

Exploring the mechanism of resistance to sorafenib in two hepatocellular carcinoma cell lines

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ABSTRACT

Sorafenib has long been the only approved systemic therapy for advanced hepatocellular carcinoma (HCC), but most patients show primary or acquired drug resistance. In the present study, RNA was extracted from sorafenib-resistant and -sensitive clones of the HCC cell lines HepG2 and Huh7. Protein-protein interaction networks of the up- and down-regulated genes common to the two sorafenib-resistant cell lines were extracted and subjected to modular analysis in order to identify functional modules. Functional enrichment analysis showed the modules were involved in different biological processes and pathways. These results indicate that sorafenib resistance in HCC is complicated and heterogeneous. The potential regulators of each functional module, including transcription factors, microRNAs and long non-coding RNAs, were explored to construct a comprehensive transcriptional regulatory network related to sorafenib resistance in HCC. Our results provide new insights into sorafenib resistance of HCC at the level of transcriptional regulation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the world's deadliest cancers [1]. HCC can be diagnosed in early stages by ultrasonography, when it may still be treated by liver resection or transplantation, resulting in a 5-year survival rate above 50%. [2] In fact, several other therapies can treat early-stage HCC, including radiofrequency

ablation, radioembolization and transarterial chemo embolization [3]. However, 70% of patients are diagnosed with HCC when the disease is already in a late stage. Only one systemic therapy is available for advanced HCC: sorafenib [4], which inhibits the proliferation of tumor cells by inhibiting Raf kinase and several receptor tyrosine kinases [5]. Unfortunately, sorafenib provides benefit to only 30% of patients with advanced HCC, in part because

many patients develop resistance to the drug within 6 months [4].

The mechanism of sorafenib resistance (SR) in HCC is poorly understood. Some studies have identified individual molecules that may confer SR, such as PHGDH [6], the Akt pathway activator SNHG1 [7] and PMPCB [8]. This led us to hypothesize that SR is caused by multiple molecular modules. To explore this possibility, we used two HCC cell lines to identify genes differentially expressed in the presence of SR, based on which we developed a comprehensive transcription regulatory network.

RESULTS

Using HepG2 and Huh7 cell lines, we compared SR and sorafenib-sensitive (SS) cells in order to identify Differentially expressed genes (DEGs) associated with SR. The DEGs common to both cell lines were then used to identify functional modules and regulatory

molecules that may help identify molecular pathways and networks associated with SR (Figure 1).

SR is associated with multiple genes and pathways in the two HCC cell lines

In the HepG2 cell line, 3075 genes were up-regulated and 638 down-regulated in association with SR. The corresponding numbers in Huh7 cells were 6946 and 2310 (Figure 2A). The sorafenib-targeted genes up-regulated in association with SR were ABCB1, ABCC4, ABCG2, BRAF and RALBP1 in the HepG2 line (Figure 2B); and ABCC2, ABCG2, CYP2B6, CYP2C19, CYP3A7, RAF1, RALBP1, and UGT1A1 in the Huh7 line (Figure 2C). Cluster analysis showed clear separation of DEGs between SR and SS cells of both lines (Figure 2D, 2E). We extracted 1630 up-regulated DEGs common to the two cell lines (Figure 2F) and 132 down-regulated genes common to the two lines (Figure 2G). Gene set enrichment analysis identified enrichment of the following pathways in SR

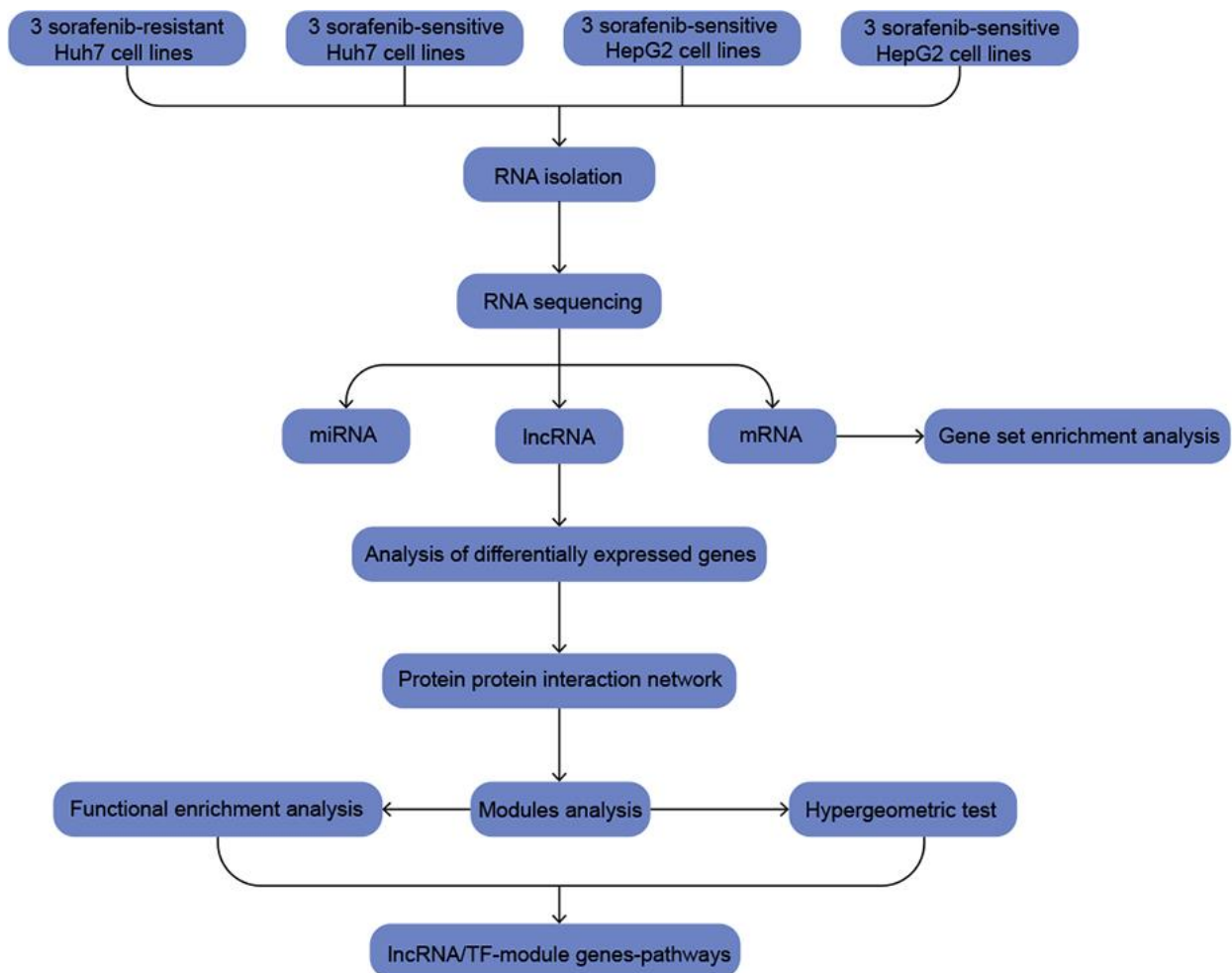
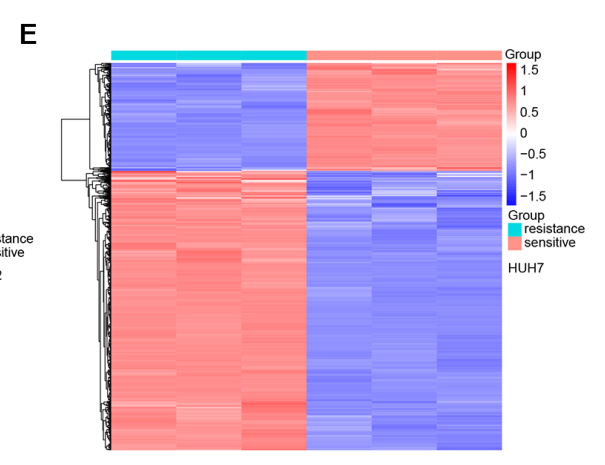
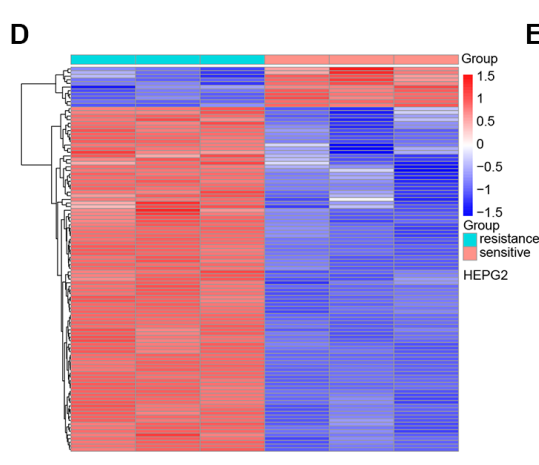
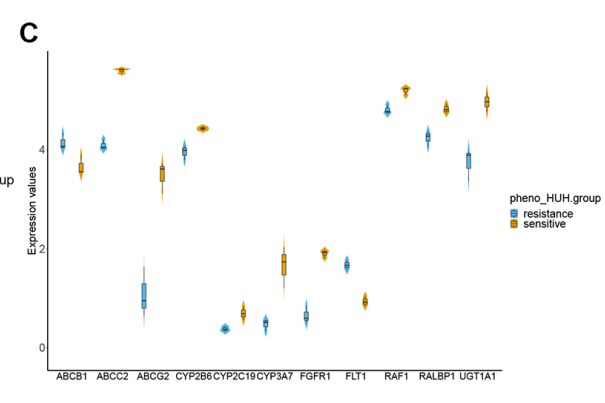
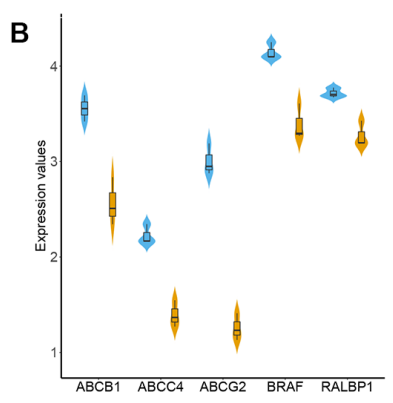
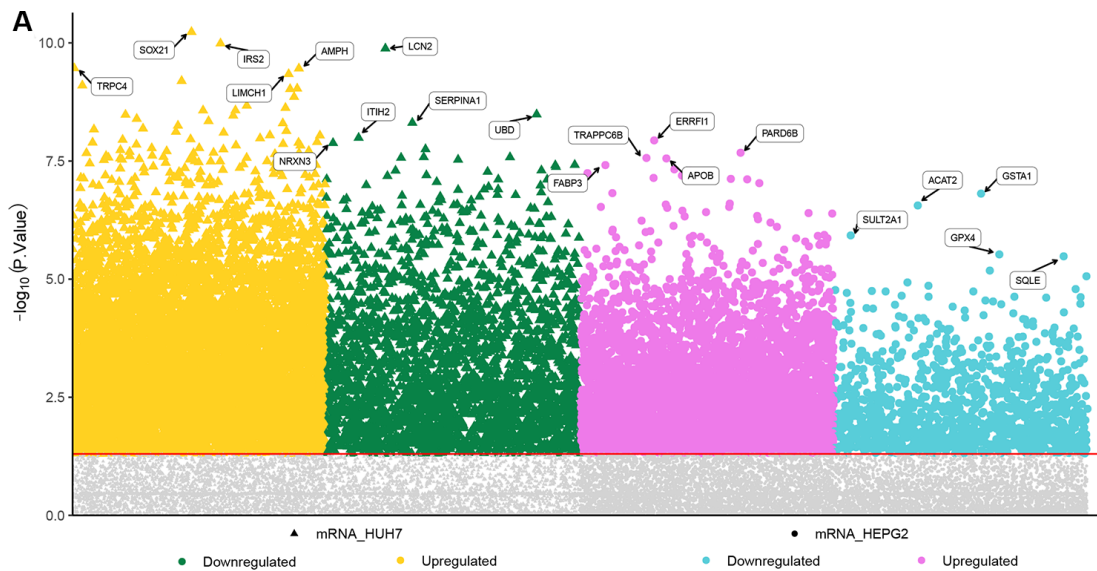


Figure 1. The workflow of the present study.

HepG2 cells: apoptosis, the MAPK signaling pathway, and the p53 signaling pathway. Pathways enriched in SR Huh7 cells were the p53 signaling pathway, pathways in cancer, and the VEGF signaling pathway (Figure 2H).

SR is associated with perturbations in multiple functional modules

Nine functional modules were identified in protein-protein interaction (PPI) networks derived from the



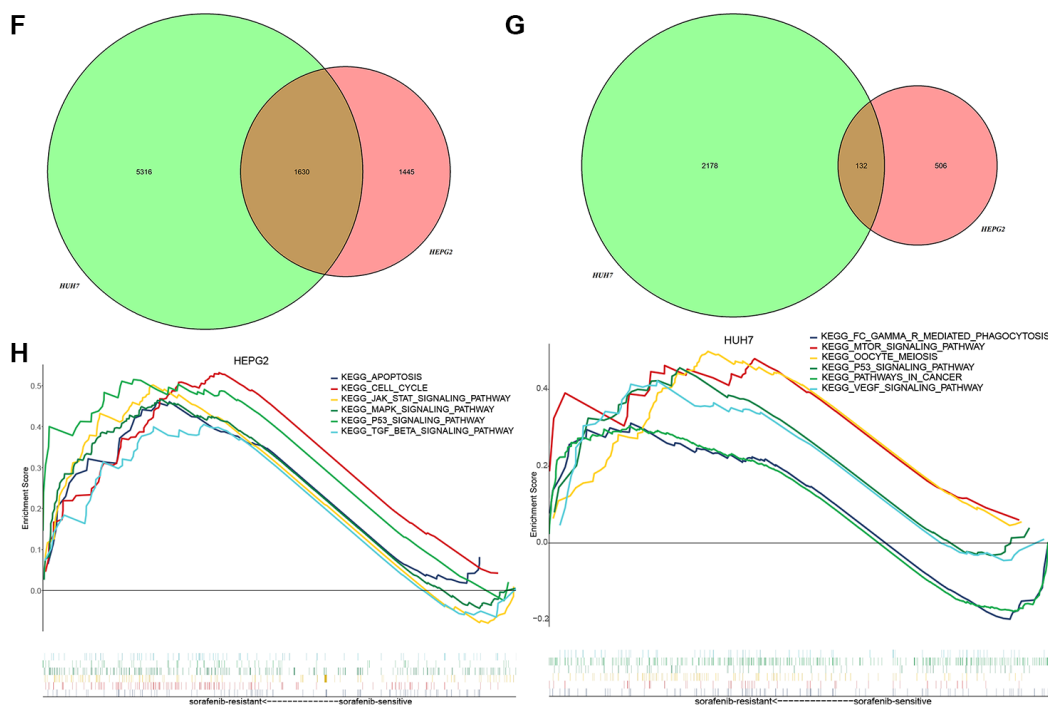


Figure 2. Identification of differentially expressed genes (DEGs) in sorafenib resistance. (A) DEGs in sorafenib resistance. (B) Expression of sorafenib-targeted genes in sorafenib-sensitive and -resistant HepG2 cells. (C) Expression of sorafenib-targeted genes in sorafenib-sensitive and -resistant Huh7 cells. (D) Cluster heatmap of DEGs between sorafenib-sensitive and -resistant HepG2 cells. (E) Cluster heatmap between sorafenib-sensitive and -resistant Huh7 cells. (F) Genes up-regulated in association with sorafenib resistance in HepG2 and Huh7 cells. (G) Genes down-regulated in association with sorafenib resistance in HepG2 and Huh7 cells. (H) Gene set enrichment analysis of genes up- or down-regulated in association with sorafenib resistance in both HCC cell lines.

common DEGs (Supplementary Figure 1, Supplementary Table 1). Nearly all module genes in these two HCC cell lines were up-regulated in SR cells (Figure 3A), suggesting that SR results from dysfunction in multiple modules. There were 2503 biological processes (BPs) (Supplementary Table 2), 560 cellular components (CCs) (Supplementary Table 3), 407 molecular functions (MFs) (Supplementary Table 4) and 57 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Supplementary Table 5). DEGs were involved in various mitosis-related BPs, such as regulation of cytoskeleton organization, microtubule-associated proteins, and the establishment of minimal bundle localization (Figure 3B). The DEGs were involved in various cancer-related pathways as well as virus-associated pathways, such as the cell cycle, Hippo signaling pathway, viral carcinogenesis, and hepatitis C (Figure 3C).

microRNAs (miRNAs) and transcription factor (TF) may promote the functional modules involved in SR

The hypergeometric test was used to predict miRNAs and TFs participating in the target functional modules. About 27 miRNAs were identified as potentially regulating functional modules involved in

SR (Figure 4A). We did not identify any long non-coding RNAs (lncRNAs) involved in SR. In the network linking TFs with target genes and KEGG pathways, the up-regulated RB1 TF was predicted to promote modules related to SR (Figure 4B). These results suggest that 27 miRNAs and one TF may help mediate SR in HCC.

Comprehensive landscape of SR in HCC

A heatmap of miRNAs identified several likely to function in SR in both HCC cell lines (Figure 5A). The comprehensive landscape of miRNAs and one TF promoting KEGG pathways (Figure 5B) identified numerous pathways that may contribute to SR in HCC (Figure 5C). These pathways included the cell cycle, hepatitis C, Hippo signaling pathway, p53 signaling pathway, protein processing in the endoplasmic reticulum, and ubiquitin-mediated proteolysis. In the p53 signaling pathway, we found that CDK1 was up-regulated, which might inhibit cell cycle arrest. We also found that in ubiquitin-mediated proteolysis, miRNAs promoted mainly different types of E3 enzymes, while the expression of UBE2G1 in the E2 enzyme was up-regulated. The E2 and E3 enzymes are involved mainly

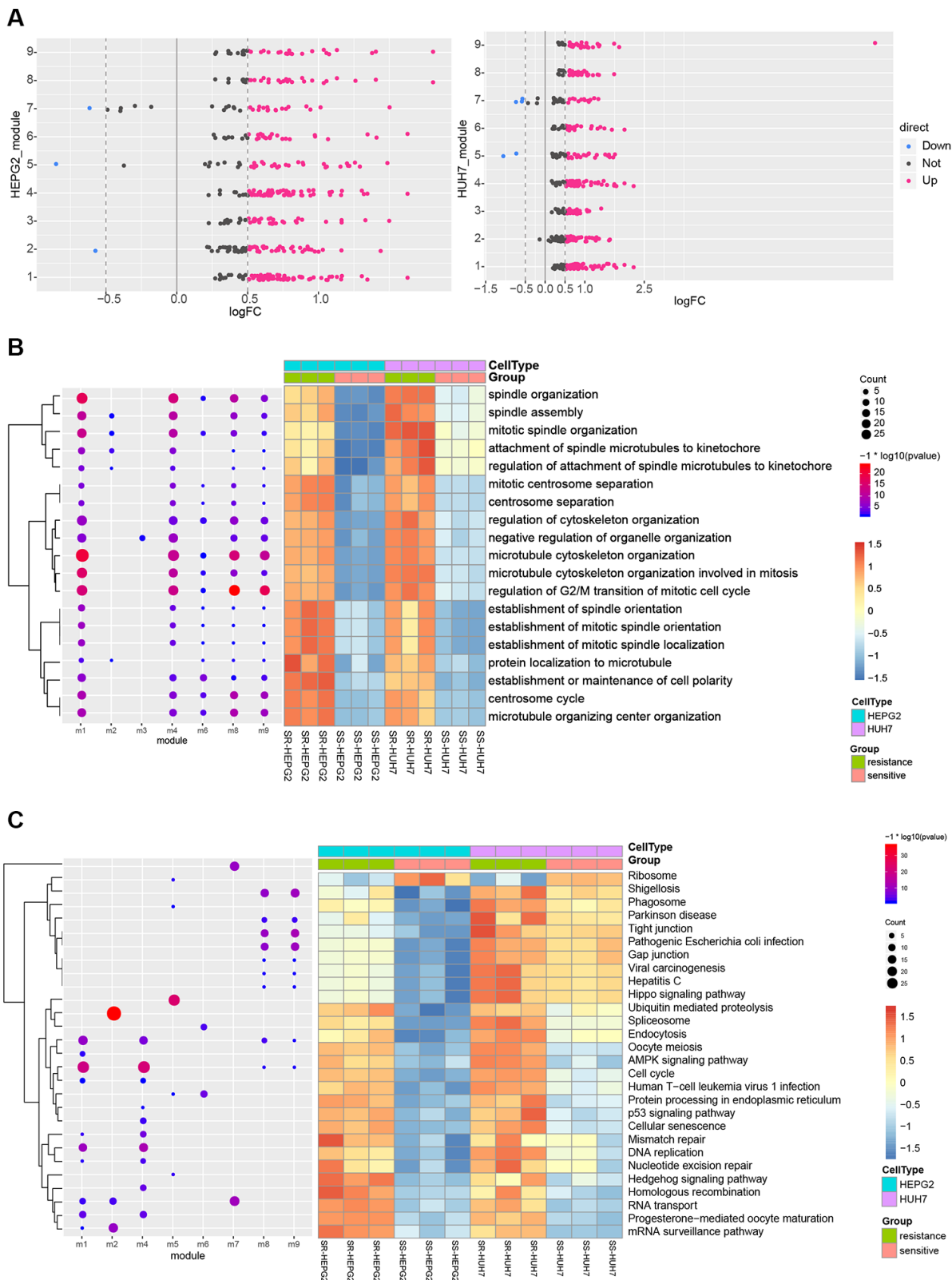


Figure 3. KEGG pathways and biological processes (BPs) predicted to be perturbed in sorafenib-resistant HCC cells. (A) Modules with similar functions in HepG2 and Huh7 cells. **(B)** Perturbed BPs in HepG2 and Huh7 cells. **(C)** Perturbed KEGG pathways in HepG2 and Huh7 cells.

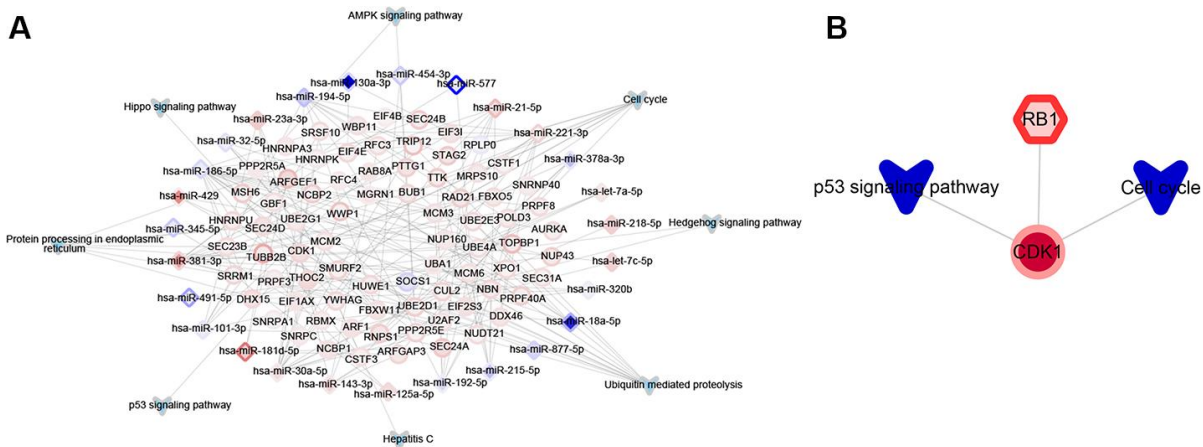


Figure 4. Significant upstream regulators of functional modules associated with sorafenib resistance in HCC cells. (A) Significant miRNA regulators. The border color of nodes reflects the log (fold change) in HepG2 cells; the inside color reflects the log (fold change) in Huh7 cells. **(B)** Significant transcription factor (TF) regulators. Nodes are colored as in panel A.

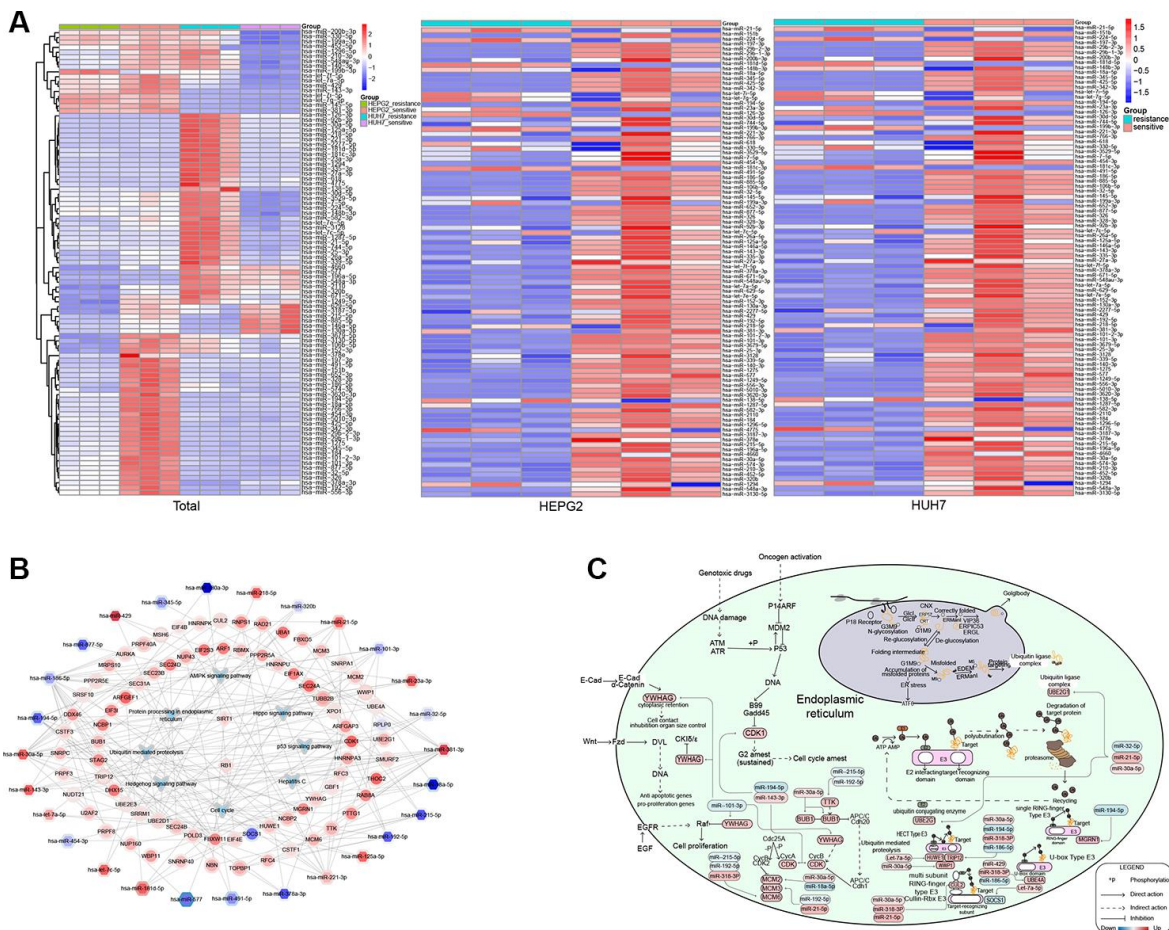


Figure 5. Potential mechanisms of resistance to sorafenib in hepatocellular carcinoma (HCC). (A) Expression of microRNAs (miRNAs) associated with resistance. **(B)** Comprehensive landscape of miRNAs and transcription factors (TFs) regulating pathways perturbed in sorafenib-resistant HCC. The border color of nodes reflects the log (fold change) in HepG2 cells; the inside color reflects the log (fold change) in Huh7 cells. **(C)** Potential mechanisms of resistance to sorafenib.

in substrate transfer. In our study, UBE2G1 was up-regulated in the ubiquitin ligase complex, which might further promote protein processing in the endoplasmic reticulum. YWHAG was also up-regulated, which might inhibit hepatitis C replication within cells, as well as promote anti-apoptotic genes, pro-proliferation genes, cell contact inhibition and organ size control by the Hippo signaling pathway. The cell cycle genes TTK, BUB1, CDK, YWHAG, MCM2, MCM3 and MCM6 were up-regulated, and nearly all were up-regulated by miRNAs, except for SOCS1 in the multi-subunit ring-finger type E3 enzyme of ubiquitin-mediated proteolysis. These miRNAs might promote and activate pathways that contribute to SR in HCC.

DISCUSSION

Sorafenib remains the only drug approved to treat late-stage HCC, yet its efficacy is limited by primary or acquired resistance. The analyses in the present study identified molecular pathways and networks, as well as their regulators, that may contribute to such resistance. This may guide efforts to reverse resistance, increasing the efficacy of sorafenib.

In the p53 signaling pathway, genetic drugs can inhibit CDK1 and promote the cell cycle. However, in HCC, miR-194-5p and miR-143-3p can mediate high expression of CDK1 and inhibit cell cycle arrest, thus promoting HCC. The increase in MCM2, MCM3, and MCM6 is related to poor performance of the tumor. Removing MCM6 can delay the S/G2 phase of hepatoma cells [9]. Simultaneously, TTK and BUB1 may promote the occurrence of HCC separately [10, 11]. We found that down-regulation of miR-192-5p was associated with SR, consistent with the ability of the miRNA to inhibit HCC [12].

Hepatitis C virus (HCV) is a major cause of HCC [13], and abnormal activation of the Hippo signaling pathway can cause HCC [14]. We found that miR-101-3p negatively regulated YWHAG in the hepatitis C pathway, and that the Hippo signaling pathway promoted HCC. Chaperones in the lumen aid endoplasmic reticulum of protein folding in that compartment. Correctly folded proteins are packaged into transport vesicles, which ferry them to the Golgi complex. In this pathway, miR-32-5p, miR-21-5p and miR-30a-5p up-regulates UBE2G1 in the ubiquitin ligase complex. Thus, abnormalities in the endoplasmic reticulum may contribute to many human diseases [15].

Studies found that SPOP can inhibit the metastasis of HCC cells through the ubiquitin-dependent proteolysis of SENP7 [16]. We identified several SR-associated

DEGs related to the E2 (ubiquitin-conjugating) enzymes and E3 (ubiquitin ligase) enzymes. These E2 and E3 DEGs are predicted to be up-regulated by miRNAs, implying that SR involves an increase in ubiquitin-mediated proteolysis. Conversely, the SOCS1 component of the E3 enzyme was down-regulated in SR cells, and lower expression of SOCS1 has been linked to deeper infiltration of HCC cells [17].

We identified several miRNAs that were associated with SR in HCC. Some studies have linked HCC to down-regulation of miR-194-5p [18] and up-regulation of miR-21-5p [19], while miR-192-5p may be clinically useful in diagnosis of the disease [20]. In contrast, miR-221-3p may be unrelated to HCC prognosis [21], whereas miR-429 may be a target in HCC treatment [22], and miR-877-5p may be involved in the pathogenesis of HCC [23]. Relatively few studies have examined miRNAs related to HCC. The present work suggests that miRNAs may promote HCC by altering gene expression, leading to SR in HCC.

Although the present study may provide new insights into SR in HCC, some limitations should be noted. First, the study was based primarily on bioinformatics analysis, so our results should be verified and extended in molecular experiments. Second, we focused on transcriptional regulatory mechanisms of SR, so further work is needed to examine additional mechanisms at the genome level.

CONCLUSIONS

We identified several functional modules and their potential miRNA and TF that may contribute to SR in HCC. Our results provide numerous leads to guide future mechanistic studies of resistance.

MATERIALS AND METHODS

Cell lines

The HCC cell lines HepG2 and Huh7 were obtained (ScienCell, San Diego, California, USA). Three SR and three SS HCC cell lines of HepG2 and Huh7 were obtained based on prior studies [24, 25] SR-HCC cell lines were established through incubation with sorafenib initially at 5 μ M, which was increased by 1 μ M per week for 1-2 months. SR-HCC cells were maintained in culture in the presence of sorafenib.

RNA isolation and characterization

Total RNA was isolated from SR- and SS-HCC cell lines using TRIzolTM (Invitrogen, USA) and the RNeasy Mini Kit (Qiagen) according to the manufacturers'

instructions. RNA concentration and purity were assessed using the RNA Nano 6000 Assay Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), the NanoDrop system (Thermo Fisher Scientific Inc. Waltham, USA) and 1% agarose gel electrophoresis.

Strand-specific RNA-seq library preparation and sequencing

Samples (1 µg) with an RNA integrity number ≥ 7 were deep-sequenced using Ribo-Zero™ rRNA removal Kit (Human/Mouse/Rat)/(Yeast)/(Bacteria) (Illumina, San Diego, USA) according to the manufacturer's protocol. Each sample was used to generate a strand-specific RNA-seq library as follows. First the ribosomal RNA in the sample of total RNA was removed, then the RNA was fragmented and reverse-transcribed using ProtoScript II Reverse Transcriptase (New England Biolabs, USA) with random primers and actinomycin D (Solarbio life sciences, USA). The second strand of cDNA was prepared using the Second Strand Synthesis Enzyme Mix with dACG-TP/dUTP (New England Biolabs, USA), then the double-stranded cDNA was purified using the AxyPrep Mag PCR Clean-up (Axygen Biosciences, Inc.), and both ends were repaired using the End Prep Enzyme Mix (NGS Fast DNA Library Prep Set for Illumina, Illumina, San Diego, USA), which added dA-tails. Subsequent T-A ligation created adaptors on both ends.

Adaptor-ligated DNA that contained inserts approximately 300 bp long and that was altogether approximately 360 bp long was isolated, and the second strand (marked with dUTP) was digested using Uracil-Specific Excision Reagent (New England Biolabs, USA). Every sample was amplified by PCR for 11 cycles using primers P5 and P7, which contained sequences that could anneal with the flow cell to perform a bridge PCR. The P7 primer also contained a six-base signature allowing for multiplexing. The PCR products were purified, and library quality was assessed. The resulting libraries were sequenced in 2×150 bp paired-end mode on a HiSeq X Ten System (Illumina, San Diego, USA).

Reading of alignments and transcript assembly

The original sequencing data were subjected to base recognition using the software Bcl2fastq 2.17.1.14 (https://support.illumina.com/content/dam/illumina_support/documents/documentation/software_documentation/bcl2fastq), yielding “pass filter data”, which were stored in FASTQ file format. Linker and low-quality sequences were deleted from the pass filter data, and the remaining data were compared to the reference genome (GRCH 37) using BWA 0.7.12.

Sequence alignment and gene expression profiling

Data were cleaned using Cutadapt 1.9.1 and short reads were compared using Hisat2 2.0.1 with default parameters, and the results were stored in SAM file format. Data were reformatted into BAM format using Samtools, and featureCount was used to profile the expression of lncRNAs, miRNAs, and messenger RNAs (mRNAs). The three profiles were normalized using the *limma* package [26] in R 3.5.3.

Differentially expressed genes (DEGs)/miRNAs/lncRNAs and KEGG pathways associated with SR in HCC

Genes whose expression differences between SR- and SS-HCC cell lines were associated with $P < 0.05$ after adjustment by the false discovery rate (FDR) were considered DEGs. Up- and down-regulated DEGs common to SR-HepG2 and SR-Huh7 lines were subjected to further analyses. Targets of sorafenib in the DrugBank database (<https://www.drugbank.ca/>) [27] were obtained. We also identified differentially expressed miRNAs and lncRNAs in SR-HepG2 and SR-Huh7 compared to SS-HepG2 and SS-Huh7 cell lines, respectively. Normalized mRNA expression profiles were subjected to gene set enrichment analysis using GSEA software [11] in JAVA. The reference gene set *c2.cp.kegg.v6.2.symbols.gmt* was taken from the Molecular Signatures Database (MSigDB) [12]. A nominal value of $P < 0.05$ was considered to be statistically significant.

Analysis of PPI networks and modules related to SR in HCC

PPI networks with a combined score > 500 based on shared DEGs were extracted from the STRING database (<https://string-db.org/>) [28]. Modules were extracted from the PPI network using the ClusterONE [29] plug-in in Cytoscape [30], with a minimum cytoscape set of 30. The *ClusterProfiler* package [31] in R was applied to explore potential BPs, CCs, MFs and KEGG pathways enriched in the modules. BPs and pathways associated with an FDR-adjusted $P < 0.05$ were considered significant.

Exploring upstream regulators of the functional modules and transcription regulatory network associated with SR in HCC

Interactions between lncRNAs or miRNAs and their target genes were obtained from the RNAInter database [32], and interactions between TFs and their target genes were obtained from the TRRUST v2 database [33]. Interactions between regulators and target

functional modules were identified using the hypergeometric test in R, and interactions showing a quantity >2 and $P < 0.05$ were considered significant. All these results were used to construct a transcription regulatory network associated with SR in HCC.

AUTHOR CONTRIBUTIONS

Zhi Zhang, Steven Mo and Cheng-Zu He performed the experiments, and acquired, analyzed, and interpreted the data; Ya-Qin Qin and Jian-Jun Liao critically revised the manuscript; Shang-Tao Huang drafted the manuscript; Hong-Mian Li and Jian-Yan Lin designed, coordinated, and supervised the study; All authors approved the final version of the article.

CONFLICTS OF INTEREST

These authors declare no conflicts of interest.

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Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 2 to 4.

Supplementary Table 1. Functional modules, identified using ClusterONE, in the protein-protein interaction network of differentially expressed genes common to HepG2 and Huh7 lines.

Cluster	Size	Members
1	84	XPO1, ZWILCH, RFC3, RFC4, PLK1, NUP160, SMC1A, RAD21, STAG2, SUN1, MAPRE1, PPP2R5A, ESCO2, WDHD1, NIPBL, WAPAL, KNTC1, INCENP, ZW10, RRM1, TTK, KPNA2, PTTG1, NUP43, RANGAP1, TPX2, DTL, PPP2R5E, STIL, SMC2, MCM3, MCM5, MCM2, MCM7, MCM6, MCM8, CDKN3, CDK1, NCAPD2, GSG2, RCC2, KIF2C, CCNA2, NCAPH, NUDC, PRC1, NSMCE2, NUF2, NDC80, MELK, KIF11, CDC45, KIF4A, CDCA8, CENPA, DLGAP5, HJURP, ECT2, CDCA7, FBXO5, C1orf112, CENPF, HMMR, CDCA2, MKI67, NEK2, NDE1, KIF20B, KIF14, FAM64A, CENPK, NEIL3, FAM83D, ERCC6L, DSN1, CKAP2, ASPM, BORA, BUB1, AURKA, ATAD2, ANLN, CKAP5, AHCTF1
2	68	SRRM1, MYBL1, U2AF2, ZC3H11A, USP39, PRPF3, SNRPA, HTATSF1, SKIV2L2, RBM7, NCBP2, NCBP1, PRPF8, NUDT21, SNRPC, SNRPA1, HNRNPDL, XAB2, SF3B3, HNRNPL, HNRNPA0, SUGP1, SRSF10, RBM10, SF3A2, RBM4, GTF2F1, CWC27, PABPN1, DDX46, DHX9, RNPS1, DHX38, DNAJC8, HNRNPA3, PRPF40A, SNRNP40, GPKOW, PCBP1, HNRNPU, WBP11, HNRNPUL1, ELAVL1, HNRNPR, RBMX, HNRNPH2, HNRNPF, HNRNPK, DHX15, ZC3H18, HNRNPH3, HNRNPAB, STOM, YTHDC1, THOC2, IGF2BP1, ZNF207, SRPK2, KHDRBS1, FYTTD1, CSTF2, CSTF3, CSTF1, ZCCHC10, SLIRP, DHX34, ASCC3, ATP6AP2
3	42	TEX10, RPL7L1, RRP12, SENP3, PPAN, PES1, RPF2, SDAD1, NMD3, PRMT3, TTC27, NOP14, NOL9, WDR3, NOC4L, NOC3L, LARP1, UTP14C, TRMT11, EXOSC10, HEATR1, NOC2L, UTP11L, RIOK3, POLR1E, RSL1D1, NOA1, TRMT6, RRP36, PUS7, BYSL, DDX50, GNL3, FTSJ1, DDX21, EBNA1BP2, GNL2, DDX47, GRWD1, GNL3L, DDX17, BMS1
4	70	ZWILCH, RFC3, SPIDR, WRN, PALB2, POLD3, RFC4, PLK1, RECQL, POT1, SMC1A, RAD21, XRCC6, NBN, WDHD1, KNTC1, RRM1, TTK, PTTG1, TOPBP1, TPX2, HAT1, DTL, USP32, STIL, SMC2, MCM3, MCM5, MCM2, MCM7, ORC2, MCM6, MCM8, CDKN3, CDK1, NCAPD2, KIF2C, CCNA2, NCAPH, PRC1, NUF2, NDC80, MELK, MSH6, KIF11, CDC45, KIF4A, CDCA8, CENPA, DLGAP5, HJURP, CLSPN, ECT2, FBXO5, CENPF, HMMR, CDCA2, MKI67, NEK2, KIF20B, KIF14, ATR, FAM64A, FAM83D, CKAP2, ASPM, BUB1, AURKA, ATAD2, ANLN
5	37	UBE2D1, WWP1, SOCS1, UBE4A, KXD1, PJA2, RLIM, RNF6, MOSPD2, TRIP12, SMURF2, FBXO36, FBXO41, UBA1, USP47, UBE2M, LMO7, MEX3C, KBTBD7, UBE2E3, KLHL5, UFL1, LTN1, SH3RF1, MGRN1, UBE2G1, HECTD1, HUWE1, KBTBD6, FBXL4, FBXW11, GPR75-ASB3, FBXL16, DTX3L, CCNF, CUL2, ARDC4
6	33	TMED2, SPTBN1, IFT80, KIF3A, KIF3B, TFG, VMA21, SEC24A, SEC31A, SEC24D, SEC24B, SEC16A, KIF21B, RINT1, DCTN1, GOLPH3, COPG1, KIF1B, ARFIP1, COPB1, COPA, ARFGEF1, GBF1, KLC2, KIF20B, ARFGAP3, COPB2, KIF13B, KIF16B, COG5, SEC23B, ARCN1, ARF1
7	30	MRPS9, MRPS10, MRPL11, RPLP0, SRP54, RPL5, RPL27, RPL7L1, RPS12, RPLP1, MRPL3, SSR3, TRAM1, EIF5A2, EIF2A, EIF5B, EIF4G1, EIF2S3, EIF4E, EIF3G, EIF3E, EIF3I, EIF3D, SECISBP2, EIF4H, EIF4B, METAP1, GSPT1, DENR, EIF1AX
8	33	TACC1, SEPT2, MAPRE2, MAPRE1, RAB8A, YWHAE, TPX2, YWHAG, IQCB1, THUMPD1, CEP104, CEP97, SASS6, TUBB4B, TUBB, TUBB2A, TUBB2B, DYNC112, TUBA4A, TUBA1C, TDRD7, CCP110, HMMR, NEK2, NDE1, BORA, AURKA, CNTRL, CKAP5, AKAP9, TMEM67, ARL13B, AHI1
9	33	SEPT2, MAPRE2, MACF1, MAPRE1, RAB8A, YWHAE, TUBB3, YWHAG, IQCB1, CEP104, CEP97, SASS6, TUBB4B, TUBB, TUBB2A, TUBB2B, INVS, DYNC112, TUBA4A, TUBA1C, CCP110, NEK2, NDE1, EEF1A2, BORA, CNTRL, CKAP5, AKAP9, TMEM67, CCDC68, WDPCP, ARL13B, AHI1

Supplementary Table 2. Biological processes enriched in sorafenib-resistant cells.

Supplementary Table 3. Cellular components enriched in sorafenib-resistant cells.

Supplementary Table 4. Molecular functions enriched in sorafenib-resistant cells.

Supplementary Table 5. The Kyoto Encyclopedia of Genes and Genomes pathways enriched in sorafenib-resistant samples.

module	ID	Description	p.adjust	geneID	Count
module1	hsa04152	AMPK signaling pathway	0.041950813	PPP2R5A/PPP2R5E/CCNA2	3
module1	hsa04110	Cell cycle	7.73E-19	STAG2/MCM2/MCM3/MCM5/MCM6/MCM7/PLK1/RAD21/BUB1/TTK/SMC1A/CDC45/CCNA2/PTTG1/CDK1	15
module1	hsa03030	DNA replication	5.10E-10	MCM2/MCM3/MCM5/MCM6/MCM7/RFC3/RFC4	7
module1	hsa05166	Human T-cell leukemia virus 1 infection	0.141651764	XPO1/CCNA2/PTTG1	3
module1	hsa03430	Mismatch repair mRNA	0.016348116	RFC3/RFC4	2
module1	hsa03015	surveillance pathway	0.141651764	PPP2R5A/PPP2R5E	2
module1	hsa03420	Nucleotide excision repair	0.049602581	RFC3/RFC4	2
module1	hsa04114	Oocyte meiosis	4.74E-09	FBXO5/PLK1/PPP2R5A/PPP2R5E/AURKA/BUB1/SMC1A/PTTG1/CDK1	9
module1	hsa04914	Progesterone-mediated oocyte maturation	0.000206509	PLK1/AURKA/BUB1/CCNA2/CDK1	5
module1	hsa03013	RNA transport mRNA	0.016348116	NUP160/NUP43/RANGAP1/XPO1	4
module2	hsa03015	surveillance pathway	1.30E-09	SRRM1/RNPS1/NUDT21/CSTF1/CSTF2/CSTF3/NCBP2/NCBP1/PABPN1	9
module2	hsa03013	RNA transport	0.003546577	SRRM1/RNPS1/NCBP2/NCBP1/THOC2	5
module2	hsa03040	Spliceosome	1.00E-35	PRPF8/USP39/SRSF10/U2AF2/DHX15/HNRNPA3/NCBP2/SF3B3/RBMX/HNRNPK/HNRNPU/NCBP1/PCBP1/WBP11/PRPF40A/XAB2/THOC2/SNRPA/SNRPA1/SNRPC/SF3A2/PRPF3/SNRNP40/DHX38/DDX46	25
module3	hsa03008	Ribosome biogenesis in eukaryotes	1.35E-13	WDR3/GNL3/GNL2/NMD3/GNL3L/HEATR1/UTP14C/BMS1	8
module3	hsa03020	RNA polymerase	0.077470859	POLR1E	1
module4	hsa04110	Cell cycle	8.91E-21	MCM2/MCM3/MCM5/MCM6/MCM7/ORC2/PLK1/ATR/RAD21/BUB1/TTK/SMC1A/CDC45/CCNA2/PTTG1/CDK1	16
module4	hsa04218	Cellular senescence	0.009542975	NBN/ATR/CCNA2/CDK1	4
module4	hsa03030	DNA replication	4.86E-12	POLD3/MCM2/MCM3/MCM5/MCM6/MCM7/RFC3/RFC4	8
module4	hsa03460	Fanconi anemia pathway	0.053740596	ATR/PALB2	2
module4	hsa03440	Homologous recombination	7.96E-05	POLD3/TOPBP1/NBN/PALB2	4
module4	hsa05166	Human T-cell leukemia virus 1 infection	0.113368193	ATR/CCNA2/PTTG1	3
module4	hsa03430	Mismatch repair	9.11E-06	POLD3/MSH6/RFC3/RFC4	4
module4	hsa03450	Non-homologous end-joining	0.113368193	XRCC6	1
module4	hsa03420	Nucleotide excision repair	0.002713314	POLD3/RFC3/RFC4	3
module4	hsa04114	Oocyte meiosis	2.87E-06	FBXO5/PLK1/AURKA/BUB1/SMC1A/PTTG1/CDK1	7
module4	hsa04115	p53 signaling pathway	0.082941236	ATR/CDK1	2
module4	hsa04914	Progesterone-mediated oocyte maturation	0.00012879	PLK1/AURKA/BUB1/CCNA2/CDK1	5
module5	hsa04340	Hedgehog signaling pathway	0.047695487	FBXW11/SMURF2	2
module5	hsa05012	Parkinson disease	0.197409651	UBA1/UBE2G1	2

module5	hsa04141	Protein processing in endoplasmic reticulum	0.207742282	UBE2D1/UBE2G1	2
module5	hsa05131	Shigellosis	0.065360875	FBXW11/UBE2D1	2
module5	hsa04120	Ubiquitin mediated proteolysis	3.36E-22	HUWE1/UBE2E3/WWP1/FBXW11/MGRN1/SMURF2/UBA1/UBE2D1/UBE2G1/CUL2/SOCS1/UBE2M/TRIP12/UBE4A	14
module6	hsa04144	Endocytosis	0.002587885	ARFGEF1/ARFGAP3/ARF1/GBF1	4
module6	hsa04141	Protein processing in endoplasmic reticulum	4.29E-05	SEC24B/SEC23B/SEC24A/SEC31A/SEC24D	5
module7	hsa03010	Ribosome	1.43E-09	MRPL3/MRPS10/RPL5/RPL27/RPLP0/RPLP1/RPS12/MRPS9/MRPL11	9
module7	hsa03013	RNA transport	1.59E-10	EIF1AX/EIF2S3/EIF4B/EIF4E/EIF4G1/EIF3E/EIF3D/EIF3G/EIF3I/EIF5B	10
module8	hsa04210	Apoptosis	0.047212144	TUBA4A/TUBA1C	2
module8	hsa04110	Cell cycle	0.046453039	YWHAE/YWHAG	2
module8	hsa04540	Gap junction	9.14E-09	TUBB4B/TUBB/TUBB2B/TUBA4A/TUBB2A/TUBA1C	6
module8	hsa05160	Hepatitis C	0.047212144	YWHAE/YWHAG	2
module8	hsa04390	Hippo signaling pathway	0.047212144	YWHAE/YWHAG	2
module8	hsa04114	Oocyte meiosis	0.003949079	AURKA/YWHAE/YWHAG	3
module8	hsa05130	Pathogenic Escherichia coli infection	1.50E-09	TUBB4B/TUBB/TUBB2B/TUBA4A/TUBB2A/TUBA1C	6
module8	hsa04145	Phagosome	6.08E-09	TUBB4B/DYNC1I2/TUBB/TUBB2B/TUBA4A/TUBB2A/TUBA1C	7
module8	hsa04530	Tight junction	0.007179351	RAB8A/TUBA4A/TUBA1C	3
module8	hsa05203	Viral carcinogenesis	0.068829321	YWHAE/YWHAG	2
module9	hsa04210	Apoptosis	0.057983071	TUBA4A/TUBA1C	2
module9	hsa04110	Cell cycle	0.057983071	YWHAE/YWHAG	2
module9	hsa04540	Gap junction	1.86E-10	TUBB3/TUBB4B/TUBB/TUBB2B/TUBA4A/TUBB2A/TUBA1C	7
module9	hsa05160	Hepatitis C	0.057983071	YWHAE/YWHAG	2
module9	hsa04390	Hippo signaling pathway	0.057983071	YWHAE/YWHAG	2
module9	hsa04114	Oocyte meiosis	0.057983071	YWHAE/YWHAG	2
module9	hsa05130	Pathogenic Escherichia coli infection	1.83E-11	TUBB3/TUBB4B/TUBB/TUBB2B/TUBA4A/TUBB2A/TUBA1C	7
module9	hsa04145	Phagosome	1.86E-10	TUBB3/TUBB4B/DYNC1I2/TUBB/TUBB2B/TUBA4A/TUBB2A/TUBA1C	8
module9	hsa04530	Tight junction	0.012086629	RAB8A/TUBA4A/TUBA1C	3
module9	hsa05203	Viral carcinogenesis	0.084210805	YWHAE/YWHAG	2