

Bioinformatic identification of IGF1 as a hub gene in hepatocellular carcinoma (HCC) and *in-vitro* analysis of the chemosensitizing effect of miR-379 via suppressing the IGF1/IGF1R signaling pathway

D.-J. HUANG^{1,2}, J.-Z. HUANG², J. YANG³, Y.-H. LI¹, Y.-C. LUO², H.-Y. HE², H.-J. HUANG²

¹Department of Interventional Radiology, Nanfang Hospital, Southern Medical University, Guangzhou, China

²Department of Interventional Radiology, The First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning China

³Department of Interventional Radiology, the Second Affiliated Hospital of Harbin Medical University, Harbin, China

De-Jia Huang and Jun-Zhen Huang are co-first author

Abstract. – OBJECTIVE: We investigated the interactions among the dysregulated genes in hepatocellular carcinoma (HCC) and identified the hub genes in the protein-protein interaction (PPI) network. Also, we also investigated the regulative effect of miR-379 on the IGF1/IGF1R signaling pathway and chemoresistance in HCC.

MATERIALS AND METHODS: Raw data of a microarray that compared transcriptional gene profile between 3-paired HCC tissue samples and adjacent normal tissues were downloaded from Expression Atlas (E-GEOD-33006). The raw data was reanalyzed to identify the significantly dysregulated genes, which were further used for PPI network and KEGG pathway analysis. The regulative effect of miR-379 on IGF1R expression was studied by dual luciferase assay and Western blotting. The functional role of miR-379 in chemosensitivity of HCC cells was studied by drug sensitivity and flow cytometric assay.

RESULTS: IGF1 is a hub gene that is mostly upregulated in HCC and it is an important player in the p53 signaling pathway. Knockdown of IGF1R significantly decreased IC50 of 5-FU, paclitaxel (PTX) and Doxorubicin (DOX) in Huh7 and HepG2 cells. MiR-379 could directly bind to the 3'UTR of IGF1R and suppress IGF1R expression. MiR-379 overexpression sensitized Huh7 and HepG2 cells to 5-FU, PTX and DOX and also enhanced DOX-induced cell apoptosis.

CONCLUSIONS: IGF1 is a hub gene in HCC and is also one of the most upregulated genes in HCC tissues compared to normal tissues. It is involved in the p53 signaling pathway regulation. MiR-379 can sensitize HCC cells to chemother-

apeutic reagents via targeting IGF1R and suppressing its expression.

Key Words:

Bioinformatics, IGF1, IGF1R, Hepatocellular carcinoma, miR-379, Chemosensitivity.

Introduction

Hepatocellular cancer (HCC) is one of the most prevalent cancers and is also a leading cause of cancer-related death globally^{1,2}. Partial hepatic resection and liver transplantation are still the curative and the most effective therapies for HCC³. However, a large proportion of the HCC patients were diagnosed at advanced stages and thus were not quantified for surgery⁴. Besides, liver transplantation is also quite limited since the liver donors are in a great shortage³. For most of the HCC patients, systemic chemotherapy remains the major treatment option. Conventional chemotherapeutic reagents such as 5-fluorouracil (5-FU), paclitaxel (PTX) and doxorubicin (DOX) have been administrated for the patients and significantly improved their survival⁵. However, the intrinsic and acquired drug resistance, especially multi-drug resistance (MDR) is still one of the toughest obstacles to successful treatment^{6,7}. The mechanism of drug resistance in HCC is quite complex and has not been fully understood⁸.

The high-throughput platforms, such as microarray, have been used as an important tool in medical oncology. With the data obtained from the gene microarray, bioinformatic analysis can be further performed to identify the key genes involved in different pathways, biological processes, or molecular functions^{9,10}. One recent microarray-based study¹¹ explored the transcriptional gene profile between 3-paired HCC tissue samples and adjacent normal liver tissues, but the interactions among the dysregulated genes, particularly the pathways in the protein-protein interaction (PPI) network, remain to be elucidated.

IGF1 is a potent mitogenic factor that exerts anti-apoptotic effects on many cell systems. Recent studies demonstrated that activation of the IGF1/IGF1R signaling pathway is associated with enhanced chemoresistance^{12,13}, while blocking the IGF1/IGF1R signaling pathway show the chemosensitizing effect on some cancer cells, including HCC cells¹⁴⁻¹⁶. MiRNAs are a class of small and conserved noncoding RNAs that regulate gene expression via degradation and translational inhibition of the targeting mRNAs¹⁷. Previous studies^{13,18} reported that several miRNAs have an inhibitive effect on the IGF1/IGF1R signaling pathway, such as miR-122 and miR-378. However, whether other miRNAs are involved in the regulation in HCC is not clear.

In this work, we reanalyzed the raw microarray data of E-GEOD-33006 downloaded from Expression Atlas¹¹ and found IGF1 is a hub gene in HCC. Then, we demonstrated that miR-379 is a novel suppressive miRNA on the IGF1/IGF1R signaling pathway, which has a chemosensitizing effect on the cancer cells.

Materials and Methods

Microarray Data

The gene microarray that compared transcriptional gene profile between 3-paired HCC tissue samples and adjacent normal liver tissues were download from Expression Atlas with Accession Number: E-GEOD-33006¹¹. The raw data file of the microarray was reanalyzed using Morpheus (<https://software.broadinstitute.org/morpheus/>).

Protein-protein Interaction (PPI) and KEGG Pathway Analysis

To identify the network of the most upregulated genes, the top 300-upregulated genes

were loaded into the Search Tool for the Retrieval of Interacting Genes (STRING) (<http://string-db.org/>) database for analysis. To ensure the validity of the network, only experimentally validated interactions with a high confidence score ≥ 0.70 were included. To further analyze the functional pathways that the genes may involve in, KEGG pathway analysis was further performed using the online tool provided by STRING. $p < 0.05$ was considered statistically significant.

Cell Culture and Transfection

Human hepatocellular carcinoma cell line Huh7 and HepG2 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U of penicillin/ml and 100 μ g of streptomycin/ml in a humidified atmosphere containing 5% CO₂ at 37°C.

IGF1R siRNA (5'-GCAGACACCUACAA-CAUCAUU-3')¹⁹, miR-379 mimics and the corresponding negative controls were synthesized and purchased from Ribobio (Guangzhou, China). Huh7 and HepG2 cells were transfected with 100 nM IGF1R siRNA, 40 or 80 nM miR-379 mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

qRT-PCR Analysis

Total RNA was extracted from cells using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and converted into cDNA using a First-Strand Synthesis Kit (Invitrogen). To detect IGF1R mRNA expression, qRT-PCR analysis was performed on an ABI 7700 sequence detector using gene specific primers and TaqMan Universal PCR Master Mix according to the directions of the manufacturer (Applied Biosystems, Foster City, CA, USA). The primers used for IGF1R were: F, 5'-CCTCCAACTTCGTCTT-TGCAA-3'; R, 5'-CAGGTCACTGGCCCAG-GA-3'. The expression of mature miR-379 was analyzed using the TaqMan MicroRNA Assay and normalized to the expression of RNU48. The 2^{- $\Delta\Delta$ Ct} method was used to calculate the relative quantities of target genes. All reactions were performed in triplicate.

Western Blot Analysis

Proteins from cell samples were extracted by using a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Then, the

protein concentration was measured using the BCA method. After that, the samples containing 20 µg protein were loaded in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation. The first antibodies used include anti-IGF1R (1:500, ab131476, Abcam, Cambridge, UK) and anti-β-actin (1: 2000, ab8227, Abcam). The second HRP conjugated secondary antibodies were obtained from Abcam. The signal intensity of the protein bands was visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA). The signal intensity was quantified using ImageQuant TL software (GE Healthcare, Piscataway, NJ, USA).

Measurement of IC50

24 h after transfection with IGF1R siRNA (100 nM) or miR-379 mimics (80 nM), Huh7 and HepG2 cells were seeded in a 96-well plate at a density of 3×10^3 cells/well. The cells were then treated with varying concentrations of 5-FU, paclitaxel (PTX) or Doxorubicin (DOX) for 72 h. After the treatment, cell viability was measured using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to manufacturer's instruction. In brief, 10 µl of CCK-8 solution was added to the medium and then incubated at 37°C for 2 h. Cell viability was reflected by the absorbance at 450 nm determined by a 96-well spectrophotometry. The IC50 value was determined by creating dose-response curves.

Dual Luciferase Assay

The binding sites between IGF1R 3'UTR and miR-379 were predicted using Targetscan 7.1. To construct luciferase reporter vectors, the wild type IGF1R 3'UTR fragment containing the predicted miR-379 binding site and the mutant sequence with mutant binding sites were chemically synthesized and inserted into the downstream of the luciferase gene of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) respectively. The reconstructed plasmids were named as pmirGLO-IGF1R-WT and pmirGLO-IGF1R-MT respectively. Huh7 and HepG2 cells cultured in the 96-well plate were co-transfected with the recombinant reporter plasmids and miR-379 mimics or the scramble negative controls using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested 24 h after transfection and then the luciferase activity was measured using a GloMaxTM 96 Microplate Luminometer (Promega) using the Dual-Luci-

ferase reporter assay system (Promega). Firefly luciferase activity was normalized to that of Renilla luciferase.

Assessment of DOX-Induced Apoptosis by Propidium Iodide (PI) Staining

24 h after the transfection of miR-379 mimics or the negative control, Huh7 and HepG2 cells were subjected to 0.5 µM DOX treatment for 24 h. Then, the cells were harvested, fixed with 70% ethanol and stained using propidium iodide (PI) staining solution (0.5 µg/ml PI, 10 µg/ml ribonuclease A, 0.1% sodium citrate and 0.1% Triton X-100). A total of 20,000 cells were acquired using the Partec flow cytometer (Sysmex Partec, Görlitz, Germany) and were analyzed using FlowJo software.

Statistical Analysis

Data were presented as mean ± SD with at least three repeats. Statistical analyses and graphical representations were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (San Diego, CA, USA) software. The Student's t-test was used to determine the statistically significant differences among indicated experimental results. $p < 0.05$ was considered as statistically significant.

Results

Identification of the Significantly Dysregulated Genes in HCC

The gene microarray (E-GEOD-33006) found that hundreds of genes were significantly dysregulated in HCC tissue samples compared to adjacent normal liver tissues (Figure 1A). By reanalyzing the raw data, we identified the top 40 upregulated genes (Figure 1B, left) and the top 40 downregulated genes (Figure 1B, right).

Protein-protein Interaction (PPI) Network and KEGG Pathway Analysis

To identify the hub genes among the most the upregulated ones, the PPI analysis was performed by using STRING. The top 20 hub nodes with the highest scores were identified, including SOCS2, IGFBP3, IGF2, IL33, MASP1, PSAT1, SOCS3, AR, CXCL12, IL8, FOS, CISH, LEPR, APOF, ESRRG, CYP1A2, IGF1, ID1, HOGA1 and THBS1 (Figure 2, red arrows). By performing KEGG pathway analysis, we found that the upregulated genes were enriched in pa-

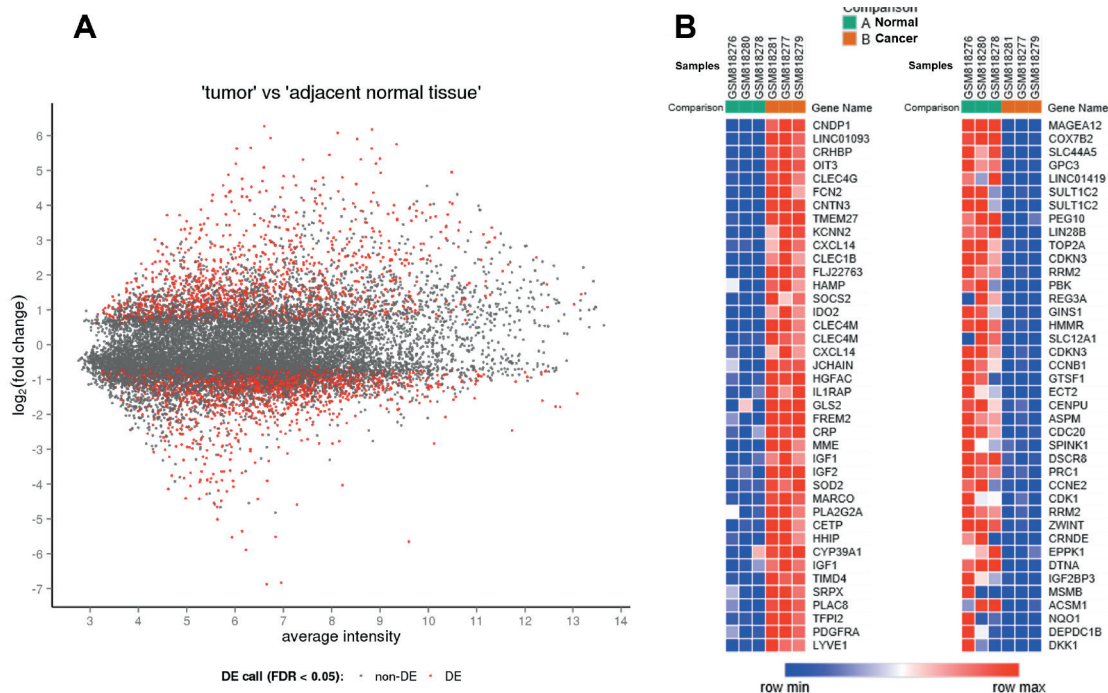


Figure 1. The dysregulated genes in HCC vs. normal tissues. **A**, The MA plot for the contrast of all genes in the array (Expression Atlas, ID: E-GEOD-33006). **B**, The heat map of the top 80 differentially expressed genes (40 up-regulated genes and 40 down-regulated genes). Red: up-regulation; Blue: down-regulation.

thways such as Cytokine-cytokine receptor interaction, p53 signaling pathway, complement and coagulation cascades, malaria, prion diseases, glycine, serine and threonine metabolism, metabolic pathways and NF-kappa B signaling pathway (Table I). Typically, we found that IGF1, one of the top 40 upregulated gene and a hub gene in PPI network, is mainly involved in the p53 signaling pathway (Table I, red font). According to previous studies, the p53 signaling pathway regulates transcription of a series of protective antioxidant genes that play important roles in extensive DNA damage response, thereby closely related to chemosensitivity regulation in HCC²⁰⁻²². Therefore, we decided to further investigate the functional role of the IGF1/IGF1R signaling pathway in HCC.

Knockdown of IGF1R Enhances Chemosensitivity of HCC Cells

To investigate how the IGF1/IGF1R signaling pathway modulates chemosensitivity of HCC cells, both Huh7 and HepG2 cells were firstly transfected with IGF1R siRNA (Figure 3A and B). By performing the CCK-8 assay, we observed that knockdown of IGF1R significantly decreased

IC50 of 5-FU, PTX and DOX in both Huh7 (Figure 3C) and HepG2 (Figure 3D) cells.

MiR-379 Suppresses IGF1R Expression in HCC and Modulates Chemosensitivity

By using the bioinformatics prediction, we found that miR-379 has a putative binding site in the 3'UTR of IGF1R (Figure 4A). By using the reconstructed luciferase reporter plasmids, we confirmed that miR-379 could effectively suppress the relative expression of firefly luciferase in the plasmids carrying the wild-type sequence (Figure 4B-C), but it had no inhibitive effect on the luciferase expression of the mutant plasmid (Figure 4B-C). Then, we further assessed whether miR-379 overexpression could directly lead to IGF1R downregulation. In both Huh7 and HepG2 cells, transfection of miR-379 mimics decreased IGF1R expression in a dose-dependent manner (Figure 4D-E). The following drug sensitivity assay also confirmed that miR-379 mimics significantly decreased IC50 of 5-FU, PTX and DOX in both Huh7 (Figure 4F) and HepG2 (Figure 4G) cells. To further verify the chemosensitizing effect of miR-379, Huh7 and HepG2 cells with or wi-

Table I. KEGG pathway analysis of the significant upregulated genes in hepatocellular cancer.

Pathway ID	Pathway description	Observed gene count	<i>p</i> -value	Matching proteins in the network (labels)
4060	Cytokine-cytokine receptor interaction	13	0.000831	CCL2, CXCL12, CXCL14, CXCL2, FAS, GHR, HGF, IL1RAP, IL8, LEPR, LIFR, PDGFRA, TNFSF14
4115	p53 signaling pathway	7	0.000831	FAS, GADD45B, IGF1, IGFBP3, SERPINE1, STEAP3, THBS1
4610	Complement and coagulation cascades	7	0.000831	C6, C7, C9, CFD, CR1, MASP1, SERPINE1
5144	Malaria	6	0.000831	CCL2, CR1, HBB, HGF, IL8, THBS1
5020	Prion diseases	5	0.00269	C6, C7, C9, EGR1, PRNP
260	Glycine, serine and threonine metabolism	5	0.00333	AGXT2, GNMT, PHGDH, PSAT1, SDS
1100	Metabolic pathways	27	0.00999	ACADL, ADH1B, AGXT2, ASPA, ASS1, CNDP1, CYP1A2, DHODH, G6PC, HAL, HAO2, HOGA1, IDO2, KMO, MAN1C1, MTHFD2L, NAT2, NNMT, OAT, PHGDH, PLA2G2A, PSAT1, PTGIS, SDS, ST3GAL6, UGT2B17, UPP2
4064	NF-kappa B signaling pathway	6	0.0163	BCL2A1, CXCL12, CXCL2, GADD45B, IL8, TNFSF14
5142	Chagas disease (American trypanosomiasis)	6	0.023	CCL2, FAS, FOS, GNA14, IL8, SERPINE1
4917	Prolactin signaling pathway	5	0.0297	CISH, ESR1, FOS, SOCS2, SOCS3
232	Caffeine metabolism	2	0.0316	CYP1A2, NAT2

thout miR-379 overexpression were subjected flow cytometric analysis of cell apoptosis after 12 h DOX treatment. The results showed that enforced miR-379 expression substantially increased the ratio of apoptotic cells after DOX treatment (Figure 4H-K).

Discussion

By performing PPI analysis, we confirmed that IGF1 is a hub gene that is mostly upregulated in HCC. Its functional role in HCC has also been gradually revealed. In detail, IGF1 overexpression can enhance cell invasion, colony formation and migration activity of HCC cells²³. Mechanistically, HCC cells can use IGF1 to prevent inter-cellular exosomal transfer of miR-122, a growth

retarding miRNA to maintain its proliferation²⁴. Also, increased autocrine of IGF1 is associated with maintenance of stemness in oxaliplatin-resistant HCC cells²⁵.

Previous studies²⁶ reported that IGF1 can antagonize Regorafenib-mediated growth, migration and invasion inhibition, as well as the drug-mediated induction of apoptosis in HCC cells. Blocking the IGF1/IGF1R signaling pathway shows both tumor suppressive and chemosensitizing effect. For example, G protein-coupled receptor kinase 2 (GRK2) can interact with IGF1R and inhibit IGF1-induced activation of IGF1R signaling pathway²³. Knockdown of IGF1R directly results in reduced growth, clonogenic survival, adhesion and migration of liver cancer cells and also sensitized the cancer cells to apoptosis-inducing reagents and chemothe-

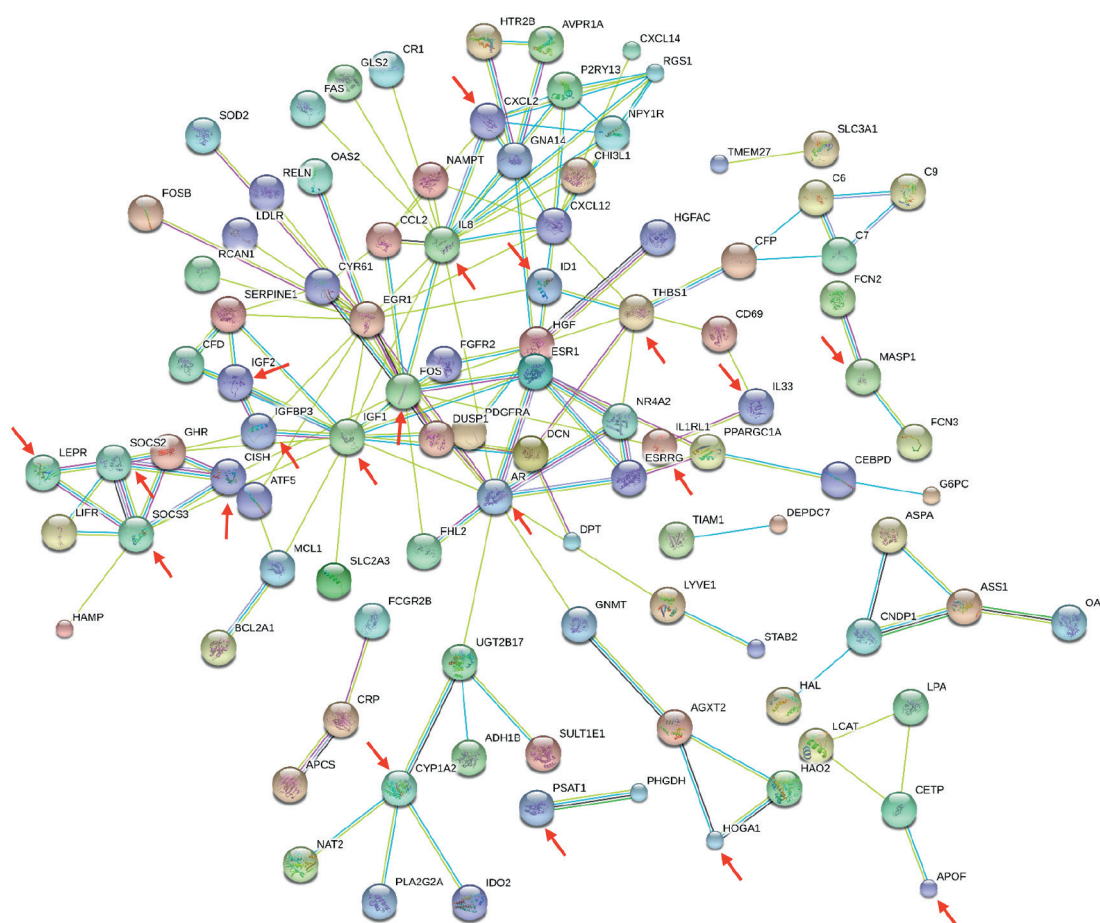


Figure 2. The protein-protein interaction (PPI) network of the upregulated genes. The protein-protein interaction (PPI) network of the upregulated genes obtained by using the (STRING) (<http://string-db.org/>) database. Only experimentally validated interactions with a high confidence score ≥ 0.70 were included in the PPI network. Red arrows indicate the top 20 hub genes in the network.

therapeutic drugs including DOX¹². Several miRNAs with inhibitive effect on the IGF1/IGF1R signaling pathway also show tumor suppressive and chemosensitizing effect on several types of cancer, including HCC²⁷. For example, miR-630 can target IGF1R and regulate HER-targeting drug sensitivity in HER2 overexpressing breast cancer¹⁵. MiR-223 can reverse the resistance of EGFR-TKIs through the IGF1R/PI3K/Akt signaling pathway¹⁶. MiR-28-5p is a critical regulator of IGF1 mRNA translation in HCC cells, while enforced miR-28-5p expression can downregulate IGF1 protein and reduce tumor growth²⁸. MiR-378 can also lower cell proliferation and colony formation of HBV-related HCC by directly targeting the IGF1R 3'UTR and inhibiting

its protein expression¹⁸. MiR-122 can inhibit tumorigenic properties of HCC cells and sensitize these cells to sorafenib by targeting IGF1R¹⁴.

By performing KEGG analysis, we confirmed that IGF1 is an important player in the p53-signaling pathway. Considering the critical role of p53 signaling in regulating chemosensitivity of cancer cells, we decided to further investigate the underlying mechanism of the IGF1/IGF1R pathway in drug responses of HCC cells. By performing drug sensitivity assay, we confirmed that knockdown of IGF1R significantly sensitized both Huh7 and HepG2 cells to 5-FU, PTX and DOX. Online bioinformatic analysis showed that miR-379 has a possible conserved binding site in the 3'UTR of IGF1R. In HCC, miR-379 has been demonstrated as a tumor sup-

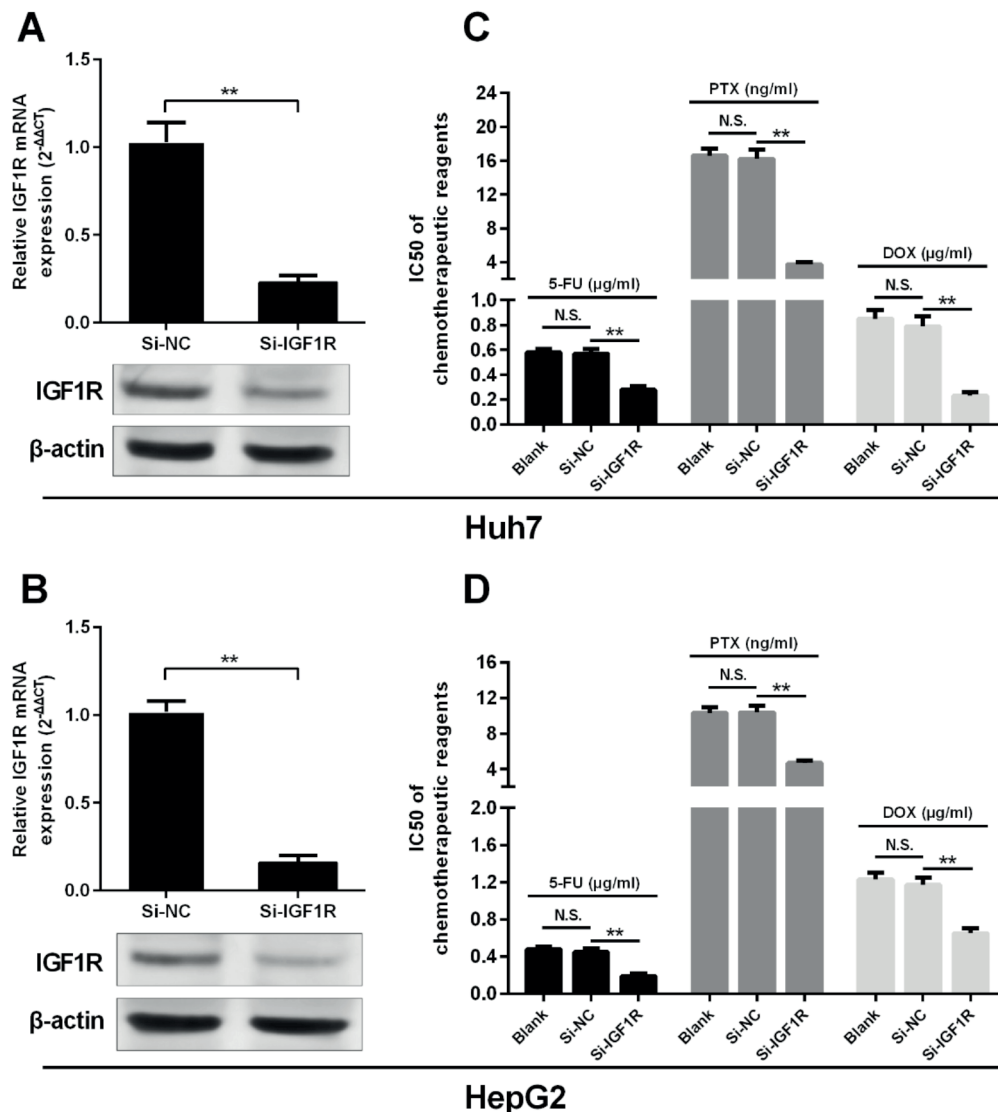


Figure 3. Knockdown of IGF1R enhances chemosensitivity of HCC cells. A-B, QRT-PCR (up) and Western blotting (down) assay of IGF1R mRNA and IGF1 protein expression in Huh7 (**A**) and HepG2 (**B**) cells after transfection of si-NC (100 nM) or si-IGF1R (100 nM). **C-D**, IC₅₀ of 5-FU, PTX and DOX in Huh7 (**C**) and HepG2 (**D**) cells with or without transfection of si-IGF1R. N.S., not significant; ** $p < 0.01$.

pressive miRNA. Enforced miR-379 expression can significantly inhibit HCC cell migration, invasion, epithelial-to-mesenchymal transition (EMT) and metastasis both *in vitro* and *in vivo*²⁹. Mechanistically, miR-379 can target focal adhesion kinase (FAK) 3'UTR and repress FAK expression, leading to suppression of the AKT signaling²⁹. In addition, it might also involve in regulation of multidrug resistance in HCC cells³⁰. In this study, we further confirmed that miR-379 can directly bind to the 3'UTR of IGF1R and suppress IGF1R expression. Therefore, we then investigated the functional role of miR-379 in chemosensitivity of HCC cells. The results suggest

that miR-379 overexpression sensitized both Huh7 and HepG2 cells to 5-FU, PTX and DOX and also enhanced DOX-induced cell apoptosis. Therefore, we infer that miR-379 can act as a chemosensitizer in HCC via suppressing the IGF1/IGF1R signaling pathway.

Conclusions

IGF1 is a hub gene in HCC and is also one of the mostly upregulated genes in HCC tissues compared to normal tissues. It is involved in the p53 si-

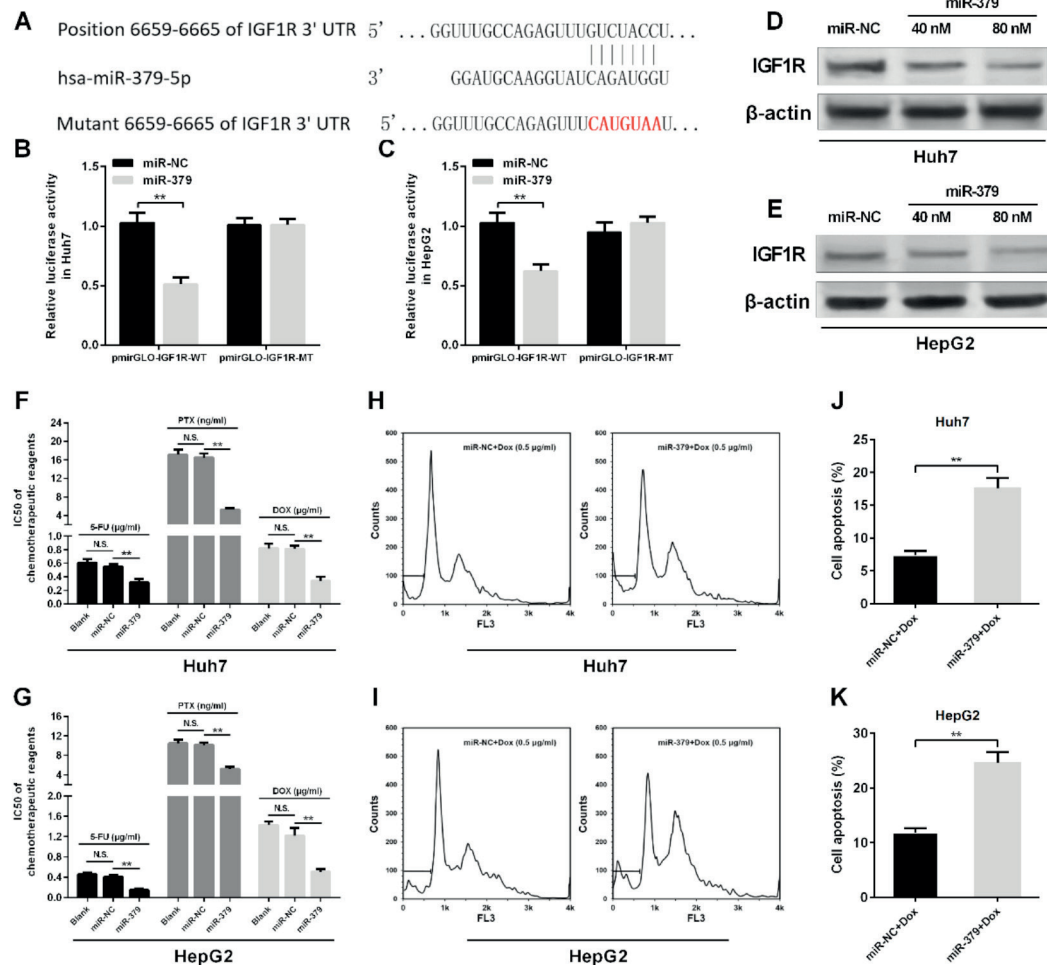


Figure 4. MiR-379 suppresses IGF1R expression in HCC and modulates chemosensitivity. **A**, Predicted binding sites between miR-379 and 3'UTR of IGF1R. The fragment with mutant binding sites was also given. **B-C**, Dual luciferase assay of the inhibitory effect of miR-379 on firefly luciferase expression of the reporters carrying wild type or mutant IGF1R 3'UTR fragments in Huh7 (**B**) and HepG2 (**C**) cells. **D-E**, Western blotting of IGF1 protein expression in Huh7 (**D**) and HepG2 (**E**) cells after transfection of 40 nM miR-NC, 40 nM miR-379 mimics or 80 nM miR-379 mimics. **F-G**, IC50 of 5-FU, PTX and DOX in Huh7 (**F**) and HepG2 (**G**) cells with or without transfection of 80 nM miR-379 mimics. **H-I**, Representative images of DOX induced apoptosis in Huh7 (**H**) and HepG2 cells (**I**) with or without transfection of 80 nM miR-379 mimics by PI staining and analyzed by flow cytometry. Percentages of cells in pre-G1 peak were represented as cells undergoing apoptosis. **J-K**, Quantitation of the ratio of apoptotic cells showed in figure H-I. N.S., not significant; ** $p < 0.01$.

gnaling pathway regulation. MiR-379 can sensitize HCC cells to chemotherapeutic reagents via targeting IGF1R and suppressing its expression.

Conflict of Interest

The authors declare no conflicts of interest.

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