoma multiforme	Increased	Oncogene(Promoting cell cycle)	Wu WN(2017) [31]
Glomangiopericytoma	Increased	Oncogene(Promoting proliferation through GAS5)	Liu Q(2018) [32]
Nasopharyngeal carcinoma	Increased	Oncogene(Enhancing mTOR pathway)	Mai SJ(2019) [33]
Hepatocellular cellular carcinoma	Increased	Oncogene(Promoting cancer occurrence through suppressing SOC5 expression)	Avencia Sanchez(2019) [34]
Breast cancer	Decreased	Tumor suppressor gene(Suppressing metastasis)	Zhang N(2019) [26]
Neuroblastoma	Decreased	Tumor suppressor gene(Promoting differentiation)	Johanna Dzieran(2018) [35]
Ovary cancer	Decreased	Tumor suppressor gene(Suppression proliferation)	Li WJ(2019) [36]

Mature sequence of miR-18a in various species

Sequences of miR-18a in various species were obtained from miRbase database. As presented in Table 2, miR-18a is highly conserved during evolution and the mature sequence is UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA. The result suggests an important role of miR-18a in organisms.

Table 2
Mature sequences of hsa-miR-18a in various species

ID	Species	microRNA name	Mature sequences
MI0000072	<i>Homo sapiens</i>	hsa-miR-18a	1-UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA-37
MI0002996	<i>Pan troglodytes</i>	ptr-miR-18a	1-UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA-37
MI0003002	<i>Macaca mulatta</i>	mml-miR-18a	1-UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA-37
MI0000567	<i>Mus musculus</i>	mmu-miR-18a	12-UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA-48
MI0000846	<i>Rattus norvegicus</i>	rno-miR-18a	12-UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA-48
MI0010324	<i>Canis familiaris</i>	cfa-miR-18a	9-UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA-45

Target genes prediction and their expression map in normal human tissues

Online databases TargetScan, miRDIP, miRWalk and miRDB were utilized and the numbers of target genes predicted were 5543, 982, 3078 and 944, respectively (Fig. 1a.). The original prediction data are provided in Table S1. Only target genes which were presented by all four databases simultaneously were received as the gene set for further research. To elevate the comprehensiveness and feasibility for further work, five genes which were proved to be the targets of miR-18a in miRecords database were added into the gene set. We obtained 90 target genes for further study ultimately as shown in Table 3.

Next, to further refine our exploration into the function of miR-18a, we depicted a rough expression map of miR-18a in normal tissues base on TiGER database(Fig. 1b). After clustering and statistic analysis, no tissue specificity was observed within target genes of miR-18a. Genes included in TiGER database were provided in Table S2. The result reinforces the importance of unveiling the association between miR-18a and neoplastic diseases including HCC.

Table 3
Gene set for further research

<i>ANKRD13C</i>	<i>DAAM2</i>	<i>GIT2</i>	<i>MAP7D1</i>	<i>PLL</i>	<i>TMEM170B</i>
<i>ATM</i>	<i>DICER1</i>	<i>HCFC2</i>	<i>MAPK4</i>	<i>PNISR</i>	<i>TRAPPC8</i>
<i>ATXN1</i>	<i>DIP2C</i>	<i>HEATR5A</i>	<i>MRPL35</i>	<i>PRKACB</i>	<i>UEVLD</i>
<i>BBX</i>	<i>DIRAS2</i>	<i>HMBOX1</i>	<i>MYLK</i>	<i>PTCHD1</i>	<i>WASF2</i>
<i>BRWD1</i>	<i>DNAJC8</i>	<i>HMGCS1</i>	<i>NACC1</i>	<i>PTP4A3</i>	<i>XYLT2</i>
<i>CA12</i>	<i>ELOVL1</i>	<i>HSF5</i>	<i>NCOA1</i>	<i>RAB9A</i>	<i>ZBTB4</i>
<i>CA13</i>	<i>ERI1</i>	<i>INO80D</i>	<i>NEDD4</i>	<i>RNF4</i>	<i>ZBTB47</i>
<i>CAD</i>	<i>ESCO2</i>	<i>IRF2</i>	<i>NEDD9</i>	<i>SH3BP4</i>	<i>ZCCHC3</i>
<i>CBX2</i>	<i>EXPH5</i>	<i>KCNH7</i>	<i>NFASC</i>	<i>SLC12A6</i>	<i>ZFP62</i>
<i>CCND1</i>	<i>FAM136A</i>	<i>KCNJ2</i>	<i>NOTCH2</i>	<i>SMAP2</i>	<i>ZNF367</i>
<i>CCND2</i>	<i>FAM3C</i>	<i>KCNS2</i>	<i>NR3C1</i>	<i>SORBS2</i>	<i>SOX6*</i>
<i>CHRM2</i>	<i>FBXL3</i>	<i>KCTD16</i>	<i>NUFIP2</i>	<i>TAOK1</i>	<i>RORA*</i>
<i>CLCC1</i>	<i>FGF1</i>	<i>KDM2A</i>	<i>OCRL</i>	<i>TBC1D9B</i>	<i>SMG1*</i>
<i>CREBL2</i>	<i>GCLC</i>	<i>KLF6</i>	<i>PARD6B</i>	<i>TGFBR3</i>	<i>CBX7*</i>
<i>CTDSPL</i>	<i>GIGYF1</i>	<i>MAP3K1</i>	<i>PDE4D</i>	<i>THBD</i>	<i>SREBP1*</i>
* Target genes obtained from miRecords database.					

GO, KEGG enrichment analysis and PPI of miR-18a target genes

In order to elucidate the mechanisms of miR-18a in cancer, GO and KEGG enrichment analyses and PPI network construction were carried out further. 85 target genes obtained GO annotations, 37 target genes obtained KEGG annotations and 44 target genes participated in PPI network.

In terms of GO enrichment, nucleus (GO:0005634) and nucleoplasm (GO:0005654) were the most represented GO terms in the cellular component subontology ($P < 0.05$). In biological molecular function, DNA binding (GO:0003667), protein complex binding (GO:0032403) and protein serine/threonine kinase activity (GO:0004647) were relatively abundant terms ($P < 0.05$). The top enriched biological process terms were transcription, DNA-templated (GO:0006351), cell cycle (GO:0007409) and protein phosphorylation (GO:0006468) ($P < 0.05$). Table 4 presented full details and Fig. 2a visualized the number and p -value of GO enrichment.

KEGG pathway analysis revealed 12 significantly enriched pathways as shown in Table 5, especially in tumorigenesis-related pathways and metabolic pathways including cell cycle (hsa04110), PI3K pathway (hsa04151), Wnt signaling pathway (hsa04310), arginine biosynthesis (hsa00220) and angiogenesis (hsa04370) ($P < 0.05$). The number and p -value of all KEGG terms were shown in Fig. 2b. Notably, we observed in the pathway map provided by KEGG database that *CCND1* and *FGF* played an essential role in cancer (Fig. 2c).

A total of 90 nodes and 51 edges were revealed in the PPI network with a PPI enrichment p -value < 0.05 (Fig. 2d). *ATM*, *CCND1*, *CCND2*, *NR3C1* were identified as hub genes which might play key roles in the network and the function of miR-18a. Crucial genes unveiled in KEGG and PPI analysis made it reasonable to believe that these genes accompanied with miR-18a could be fruitful in the association with cancer.

Table 4
Gene Ontology (GO) enrichment of target genes

GO ID	GO Term	P-value	Gene number	Annotated genes
Biological processes				
GO:0006351	transcription, DNA-templated	3.51E-04	22	NCOA1, CBX7, INO80D, KDM2A, ZBTB47, CBX2, CREBL2, RORA, NR3C1, RNF4, ZBTB4, HMBOX1, KLF6, ATXN1...
GO:0007049	cell cycle	4.19E-03	6	PARD6B, CCND2, CCND1, CREBL2, ESCO2, MAPK4
GO:0007188	adenylate cyclase-modulating G-protein coupled receptor signaling pathway	1.40E-02	3	CHRM2, WASF2, PRKACB
GO:0030522	intracellular receptor signaling pathway	1.48E-02	3	NOTCH2, NCOA1, RORA
GO:0007595	lactation	1.79E-02	3	NCOA1, CCND1, CAD
GO:0071805	potassium ion transmembrane transport	2.14E-02	4	KCNH7, KCNS2, SLC12A6, KCNJ2
GO:0006468	protein phosphorylation	2.40E-02	7	MAP3K1, CCND1, TAOK1, ATM, PRKACB, MAPK4, MYLK
GO:0071476	cellular hypotonic response	3.37E-02	2	SLC12A6, MYLK
GO:0042921	glucocorticoid receptor signaling pathway	4.31E-02	2	NEDD4, NR3C1
GO:0071260	cellular response to mechanical stimulus	4.71E-02	3	GCLC, MAP3K1, KCNJ2
Cellular components				
GO:0005634	nucleus	7.31E-05	43	NOTCH2, SMG1, INO80D, ZBTB47, NUFIP2, NEDD9, RORA, CREBL2, NR3C1, RNF4, ZBTB4, HMBOX1, PARD6B...
GO:0005654	nucleoplasm	2.20E-04	27	NOTCH2, RORA, FGF1, NR3C1, RNF4, ATXN1, CCND2, CCND1, BBX, SOX6, ZNF367, PRKACB, MAPK4, NCOA1...
GO:0005819	spindle	1.84E-02	4	MAP7D1, NEDD9, ATM, NR3C1

GO ID	GO Term	P-value	Gene number	Annotated genes
GO:0005938	cell cortex	1.92E-02	4	PARD6B, NEDD4, NEDD9, FGF1
GO:0005737	cytoplasm	2.30E-02	34	SMG1, NUFIP2, NEDD9, NR3C1, RNF4, MYLK, HMBOX1, PARD6B, ATXN1, CCND1, ERI1, NACC1, BBX, SH3BP4...
GO:0030027	lamellipodium	3.78E-02	4	NEDD9, SORBS2, WASF2, MYLK
Molecular functions				
GO:0032403	protein complex binding	3.80E-04	7	NCOA1, GIT2, HMBOX1, CCND1, ATM, NR3C1, WASF2
GO:0031698	beta-2 adrenergic receptor binding	1.84E-02	2	PDE4D, NEDD4
GO:0004674	protein serine/threonine kinase activity	3.00E-02	6	SMG1, MAP3K1, TAOK1, ATM, PRKACB, MAPK4
GO:0019901	protein kinase binding	3.00E-02	6	MAP3K1, CCND2, CCND1, SLC12A6, ZBTB4, MAPK4
GO:0003677	DNA binding	4.24E-02	14	KDM2A, ZBTB47, CBX2, RORA, NR3C1, RNF4, HMBOX1, KLF6, ATXN1, IRF2, BBX, ATM, SOX6, ZNF367
GO:0008144	drug binding	4.82E-02	3	CHRM2, HMGCS1, PDE4D
GO:0046872	metal ion binding	5.00E-02	16	GIT2, SMG1, ZBTB47, PDE4D, CAD, CTDSPL, SORBS2, ESCO2, DICER1, ZBTB4, MYLK, KLF6, ZFP62, ERI1...

Table 5
Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment of target genes

KEGG ID	KEGG Term	P-value	Gene number	Annotated genes
hsa04110	Cell cycle	5.17E-03	2	CCND1, CCND2
hsa04919	Thyroid hormone signaling pathway	1.87E-02	4	NOTCH2, NCOA1, CCND1, PRKACB
hsa04151	PI3 kinase pathway	2.90E-02	2	CCND1, CCND2
hsa04310	Wnt signaling pathway	3.00E-02	4	CCND2, CCND1, DAAM2, PRKACB
hsa00220	Arginine biosynthesis	3.11E-02	1	CAD
hsa04725	Muscarinic acetylcholine receptor 2 and 4 signaling pathway	3.27E-02	2	CHRM2, PRKACB
hsa5166	HTLV-I infection	3.53E-02	5	MAP3K1, CCND2, CCND1, ATM, PRKACB
hsa04921	Oxytocin signaling pathway	3.71E-02	4	CCND1, PRKACB, KCNJ2, MYLK
hsa04390	Hippo signaling pathway	3.78E-02	4	PARD6B, CCND2, CCND1, FGF1
hsa05010	Alzheimer disease-amyloid secretase pathway	3.86E-02	2	CHRM2, MAPK4
hsa04115	p53 signaling pathway	4.29E-02	3	CCND2, CCND1, ATM
hsa04370	Angiogenesis	4.63E-02	3	NOTCH2, MAP3K1, FGF1

Published researches of target genes and the latent correlation between miR-18a and HCC

A literature research was performed based on PubMed database until March 20, 2021, to retrieve information on the target genes (n = 14) and pathways (n = 3) modulated by miR-18a (Table 6). Entries retrieved for our literature research were connected by “OR” and were provided in Table S3. 11 out of 14 target genes validated by published studies focused on the function of tumorigenesis and progress while all three pathways identified participated in tumor-promoting functions including promoting EMT, metastasis and inhibiting DNA damage repair and apoptosis. Of note, a recent research conducted by Zheng *et. al*/ demonstrated that miR-18a was involved in tumorigenesis and progression of HCC via directly targeting NEDD9, which suggested miR-18a of holding latent value in its association with HCC.

Table 6
Target genes (n = 14) and signal transduction pathways (n = 3) modulated by miR-18a in PubMed database

Target	Influenced pathway	Associated function	Cell or tissue type	Author (Year)
Target genes				
SOX6	NA	Enhancing proliferation and invasion	Esophageal squamous carcinoma cells	Yan Z (2021) [37]
NOTCH2	Notch2 pathway	Enhancing proliferation and migration	Pulmonary arterial smooth muscle cells	Miao RY (2020) [33]
RORA	TNF- α /NF- κ B signaling pathway	Enhancing proliferation and tumorigenesis	Glioma stem cells	Jiang Y (2020) [38]
NEDD9	NA	Tumorigenesis and progression	HCC tissues and cells	Zheng YW (2020) [39]
FBXL3	NA	Suppressing cell growth	Hemangioma cells	Dai YX (2020) [40]
ATM	ATM/LC3B signaling pathway	Inhibiting autophagy	Macrophages	Yuan QL (2020) [41]
FGF1	NA	Promoting proliferation	Myoblast cells	Liu CC (2018) [42]
SREBP1	NA	Initiating EMT	Breast cancer cells	Zhang N (2019) [26]
IRF2	IRF2 signaling pathway	Promoting invasion and migration	Osteosarcoma cells	Lu C (2018) [43]
ATM	NA	Radiosensitizing	Lung cancer stem-like cells	Chen X (2018) [44]
SOX6	p53 signaling pathway	Preventing hypoxia injury in cerebral ischemia	Neuronal pheochromocytoma cells	Tian JY (2017) [45]
NOTCH2	Notch2 pathway	Inhibiting EMT and cardiac fibrosis	Aortic valvular endothelial cells	Geng HZ (2017) [46]
CBX7	NA	Enhancing malignancy	Glioblastoma cells	Wu WN (2017) [31]
ATM	NA	Inhibiting proliferation and migration	Colon cancer cells	Liu MJ (2017) [47]
Participated pathways				
SOCS5	PI3K/AKT pathway	Inhibiting apoptosis and promoting EMT	Osteosarcoma cells	Ye F (2019) [48]

Target	Influenced pathway	Associated function	Cell or tissue type	Author (Year)
Wnt	Wnt/ β -Catenin signaling pathway	Promoting metastasis	Gastric cancer cells	Yuan JS (2019) [30]
ATM kinase	ATM induced DNA damage response	Inhibiting DNA damage repair	Breast cancer tissues and cells	Song LB (2011) [49]

Potential of miR-18a and target gene CHRM2 as prognostic predictors of HCC patients

To explore the association between miR-18a with HCC, differentially expressed genes (DEGs) between HCC cases ($n = 371$) and controls ($n = 50$) were deposited ($| \log_{FC} | > 2$ and $FDR < 0.05$) based on the TCGA transcriptomic datasets (Fig. 3a,b). 144 un-regulated DEGs and 7 down-regulated DEGs were identified, respectively (Table S4). Consistent with the predicted target genes of miR-18a, the KEGG enrichment of up-regulated DEGs presented a latent HCC-promoting feature by harboring significant annotations in cell cycle and p53 signaling pathway ($P < 0.05$) (Fig. 3c). Other characteristics were also provided by GO and KEGG enrichment on DEGs, including some down-regulated liver-specific metabolism pathways (xenobiotic metabolism, P450 activities, gluconeogenesis, *etc*) and elevated tumor-promoting features (DNA replication, nuclear division, cell cycle phase transition, *etc*) (Fig. 3d ~ f).

Next, the intersection between DEGs compared to predicted target genes was examined and the only gene obtained was Cholinergic Muscarinic Receptor 2 (*CHRM2*), dramatically (Fig. 4a). The expression level of *CHRM2* in HCC tissues was significantly lower than in paired adjacent normal tissues (Fig. 4b). The Kaplan-Meier Plotter online tool was used to evaluate the prognostic role of miR-18a and *CHRM2* in HCC based on *RNA-seq* data (Fig. 4c, d). Notably, a high level of miR-18a and low level of *CHRM2* were authenticated as prognosticators for poor overall survival (OS) and the results were statistically significant ($P < 0.01$). In order to unravel the possible mechanism of *CHRM2* in affecting the HCC prognosis, PPI network of *CHRM2* was constructed which contained 11 proteins (enrichment P -value = $3.84e-13$). Intriguingly, proteins interacting with *CHRM2* indeed engaged PI3K/AKT pathway (GNB1, GNB5, GNG2, *CHRM2*), Ras signaling pathway (GNB1, GNB5, GNG2) and Pathways in cancer (GNB1, GNB5, GNG2, GNAI1) after KEGG enrichment analysis from STRING database (Fig. 4e). Taken together, these results highlighted a potential impact of miR-18a and *CHRM2* on HCC prognosis and indicated a latent value as prognostic biomarkers in HCC tissues.

Discussion

Hepatocellular carcinoma is the most prevalent form of liver cancer and accounts for the fourth most common malignant tumor in China[50]. Bearing the third highest cause of cancer-related mortality, HCC poses a gigantic threat on the public health[51]. Due to the smoldering onset and slowly progressive course, the early manifestations of HCC are difficult to detect which leads to the dilemma that a large proportion of HCC patients cannot be diagnosed properly until the mid-late stage, missing the best period

for cures[52]. Moreover, the heterogeneity between HCC patients has been greatly appreciated recently. As displayed in the study of Gao *et. al*[53], differences in clinical features and prognosis of HCC patients can be finely demonstrated through transcriptomic and proteomic discrepancies. Thus, the individual variability suggests the clear demand for biomarkers more efficacious for HCC prognostic prediction, which is essential for guiding the treatment strategies.

A huge number of miRNAs have been reported to be involved in cancer onset and progression and are differentially expressed among various cancer tissues[54–56]. Through participating in various biological processes including modulating oxidative stress, enhancing Warburg effect, blocking apoptosis, *etc*, miRNAs play crucial roles in cancer onset, invasion and metastasis[57–59]. However, it is important to note the truth that miRNAs can serve as both oncogenes and tumor-suppressing genes depending on several parameters such as miRNA types and tumor classification[28–31, 34–36]. Owing to the characteristics above, miRNAs and their panels have been receiving ascending insights into their potential as prognosticators[60, 61].

Through literature research, we found that miR-18a has been reported up-regulated in several cancers in previous studies. Yuan *et. al*/validated an activated Wnt/ β -catenin signaling pathway mediated by up-regulated miR-18a, which elevated gastric cancer metastasis[30]. Similar tumor-promoting progresses corresponding to high miR-18a levels were unveiled in cancers including HCC. The study of Avencia *et. al* uncovered that up-regulated miR-18a led to a low SOCS5 level, contributing to the HCC tumorigenesis through enhancing the activity and stability of mTOR signaling pathway[34]. However, the underlying mechanism of miR-18a on the HCC prognosis, which is determinant to prognosticator screening, has not yet been revealed clearly. Hence, carrying out bioinformatic analysis on miR-18a might help to address this problem.

Our mature sequence identification of miR-18a depicted a well-conserved orthologous sequence UGUUCUAAGGUGCAUCUAGUGCAGAUAGUGAAGUAGA across several species, underscoring the evolutionarily conserved and important functions.

Then by conducting GO analysis, we found that target genes of miR-18a were mainly functioning in the nucleus, and were enriched in biological processes involving cell cycle, transcription modulating, protein phosphorylation, *etc*. KEGG pathway analysis resulted in reliable enrichments of cell cycle, PI3K signaling pathway, Wnt signaling pathway, arginine biosynthesis, angiogenesis, *etc*. According to the constructed PPI network, *ATM*, *CCND1*, *CCND2* and *NR3C1* were recognized as hub genes, harboring critical functions downstream of miR-18a.

Our attentions may wish to temporarily focus on the pathways and crucial genes mentioned above. Protein phosphorylation plays a key role in protein function modulating. A phosphoproteomic study carried out by Ren *et. al*/revealed a kinome reprogramming in HCC, characterized by elevated activity of MAPKs and PKCs, leading to a swifter growth of HCC cells[62]. Arginine metabolism is considered indispensable in some cancers and its biosynthesis maintains the unique liver functions such as urea cycle[63]. Thongkum *et. al*/proved that blocking arginine synthesis might yield better therapeutic effects

on HCC by impacting urea cycle and pyrimidine metabolism[64]. Hub genes including *ATM*, *CCND1* and *CCND2* identified by our PPI analysis have been the focus in investigations, and their links with HCC were already established in many previous studies based on the in-depth researches mainly into their cell cycle-promoting efficacy[65–68]. Taken together, potential impacts of miR-18a on HCC might exist, for multifaced ways such as metabolism, transcription, angiogenesis and protein phosphorylation crucial for HCC were suggested regulated by miR-18a based on our GO and KEGG analysis.

However, given the small number of researches directly focused on the association between miR-18a and HCC, we tried to obtain a rough estimate of the possible target gene-dependent functions of miR-18a through investigating published studies in PubMed database. As expected, several target genes predicted in our analysis involving *SOX6*, *NOTCH2*, *RORA*, *NEDD9* [33, 37–39], *etc*, have been validated to mediate the effects of miR-18a in multiple types of cell lines and tissues mainly through promoting proliferation and migration. Notably, in the study of Zheng *et. al*, it was demonstrated that miR-18a/NEDD9 axis played a directly promoting role in HCC progression, making the association between miR-18a and HCC more convincible.

For further exploring the value of miR-18a as an in-situ biomarker of HCC, TCGA data were acquired to support our analyses. The down-regulated gene *CHRM2* was present in both TCGA DEGs in HCC and predicted target genes of miR-18a. Astonishingly, a significant prognostic influence of the muscarinic receptor, *CHRM2*, was observed through the Kaplan-Meier Plotter overall survival data in HCC that low level of *CHRM2* meant a better prognosis. Actually, the correlation between *CHRM2* and cancer has received little study at present. The establishment of the linkage between *CHRM2* and non-small cell lung cancer was implemented by Zhao *et. al* through NF- κ B signaling pathway[69]. However, in their study, inactivation of *CHRM2* led to a suppressed NF- κ B signaling pathway, finally weakened the epithelial-mesenchymal transition process, which indicated a tumor-promoting role of *CHRM2*. To explain this contradiction, proteins interacting with *CHRM2* was identified and their KEGG enrichment suggested that they might be involved in PI3K/AKT signaling pathway and Ras signaling pathway, giving the possibility for *CHRM2* of participating in the miR-18a-mediated cancer-related biological processes because of the targeting of miR-18a and the shared pathway with miR-18a. Indispensably, survival analysis of miR-18a was conducted and high miR-18a level was consistent with a significantly poor prognosis, suggesting miR-18a as a potential in-situ prognostic factor, which was comparatively apprehensible based on the results above. Despite the prognostic significance of *CHRM2* and miR-18a in HCC, we should recognize the limitation of our study that mechanisms of *CHRM2* in HCC remain largely unknown, and the pro-cancer effect of miR-18a lacks holistic uncovering. The boundedness of our study needs further in vivo and in vitro verifications hereafter.

Conclusions

Altogether, miR-18a might be involved in pathways contributing to the onset and progression of HCC. Its target gene *CHRM2* is significantly down-regulated in HCC. What's more, up-regulated miR-18a and down-regulated *CHRM2* are indicative of poor prognosis in HCC. We speculated that *CHRM2* might participate

in the modulation of miR-18a in HCC based on KEGG analysis of related proteins. The expression level of miR-18a could thus be introduced as a biomarker for HCC prognosis.

Abbreviations

HCC: hepatocellular carcinoma; miRNAs: microRNAs; miR-18a: hsa-miR-18a; TCGA: The Cancer Genome Atlas; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genome; PPI: protein-protein interact; EMT: epithelial-mesenchymal transition; DEGs: differentially expressed genes; OS: overall survival.

Declarations

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Author contributions

CS designed the study; FZ, WS and WT conducted the data analysis; FZ and GR visualized the data; FZ and WS organized the results; CS and FZ ensured the data curation; FZ wrote the manuscript; CS supervised the manuscript. FZ, WS, WT contributed equally in this study. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All data sets were derived from The Cancer Genome Atlas (TCGA) data portal and are publicly accessible from the TCGA website.

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Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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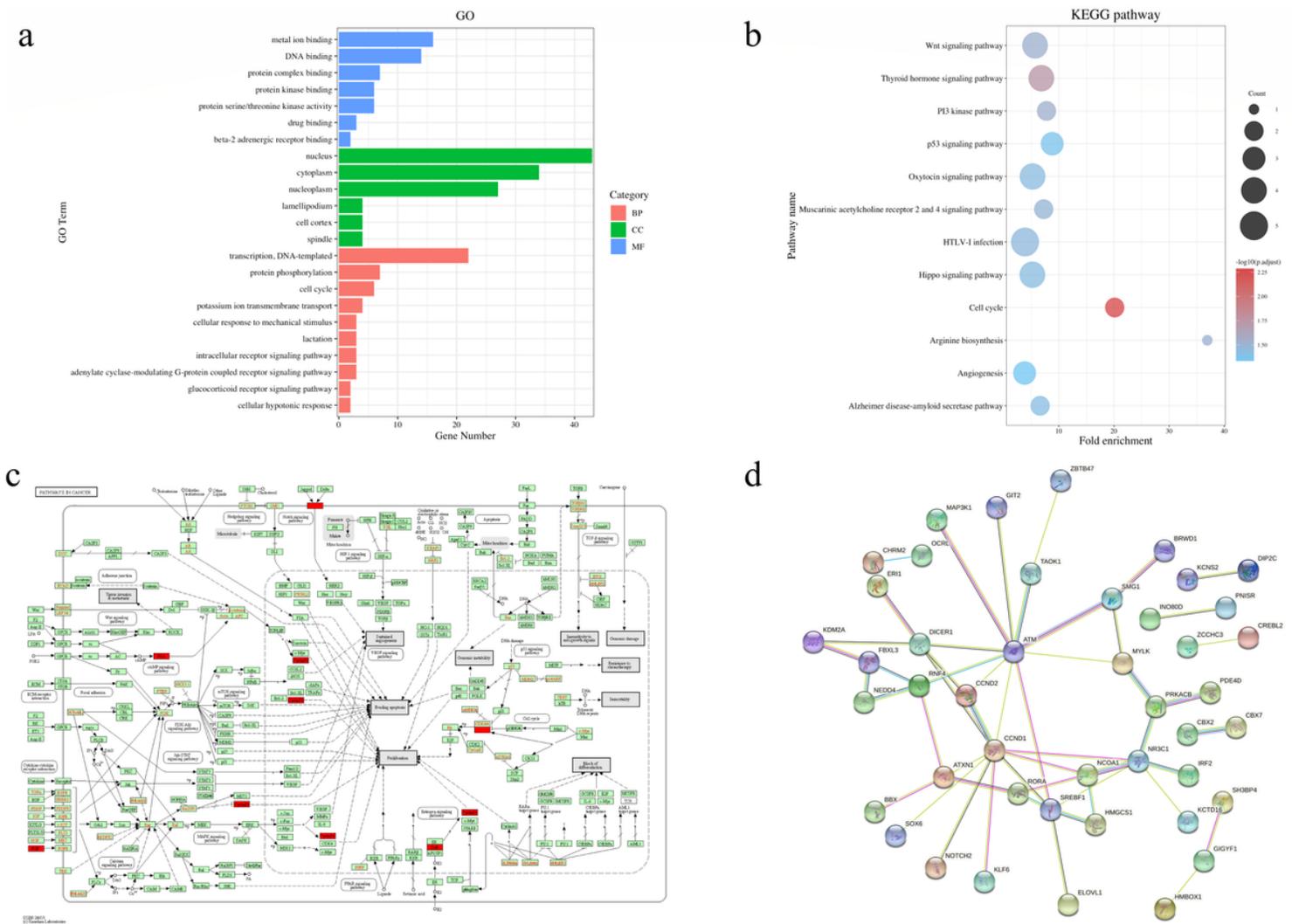


Figure 2

Gene Ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genome (KEGG) analysis and protein-protein interaction (PPI) network of predicted target genes of hsa-miR-18a. a. GO enrichment of target genes. B. KEGG pathway enrichment of target genes. C. Pathway in cancer provided by KEGG database. D. PPI network of target genes.

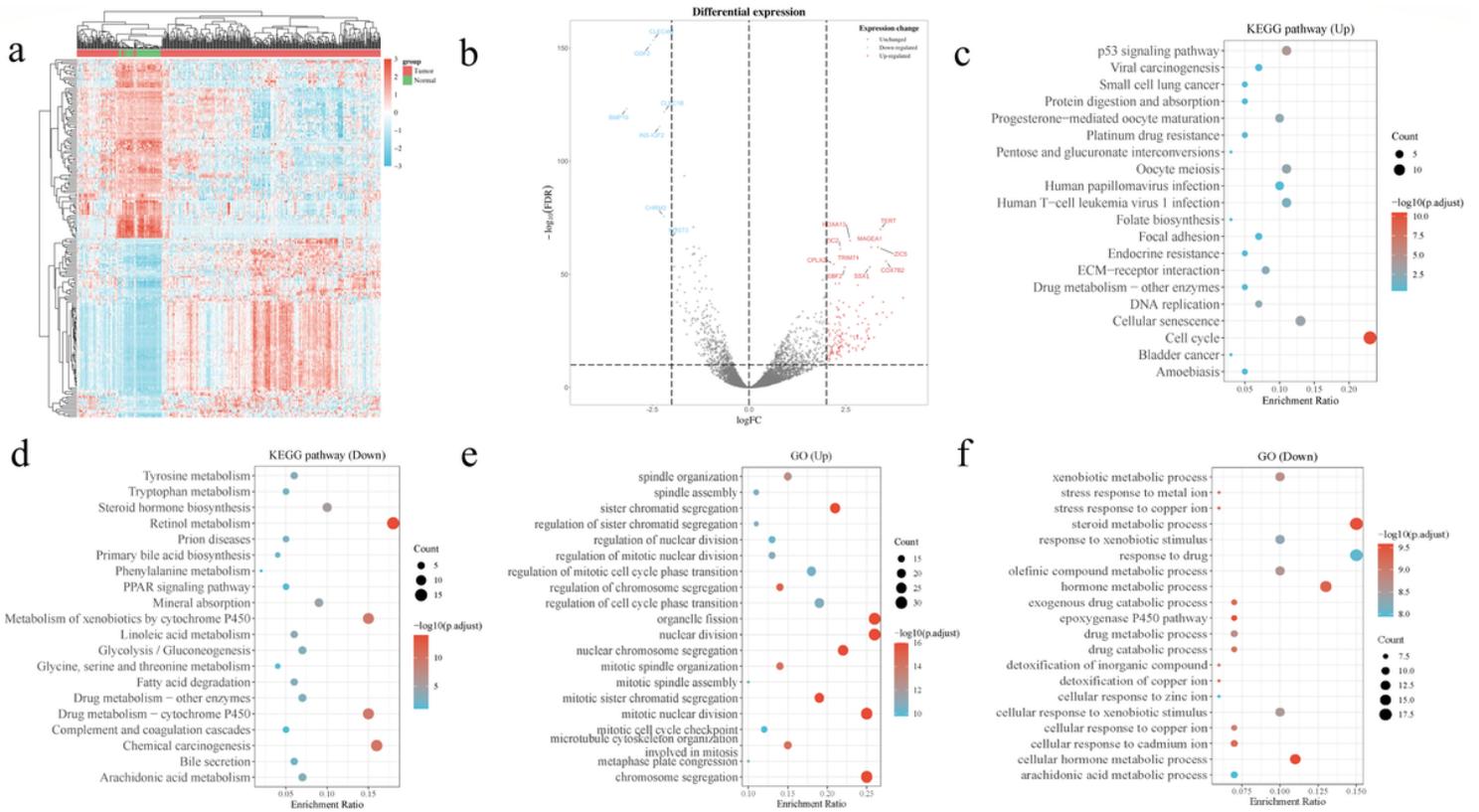


Figure 3

Differentially expressed genes (DEGs) in TCGA HCC datasets together with their Go and KEGG enrichment analysis. a. Heatmap of gene expression in TCGA HCC data. b. Volcano plot of DEGs between HCC cases and controls that were upregulated (red dots) or downregulated (blue dots) ($|\logFC| > 2$ and $\text{FDR} < 0.05$). (c~f) KEGG enrichment analysis (c, d) and GO enrichment analysis (e, f) of DEGs.

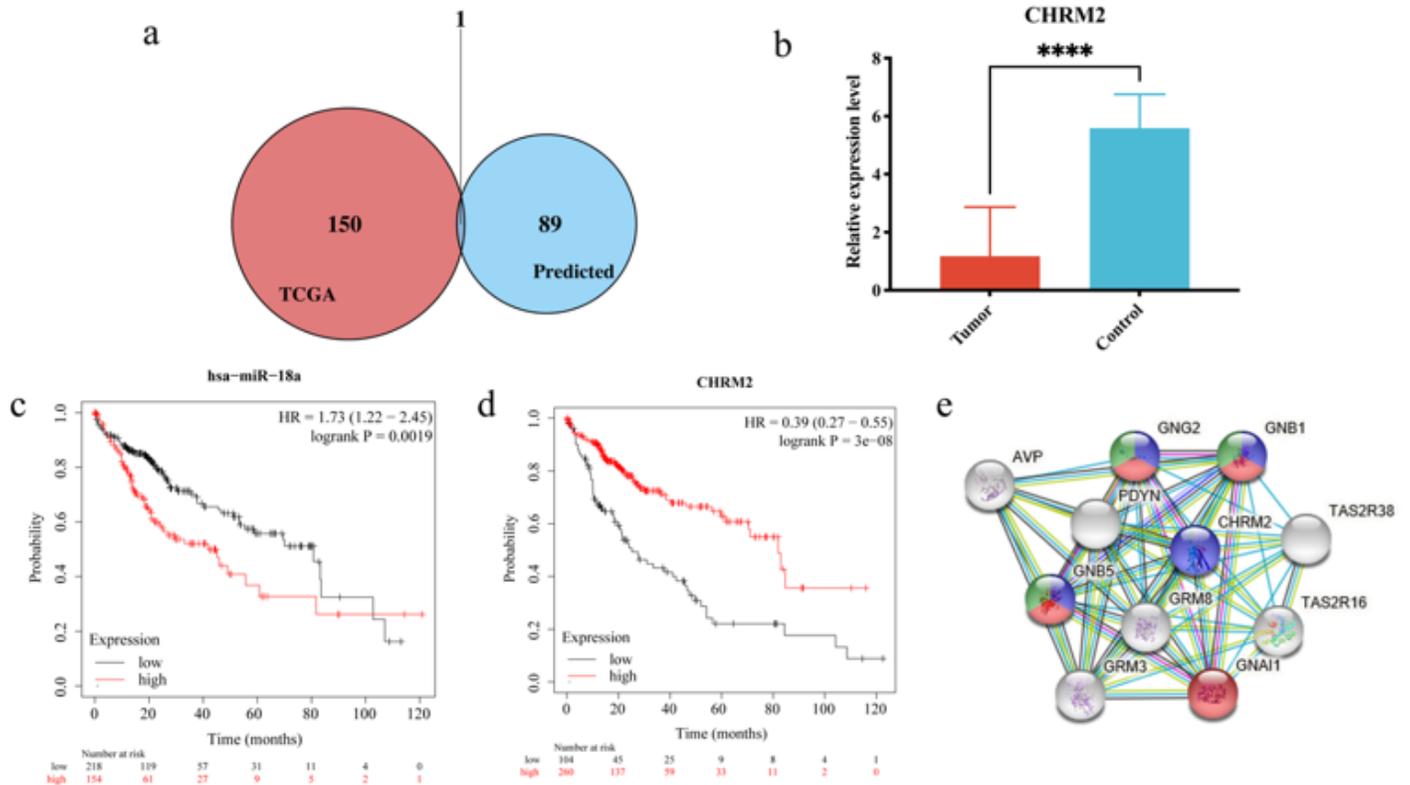


Figure 4

CHRM2 is a potential prognostic predictor in HCC. a. Venn diagram presenting the intersection of TCGA DEGs and predicted target genes of miR-18a. b. CHRM2 expression level in HCC tissues and adjacent normal tissues based on TCGA transcriptomic data. **** $P < 0.00001$. Prognostic analysis of miR-18a c. and CHRM2 d. were obtained from the Kaplan-Meier Plotter website. e. PPI network of CHRM2. Blue, green and red colors indicate proteins participated in PI3K/AKT pathway, Ras signaling pathway and Pathways in cancer, respectively.

Supplementary Files

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