Bioinformatics analysis of gene expression alterations in microRNA-122 knockout mice with hepatocellular carcinoma

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Abstract. Reduced microRNA (miR)-122 expression levels are frequently observed in hepatocellular carcinoma (HCC). The present study was conducted to investigate potential targets of miR-122 and determine the underlying regulatory mechanisms of miR-122 in HCC development. The public dataset GSE31731 was utilized, consisting of 8 miR-122 knockout (KO) mice (miR-122 KO) and 8 age-matched wild-type mice (WT group). Following data preprocessing, the differentially expressed genes (DEGs) were selected, followed by enrichment analysis. A protein-protein interaction (PPI) network was established, and a module network was further extracted. Combining the DEGs with microRNA targeting databases permitted the screening of the overlapping targets of miR-122. Furthermore, previously reported genes were screened out by literature mining. Transcription factors (TFs) of the targets were subsequently investigated. DEGs between miR-122 KO and WT groups were selected, including 713 upregulated and 395 downregulated genes. Of these, upregulated genes were enriched in cell cycle-associated processes [including nucleolar and spindle associated protein 1 (*NUSAP1*)], the cytokine-cytokine receptor interaction pathway [including C-X-C motif chemokine receptor 4 (*CXCR4*) and C-C motif chemokine receptor 2 (*CCR2*)], and the extracellular matrix-receptor interaction pathway [including integrin subunit alpha V (*ITGAV*)]. In addition, multiple overlapping targets were highlighted in the PPI network, including *NUSAP1, CXCR4, CCR2* and *ITGAV*. Notably, *CXCR4* and *CCR2* were linked in module C, enriched in the cytokine-cytokine receptor interaction pathway. Furthermore, upregulated sex determining region Y-box 4 (*SOX4*) was identified as a TF. The results of the present study may provide a theoretical basis for further studies on the mechanisms of miR-122 in the development of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the most frequent liver cancer globally (1). The majority of HCC cases occur in cirrhotic liver, and the primary risk factors are chronic hepatitis B virus or chronic hepatitis C virus (HCV) infection, which account for almost all HCC cases (2). The incidence of HCC varies between different geographical areas; however, it is increasing globally, particularly in Asia, with 6-11 per 100,000 people with the disease (3,4). A study of HCC epidemiology in Germany indicated that, despite the availability of various advanced chemotherapies and radiotherapies, including chemoembolization with drug-eluting beads, sorafenib and selective internal radiotherapy, the overall survival rate has not improved (5). Therefore, the development of more effective therapeutic methods, including molecular targeting therapy, is necessary.

Multiple studies have been conducted to investigate the molecular mechanisms underlying HCC pathogenesis and numerous gene markers have been identified in HCC, including alpha-fetoprotein, glypican-3 (a serum and histochemical marker) and transforming growth factor-β (6-8). As small, non-coding RNAs, microRNAs (miRNAs) are important

regulators of cellular function and physiology (9). Controlling miRNA expression is essential for the maintenance of the steady state of cellular machinery (10). Various microRNAs (miRNAs) have been proposed as novel biomarkers of HCC prognosis, including the chromosome 19 miRNA cluster, which is overexpressed in HCC (11). HCV-induced alteration of miRNA expression regulates inflammation, leading to liver fibrosis. In addition, miRNA (miR)-449a has been reported to serve as an inhibitor in HCV patients, acting via the downregulation of chitinase-3-like protein 1 expression, which is an inflammatory marker for chronic liver diseases with fibrosis (12). Dysregulation of other miRNAs has been detected in HCC, including upregulated miR-23a, -146a and -181a, and downregulated miR-17, -338-3p and -378 (13). Notably, miR-122 is involved in HCC pathogenesis. It is commonly downregulated in HCC and the loss of miR-122 contributes to hepatocarcinogenesis in mice (14). miR-122 has additionally been reported to induce apoptosis in human HCC cell lines via targeting the anti-apoptosis gene B-cell lymphoma-2-like 2 (15). Furthermore, miR-122 inhibits cell proliferation in HCC by targeting the Wnt/β-catenin signaling pathway (16). To further understand the modulation of miR-122 in HCC development, previous studies have investigated the consequences of miR-122 deletion. Hsu *et al* (17) revealed that deletion of mouse mir-122 resulted in hepatocarcinogenesis and HCC-like tumor development. Although increased expression of multiple targets of miR-122 has been detected in miR-122 knockout (KO) mice, including aldolase, fructose bisphosphate A (*ALDOA*), solute carrier family 7 member 1, citrate synthase and cyclin G1, the functions and pathways of the targets, and the potential associations between them at the protein level, remain to be elucidated.

In the present study, the expression profile dataset generated by Hsu *et al* (17), GSE31731, was reanalyzed and differentially expressed genes (DEGs) were identified between miR-122 KO mice and age-matched wild-type (WT) mice. Enrichment analysis of the identified DEGs was subsequently conducted, followed by protein-protein interaction (PPI) and module analysis. Furthermore, targets of miR-122 of these DEGs were selected by combining the results with relevant databases and literature mining, followed by transcription factor (TF) analysis. By means of these comprehensive bioinformatics analyses, the present study aimed to further elucidate the involvement of miR-122 in HCC, and identify potential regulators among its targets.

Materials and methods

Data resources. Gene expression of GSE31731 of HCCs, which was deposited by Hsu *et al* (17) in the public Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) database, was utilized in the present study. The miR-122 KO mice (liver tumor samples) and age-matched WT mice (healthy liver samples) were contained in this dataset. There were 8 biological replicates. Two-channel microarray experiments were conducted for generation of the dataset, based on the platform GPL13912 (accession no. Agilent-028005 SurePrint G3 Mouse GE 8x60 K Microarray; Agilent Technologies, Inc., Santa Clara, CA, USA).

Data pretreatment and differential expression analysis. Raw data were preprocessed using R package in Bioconductor (version 3.4; www.bioconductor.org/packages/3.0/bioc/). Following background correction and normalization, the expression value was converted from the probe level to the gene level. Subsequently, DEGs between liver tumor samples and healthy liver samples were screened out, based on the Student's *t*-test in Linear Models for Microarray Analysis package (version 3.30.3; www.bioconductor.org/packages/release/bioc/html/limma.html) (18). The Benjamini-Hochberg method (19) was used to adjust the P-value. The selection criteria for significant DEGs were a false discovery rate <0.01 and a $\log_2$ fold change >1.5.

Enrichment analysis for DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID; david.ncifcrf.gov/home.jsp) is a common tool for gene function and pathway annotation (20-22). To examine biological functions and pathways of the identified DEGs, Gene Ontology (GO; www.geneontology.org/) (23) and Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/pathway.html) (24) pathway enrichment analyses were performed using DAVID (version 6.8). Cut-off values for significant GO and KEGG pathway terms were P<0.05 and enriched gene number ≥2.

PPI network construction. To predict potential interactions between DEGs at the protein level, the DEGs were entered into the Search Tool for the Retrieval of Interacting Genes (string-db.org/) database (25). The parameter of interplayed PPIs was set as 0.7, and a prerequisite for the network construction was that all the PPI nodes were DEGs. Finally, the PPI network was visualized by Cytoscape (version 3.4.0; cytoscape.org/) software (26).

Furthermore, module analysis was performed for the PPI network using ClusterONE (version 1.0; www.paccanarolab.org/clusterone/) (27), followed by KEGG pathway enrichment analysis. The threshold for significant module selection was $P<1.0x10^{-6}$.

Analysis of miR-122 targets. Initially, targets of miR-122 in mouse were downloaded from three databases: miRecords (c1.accurascience.com/miRecords/) (28), TargetScan (www.targetscan.org/) (29) and microrna.org (www.microrna.org) (30), and only genes that appeared in at least two databases were deemed to be targets of miR-122. These predicted targets were compared with the DEGs, and the overlapping genes were screened out. Following this, TFs of the overlapped targets were predicted by the iRegulon plugin of Cytoscape (iregulon.aertslab.org), which integrates a set of TF databases including Transfac, Jaspar, Encode, Swissregulon and Homer to detect enriched TF motifs and their optimal sets of direct targets (31). Normalized Enrichment Score (NES) was the measurement index for TFs of the targets and the threshold used was NES >3. Furthermore, the Agilent Literature Search plugin (Agilent Technologies, Inc.) (32), which is complementary for protein interaction data, was used to analyze the literature mining association network. In the present study, the search terms were set as 'targets of miR-122', context 'liver cancer' and

Table I. Functions altered by differentially expressed genes.

Category	Term	Count	P-value
Upregulated			
BP	GO:0007049~cell cycle	57	4.96×10^{-11}
	GO:0000279~M phase	34	1.64×10^{-09}
	GO:0000278~mitotic cell cycle	31	2.64×10^{-09}
	GO:0007067~mitosis	27	3.21×10^{-09}
	GO:0000280~nuclear division	27	3.21×10^{-09}
CC	GO:0005576~extracellular region	133	9.92×10^{-18}
	GO:0044421~extracellular region part	74	2.00×10^{-13}
	GO:0005578~proteinaceous extracellular matrix	35	7.33×10^{-09}
	GO:0031012~extracellular matrix	35	2.01×10^{-08}
	GO:0044420~extracellular matrix part	18	4.96×10^{-08}
MF	GO:0005509~calcium ion binding	60	1.62×10^{-07}
	GO:0008009~chemokine activity	10	4.56×10^{-06}
	GO:0008201~heparin binding	14	5.26×10^{-06}
	GO:0042379~chemokine receptor binding	10	5.74×10^{-06}
	GO:0001871~pattern binding	17	9.14×10^{-06}
Downregulated			
BP	GO:0055114~oxidation-reduction	64	9.82×10^{-28}
	GO:0006631~fatty acid metabolic process	24	4.36×10^{-13}
	GO:0008202~steroid metabolic process	21	1.75×10^{-11}
	GO:0006694~steroid biosynthetic process	15	3.73×10^{-11}
	GO:0006956~complement activation	10	1.37×10^{-08}
CC	GO:0005777~peroxisome	22	4.28×10^{-15}
	GO:0042579~microbody	22	4.28×10^{-15}
	GO:0005792~microsome	26	5.15×10^{-14}
	GO:0042598~vesicular fraction	26	1.13×10^{-13}
	GO:0005739~mitochondrion	66	7.86×10^{-11}
MF	GO:0009055~electron carrier activity	34	4.36×10^{-21}
	GO:0020037~heme binding	23	9.42×10^{-14}
	GO:0046906~tetrapyrrole binding	23	2.57×10^{-13}
	GO:0005506~iron ion binding	33	3.02×10^{-13}

The top 5 functions are presented for each category, ranked by the enrichment significance. Up, upregulated differentially expressed genes; down, downregulated differentially expressed genes; BP, biological process; CC, cellular component; MF, molecular function; GO, gene ontology; count, gene numbers enriched in a specific gene ontology term.

species 'Mus', to select the reported HCC-associated literature involving miR-122.

Results

DEGs between miR-122 KO and WT groups. Using predefined criteria, DEGs between miR-122 KO and WT groups were screened out, including 713 upregulated and 395 downregulated genes.

Altered functions and pathways of DEGs. Based on GO and KEGG enrichment analyses, upregulated DEGs were identified to be significantly enriched in cell cycle associated biological processes (BPs), including the cell cycle, M phase and mitotic cell cycle [for example nucleolar and spindle associated protein 1 (*NUSAP1*); Table I], the cytokine-cytokine receptor interaction pathway [for example C-X-C motif chemokine receptor 4 (*CXCR4*) and C-C motif chemokine receptor 2 (*CCR2*); Table II], and various cancer-associated pathways, including small cell lung cancer and pathways in cancer [for example integrin subunit alpha V (*ITGAV*); Table II]. The downregulated DEGs were associated with oxidation-reduction (Table I) and metabolism-associated pathways, including drug metabolism, linoleic acid metabolism and retinol metabolism (Table II).

PPI network and functional module analysis. A PPI network was established, involving 549 nodes and 2,243 interplayed protein interactions (Fig. 1). Three sub-networks [modules A (Fig. 2A), B (Fig. 2B) and C (Fig. 2C)] were extracted from the PPI network. Enrichment analysis revealed that the majority of the genes in module A were upregulated and enriched in DNA replication and cell cycle-associated pathways, whereas the majority of genes in module B were downregulated and enriched in metabolism of xenobiotics by cytochrome P450 pathway (Fig. 3). In module C, the majority of the genes were upregulated and involved with the chemokine signaling

Table II. Pathways altered by differentially expressed genes.

Term	Count	Genes	P-value
Upregulated			
mmu04060:Cytokine-cytokine receptor interaction	27	CCL2, CXCL5, CXCR4, CXCL14, CCR2	4.82×10^{-06}
mmu04512:ECM-receptor interaction	15	COL3A1, LAMA2, ITGAV, COL1A2, LAMC1	4.87×10^{-06}
mmu04510:Focal adhesion	22	COL3A1, LAMA2, ITGAV, LAMC1	4.43×10^{-05}
mmu00480:Glutathione metabolism	10	GPX2, GSTA1, GSTA2, GSTM3, G6PDX	2.05×10^{-04}
mmu04110:Cell cycle	14	CCNB2, KMYT1, BUB1B, ESPL1, CDC20	0.001940583
mmu05222:Small cell lung cancer	11	LAMA2, COL4A1, ITGAV, LAMC2, LAMC1	0.002161153
mmu04810:Regulation of actin cytoskeleton	19	ITGAX, ITGAV, PDGFRB, PAK1, DIAP3	0.002891419
mmu04062:Chemokine signaling pathway	16	CCL2, CXCR4, CXCL16, CCR2, CX3CR1	0.006803694
mmu05200:Pathways in cancer	23	COL4A1, LAMA2, ITGAV, LAMC2, LAMC1	0.011257034
mmu00590:Arachidonic acid metabolism	9	GPX2, CBR1, CYP4F18, GPX3, GGT1 *et al*	0.018763259
Downregulated			
mmu00982:Drug metabolism	18	CYP2C37, CYP3A16, CYP2C54, CYP2C44, ADH4	1.83×10^{-12}
mmu00980:Metabolism of xenobiotics by cytochrome P450	17	CYP2C37, CYP3A16, CYP2C54, CYP2C44, CYP2C68	2.81×10^{-12}
mmu00591:Linoleic acid metabolism	14	CYP2J5, CYP2C37, CYP3A16, CYP2C54, CYP2C44	4.16×10^{-11}
mmu00830:Retinol metabolism	16	CYP2C37, CYP3A16, CYP2C54, CYP2C44, CYP2C68	6.09×10^{-11}
mmu03320:PPAR signaling pathway	16	ACOX1, ACSL1, CYP4A12A, HMGCS2, SCP2	5.86×10^{-10}
mmu00590:Arachidonic acid metabolism	15	CYP2J5, CYP2C37, CYP2C54, CYP2C44, CYP2J8	1.14×10^{-08}
mmu00120:Primary bile acid biosynthesis	8	CYP7B1, HSD3B7, CYP7A1, CYP8B1, SCP2	2.81×10^{-08}
mmu00071:Fatty acid metabolism	9	CYP4A12B, GCDH, ACOX1, ACSL1, ADH4	1.21×10^{-05}
mmu00140:Steroid hormone biosynthesis	9	CYP7B1, CYP3A16, HSD3B6, HSD17B2, CYP7A1	1.21×10^{-05}
mmu04610:Complement and coagulation cascades	11	MBL1, C8A, MBL2, C8B, CD55	1.40×10^{-05}

The top 10 pathways are presented, ranked by the enrichment significance. Up, upregulated differentially expressed genes; down, downregulated differentially expressed genes; count, gene numbers enriched in a specific pathway term.

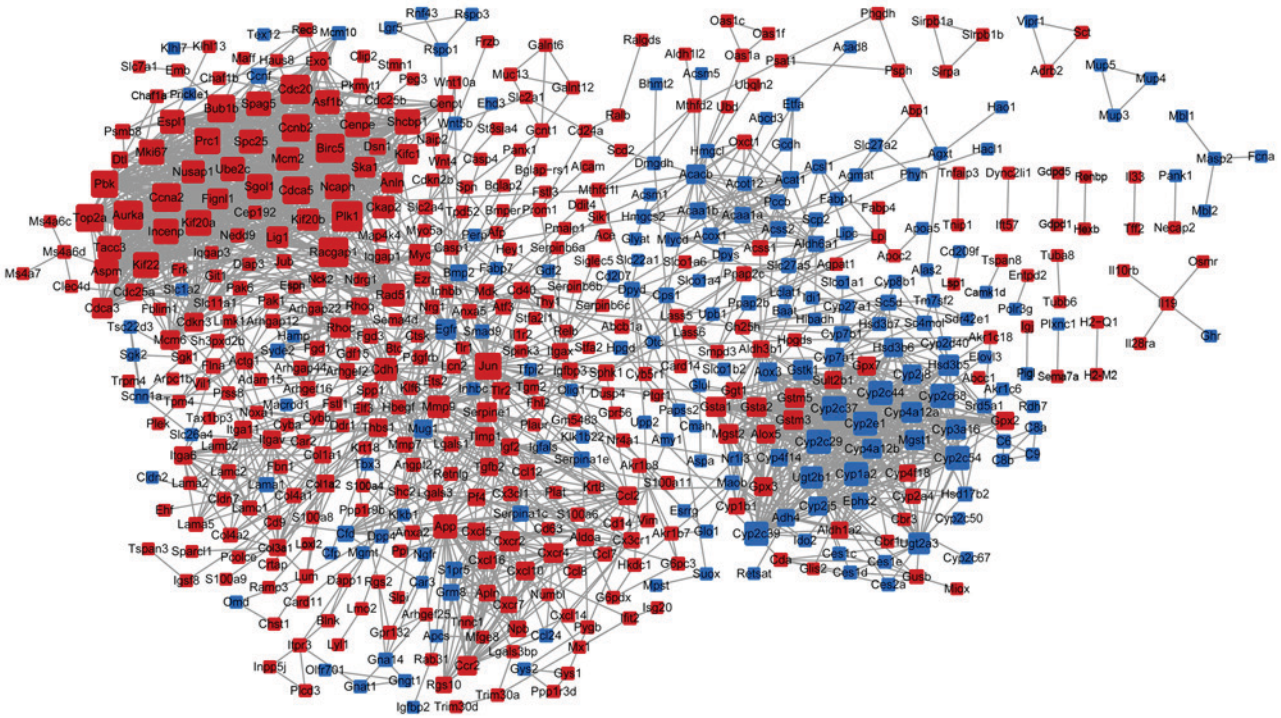

Figure 1. Protein-protein interaction network of differentially expressed genes. Red represents upregulated genes and blue represents downregulated genes. Lines between two genes denote interactions between them.

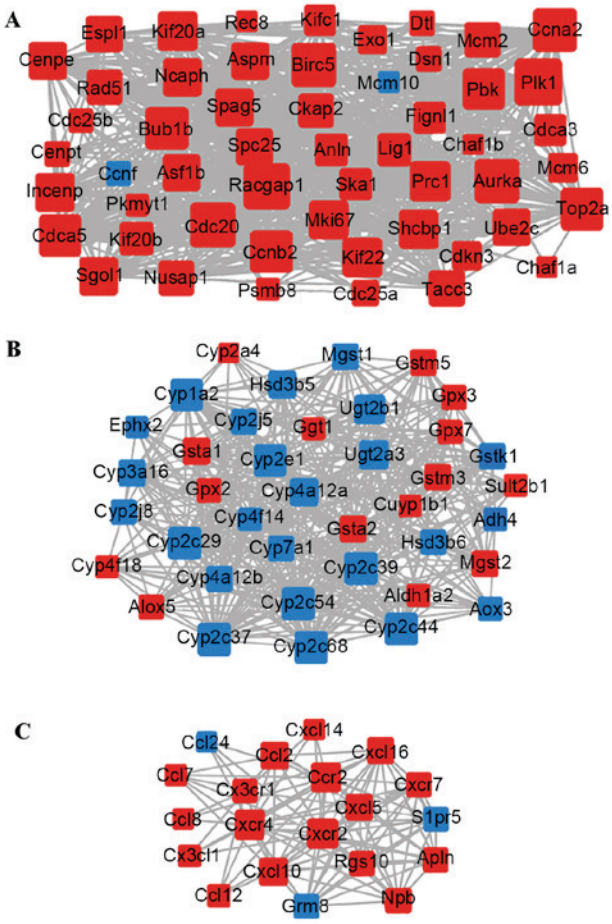

Figure 2. Module network of the protein-protein interaction network. (A) Module A, (B) module B and (C) module C networks. Red represents upregulated genes and blue represents downregulated genes. Lines between two genes denote interactions between them.

pathway and the cytokine-cytokine receptor interaction pathway (Fig. 3).

Targets of miR-122. Integrating the information from miRNA databases with identified DEGs, a total of 76 overlapping genes were selected as the targets of miR-122. Enrichment analysis indicated that these genes were significantly involved in pathways in cancer (for example *ITGAV*; Table II), regulation of actin cytoskeleton (for example *ITGAV*; Table II) and cytokine-cytokine receptor interaction (for example *CXCR4* and *CCR2*; Table II).

Notably, 39 genes of the 76 overlapping targets were additionally the predominant nodes with high degree in the PPI network, including upregulated *NUSAP1* (degree=30), *CXCR4* (degree=21), *CCR2* (degree=20), *ITGAV* (degree=17) and *ALDOA* (degree=14); and the downregulated acyl-CoA synthetase short-chain family member 2 (degree=10). *NUSAP1* was also highlighted in module A, whereas *CXCR4* and *CCR2* were prominent in module C.

In total, 12 TFs targeting 62 overlapping genes were predicted, including sex determining region Y-box 4 (*SOX4*), heterogeneous nuclear ribonucleoprotein H3, NK2 homeobox 1, inhibitor of growth family member 4, early B-cell factor 1, sex determining region Y-box 15, nuclear receptor subfamily 3 group C member 1, zinc finger protein 263, IKAROS family zinc finger 2, paired like homeodomain 3, eukaryotic translation initiation factor 5A2 and chromobox 7. The TF-target regulatory network is presented in Fig. 4. Notably, of the TFs, SOX4 was additionally an upregulated DEG.

According to literature mining, a total of 47 genes of the 76 overlapping targets were reported to be associated with HCC, including *ITGAV* and *CXCR4*. Furthermore, significantly altered expression of these genes was detected following miR-122 KO, which suggested the involvement of miR-122 in HCC development.

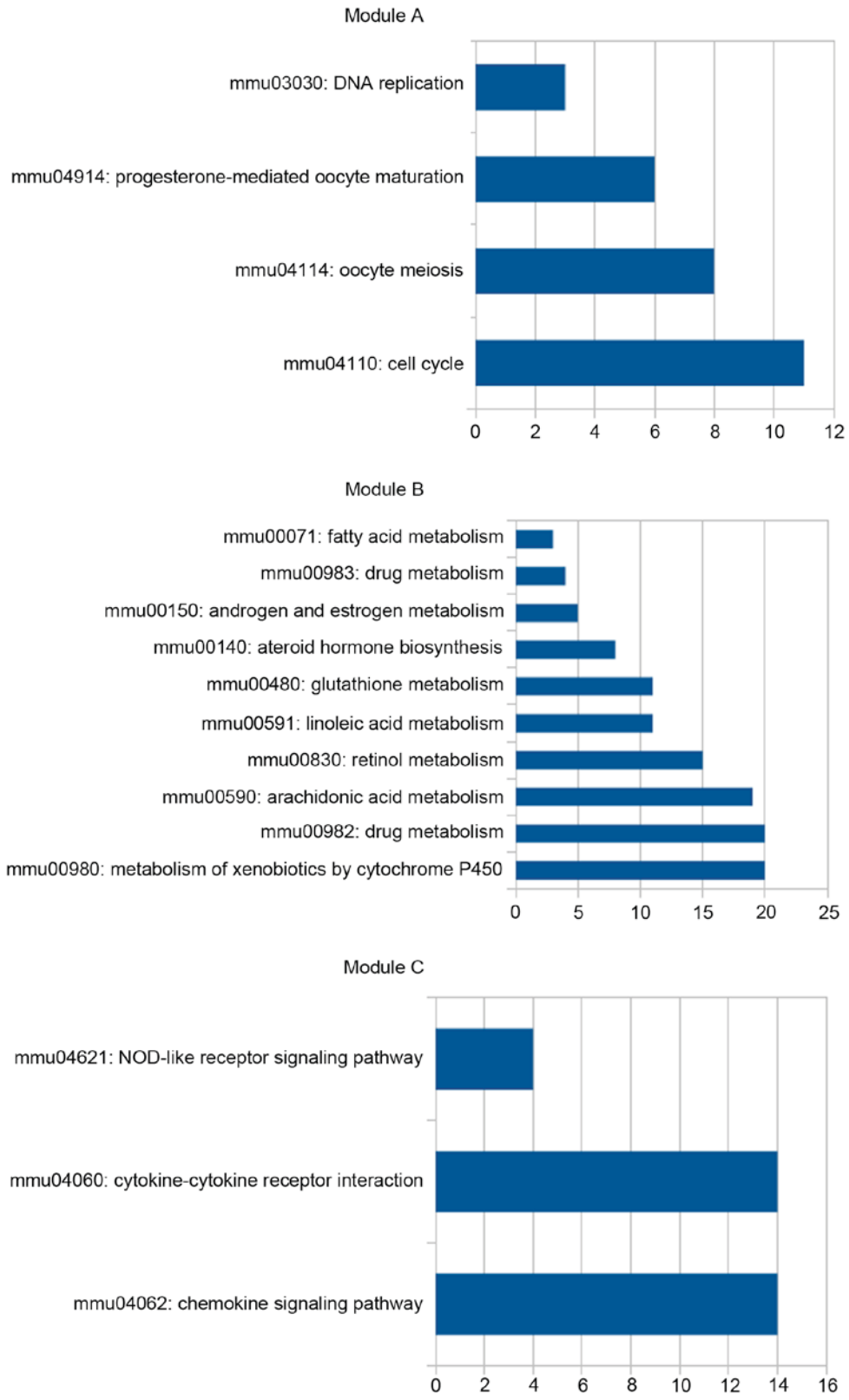

Figure 3. Functional enrichment analysis of the genes in each module network.

Discussion

miR-122 deficiency results in chronic steatohepatitis and spontaneous HCC (14). By re-analyzing the dataset GSE31731, numerous DEGs were identified between miR-122 KO and WT groups. Of these, upregulated genes were significantly enriched in cell cycle-associated processes (for example *NUSAP1*), cytokine-cytokine receptor interaction pathways (for example *CXCR4* and *CCR2*), and extracellular matrix (ECM) -receptor interactions (for example *ITGAV*). Various overlapping targets were highlighted in the PPI network, including *NUSAP1*, *CXCR4*, *CCR2* and *ITGAV*. Notably, *NUSAP1* was

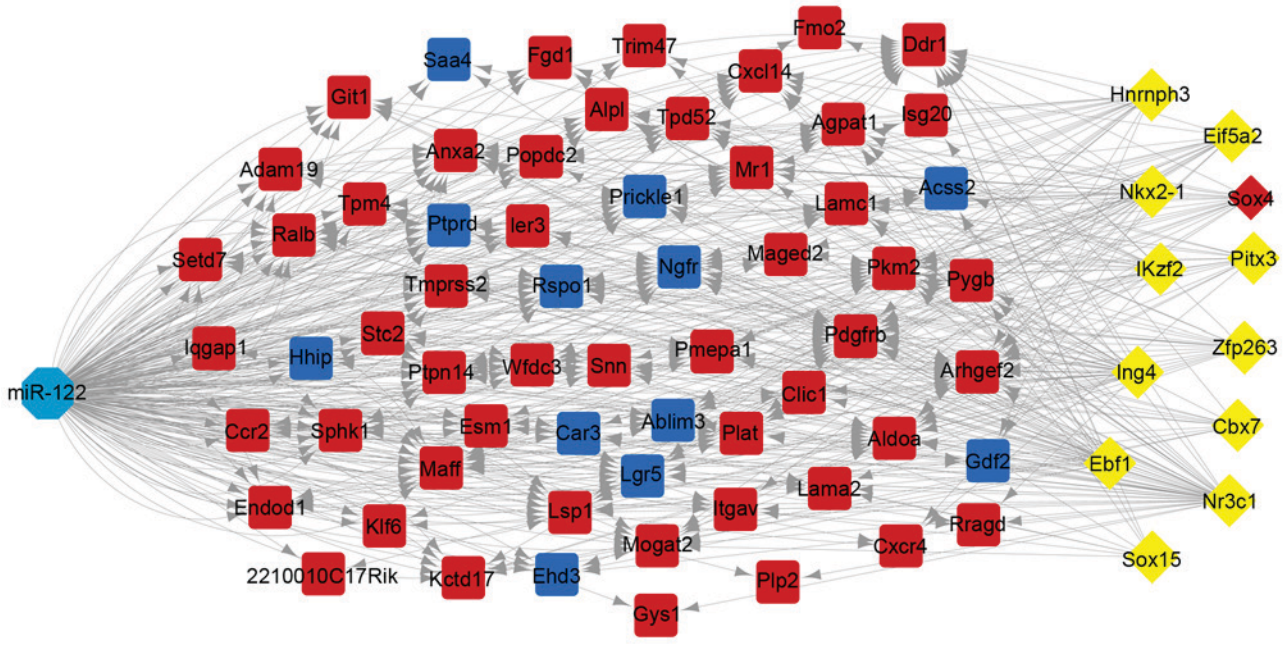

Figure 4. Targets of microRNA-122 and TFs of the targets. Squares represent targets (red, upregulated; blue, downregulated), diamonds represent TFs (red, upregulated; yellow, expression without significant difference). TF, transcription factor.

predominant in module A, which was associated with the cell cycle-associated pathway, whereas *CXCR4* and *CCR2* were linked in module C, enriched in the cytokine-cytokine receptor interaction pathway. Furthermore, upregulated *SOX4* was identified as a TF.

Restoration of miR-122 suppressed HCC tumor cell growth, and the antitumor activity was closely associated with cell cycle arrest (33). The protein encoded by *NUSAP1* is a nucleolar-spindle-associated protein that acts as a positive regulator of mitosis (34). It has been identified as a cell cycle progression gene in numerous cancer types, including prostate and lung cancer (35,36). In aggressive HCC, expression of *NUSAP1* is affected by other cell cycle-associated genes, including *L2DTL* (37). In the present study, upregulated *NUSAP1* in the miR-122 KO group was significantly enriched in cell cycle-associated BPs, and additionally served as a node in module A of the PPI network, as well as one of the overlapped targets of miR-122. However, *NUSAP1* has not been previously reported to be directly involved in HCC based on the literature mining results, suggesting that this gene may be a novel target of miR-122 involved in cell cycle-associated processes in HCC progression.

Dysregulation of the cytokine-cytokine receptor interaction pathway has been detected in HCC development (38,39). Activation of various chemokines in this pathway is involved in the development of numerous cancers, including HCC (40). As a chemokine receptor, the function of *CXCR4* has been extensively investigated. Increased expression of *CXCR4* has previously been reported to have a close association with the progression of HCC (41). In addition, *CXCR4* inhibition results in antitumor effects, including the inhibition of tumor growth and the improvement of survival in mice with HCC (42). Elevated expression of *CXCR4* in the miR-122 deficiency group, combined with the target information in miRNA databases, indicated that *CXCR4* may be a potential target of miR-122 in HCC. *CCR2* is the chemokine receptor of C-C motif chemokine ligand 2 (*CCL2*). Increased expression of *CCR2* has previously been observed in liver leukocytes from patients with HCC (43). *CCL2* is also involved in the cytokine-cytokine receptor interaction pathway and its expression is associated with HCC progression (38). Notably, *CCL2* has been identified as a target of miR-122, and restoration of miR-122 results in the suppression of *CCL2* in HCC (44). The results of the present study indicated that the DEG *CCR2* was upregulated in the miR-122 KO group and significantly enriched in the cytokine-cytokine receptor interaction pathway. Notably, it was additionally identified as an overlapping target based on the miRNA targeting database, and was linked to *CXCR4* in module C of the PPI network. These data collectively suggested that *CCR2* may be a target of miR-122, and co-regulate the cytokine-cytokine receptor interaction pathway with *CXCR4* in HCC development.

ECM-receptor interaction is a common pathway that is disturbed by altered gene expressions in various cancers (45,46). The gene *ITGAV* encodes a protein that belongs to the integrin superfamily. It has been reported to be involved in the ECM-receptor interaction pathway. It is a part of the ECM system (47), suggesting its involvement in the ECM-receptor interaction pathway. In other cancer types, including gastric cancer, *ITGAV* is enriched in this pathway (48). Notably, overexpressed *ITGAV* is induced by the gene forkhead box Q1 (*FOXQ1*), a member of the forkhead TF family that influences HCC metastasis (49), suggesting the potential involvement of this gene in HCC. Upregulated *ITGAV* in the miR-122 KO group, combined with enrichment analysis and the overlapped prediction target, indicated that this gene may be a target of miR-122 that modulates the ECM-receptor interaction pathway in HCC progression.

The intron-lacking gene *SOX4* contributes to hepatocarcinogenesis and its overexpression may be a useful prognostic

marker for survival after surgical resection (50). It has been demonstrated *in vitro* that overexpressed *SOX4* is involved in p53-mediated apoptosis in HCC (50). In addition, *SOX4* overexpression has previously been reported to control the metastasis of HCC (51). Thus, *SOX4* has been identified as a marker gene for HCC (52). Furthermore, *SOX4* serves as a TF that regulates cell differentiation. In HCC, various targets have been experimentally validated using chromatin immunoprecipitation and small interfering RNA assays, including aldo-keto reductase family 1 member B10, coiled-coil domain containing 97, dickkopf Wnt signaling pathway inhibitor 1, *FOXQ1* and microtubule associated protein 4 (51). miR-191 inhibition has previously been reported to result in the upregulation of *SOX4*, and increased *SOX4* expression promotes cell apoptosis and suppresses tumorigenesis of HCC (53). The present study indicated that the TF *SOX4* may additionally be a target of miR-122.

Despite these comprehensive bioinformatics analyses, the present study has limitations. The data was downloaded from the GEO database, and the sample size was relatively small. In addition, experimental validation of the associations between miR-122 and the predicted targets was lacking, and will be addressed in follow-up studies. Furthermore, the target expression levels following miR-122 restoration were not investigated, although this would have further confirmed the targeting associations. However, the present study has significant value as it provides novel insights into the consequences of miR-122 KO in HCC progression.

In conclusion, various crucial targets of miR-122 in HCC progression were identified, including *NUSAP1*, *CXCR4*, *CCR2* and *ITGAV*. Cell cycle-associated processes, the cytokine-cytokine receptor interaction pathway, which may be co-regulated by *CXCR4* and *CCR2*, and the ECM-receptor interaction pathway were altered by these targets. In addition, the target *SOX4* may be a TF. The results of the present study may provide a theoretical basis for further studies on the mechanisms of miR-122 in the development of HCC.

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