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.

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 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 (cl.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
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).

| Category | Term | Count | P-value |
|----------|------|-------|---------|
| **Upregulated** | | | |
| BP | GO:0007049–cell cycle | 57 | 4.96x10^{-11} |
| | GO:0000279–M phase | 34 | 1.64x10^{-9} |
| | GO:000278–mitotic cell cycle | 31 | 2.64x10^{-9} |
| | GO:0007067–mitosis | 27 | 3.21x10^{-9} |
| | GO:0000280–nuclear division | 27 | 3.21x10^{-9} |
| CC | GO:0005576–extracellular region | 133 | 9.92x10^{-18} |
| | GO:004421–extracellular region part | 74 | 2.00x10^{-13} |
| | GO:0005578–proteinaceous extracellular matrix | 35 | 7.33x10^{-10} |
| | GO:0031012–extracellular matrix | 35 | 2.01x10^{-10} |
| | GO:004420–extracellular matrix part | 18 | 4.96x10^{-10} |
| MF | GO:0005509–calcium ion binding | 60 | 1.62x10^{-7} |
| | GO:0008099–chemokine activity | 10 | 4.56x10^{-6} |
| | GO:0008201–heparin binding | 14 | 5.26x10^{-6} |
| | GO:0042379–chemokine receptor binding | 10 | 5.74x10^{-6} |
| | GO:0001871–pattern binding | 17 | 9.14x10^{-6} |
| **Downregulated** | | | |
| BP | GO:0055114–oxidation-reduction | 64 | 9.82x10^{-28} |
| | GO:0006631–fatty acid metabolic process | 24 | 4.36x10^{-13} |
| | GO:0008202–steroid metabolic process | 21 | 1.75x10^{-11} |
| | GO:0006694–steroid biosynthetic process | 15 | 3.73x10^{-11} |
| | GO:006956–complement activation | 10 | 1.37x10^{-11} |
| CC | GO:0005777–peroxisome | 22 | 4.28x10^{-15} |
| | GO:0042579–microbody | 22 | 4.28x10^{-15} |
| | GO:0005792–microsome | 26 | 5.15x10^{-14} |
| | GO:0042598–vesicular fraction | 26 | 1.13x10^{-13} |
| | GO:0005739–mitochondrion | 66 | 7.86x10^{-11} |
| MF | GO:0009055–electron carrier activity | 34 | 4.36x10^{-21} |
| | GO:0020037–heme binding | 23 | 9.42x10^{-14} |
| | GO:0046906–tetrapyrrole binding | 23 | 2.57x10^{-13} |
| | GO:0005506–iron ion binding | 33 | 3.02x10^{-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.
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                                     | 4.82x10^{-6}|
| mmu04512: ECM-receptor interaction                   | 15    | COL3A1, LAMA2, ITGAV, COL1A2, LAMC1                                  | 4.87x10^{-6}|
| mmu04510: Focal adhesion                             | 22    | COL3A1, LAMA2, ITGAV, LAMC1                                          | 4.43x10^{-5}|
| mmu00480: Glutathione metabolism                     | 10    | GPX2, GSTA1, GSTA2, GSTM3, G6PDH                                       | 2.05x10^{-4}|
| mmu04110: Cell cycle                                 | 14    | CCNB2, KMYT1, BUB1B, ESPL1, CDC20                                     | 0.001940583 |
| mmu05222: Small cell lung cancer                     | 11    | LAMA2, COL4A1, ITGAV, LAMC2, LAMC1                                   | 0.002161135 |
| 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                              | 1.83x10^{-12}|
| mmu00980: Metabolism of xenobiotics by cytochrome P450| 17    | CYP2C37, CYP3A16, CYP2C54, CYP2C44, CYP2C68                            | 2.81x10^{-12}|
| mmu00591: Linoleic acid metabolism                   | 14    | CYP2J5, CYP2C37, CYP3A16, CYP2C54, CYP2C44                            | 4.16x10^{-11}|
| mmu00830: Retinol metabolism                         | 16    | CYP2C37, CYP3A16, CYP2C54, CYP2C44, CYP2C68                            | 6.09x10^{-11}|
| mmu03320: PPAR signaling pathway                     | 16    | ACOX1, ACSL1, CYP4A12A, HMGCS2, SCP2                                 | 5.86x10^{-10}|
| mmu00590: Arachidonic acid metabolism                | 15    | CYP2J5, CYP2C37, CYP2C54, CYP2C44, CYP2J8                             | 1.14x10^{-9} |
| mmu00120: Primary bile acid biosynthesis             | 8     | CYP7B1, HSD3B7, CYP7A1, CYP8B1, SCP2                                  | 2.81x10^{-9} |
| mmu00071: Fatty acid metabolism                      | 9     | CYP4A12B, GCDH, ACOX1, ACSL1, ADH4                                   | 1.21x10^{-9} |
| mmu00140: Steroid hormone biosynthesis               | 9     | CYP7B1, CYP3A16, HSD3B6, HSD17B2, CYP7A1                              | 1.21x10^{-9} |
| mmu04160: Complement and coagulation cascades        | 11    | MBL1, C8A, MBL2, C8B, CD55                                          | 1.40x10^{-9} |

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.
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). NUSAPI 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.
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 NUSAPI), 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 NUSAPI, CXCR4, CCR2 and ITGAV. Notably, NUSAPI was
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 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.

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

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.
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 NUSAPI, 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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