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Identifying hub genes and dysregulated pathways in hepatocellular carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to identify the hub genes and dysregulated pathways of hepatocellular carcinoma (HCC) and explore the molecular mechanism of the biological process associated with HCC.

MATERIALS AND METHODS: Microarray data were got from NCBI Gene Expression Omnibus (GEO) database. The most significant top 100 up-regulated gene signatures and top 100 down-regulated gene signatures were identified by integrated analysis of the multiple microarray datasets using a novel model genome-wide relative significance (GWRS) and genome-wide global significance (GWGS). Gene Ontology (GO) enrichment analysis and pathway analysis of those genes were performed based on Gene Ontology website and Kyoto Encyclopedia of Genes and Genomes (KEGG). Protein-protein interaction (PPI) network was constructed using Cytoscape 2.1. In addition, we analysed the significantly dysregulated signaling pathways across the PPI network and KEGG pathway analysis.

RESULTS: We screened 2920 up-regulated and 2231 down-regulated gene signatures across multiple studies by GWRS and GWGS. The top 100 up-regulated and top 100 down-regulated gene signatures were selected for further research. GO enrichment analysis showed that these genes significantly enriched in terms of mitosis (p = 5.83×10-20), nuclear division (p = 5.83×10-20) and M phase of mitotic cell cycle (p = 9.39×10-20). The most significant terms of KEGG pathway included cell cycle (p = 1.33×10-8), oocyte meiosis (p = 1.41×10-4), drug metabolism (p = 2.15×10-4) and p53 signaling pathway (p = 3.57×10-4). PPI network suggested that BIRC5, CDC20, CCNB1, BUB1B, MAD2L1 and CDK1 were important significant genes which were considered as hub genes. Across the PPI and pathway, cell cycle, oocyte meiosis and p53 signaling pathway were the significantly dysregulated pathways.

CONCLUSIONS: Our study displayed robust gene signatures in HCC. It showed that the dysregulations of cell cycle, oocyte meiosis, p53 signaling pathway and progesterone-mediated oocyte maturation pathway were closely associated to the development and progression of HCC. Besides, genes BIRC5, CDC20, CCNB1, BUB1B, MAD2L1 and CDK1 as the hub genes might play important roles for diagnosing and therapy of HCC.

Key Words:
Hepatocellular carcinoma, Hub genes, Signaling pathway, Bioprocess.

Introduction

Hepatocellular carcinoma (HCC) is a cancerous growth arising from the liver. It is the eighth most common malignancy in women and the fifth most common malignancy in men worldwide[1], and the third most frequent oncological cause of cancer death[2]. The incidence of HCC in developed countries is increased recently years[3], and becomes a pressing sociomedical problem. It had a poor prognosis because of the high intrahepatic recurrence rate[4] and the frequently associated cirrhosis[5,6]. With the progress in diagnostic procedures in recent years, many new approaches, especially molecular and bioinformatic method, have come to be detected and considered for the therapy of HCC.

HCC is a highly complex and heterogeneous tumor with several genomic alterations[7] and a multifaceted molecular pathogenesis[8]. It is a slow process during which genomic changes progressively alter the hepatocellular phenotype to produce cellular intermediates that evolve into HCC[9,10]. Research on the regulation of gene expression would help us understand the pathogenesis of HCC. Microarray analysis has become a widely used tool for the generation of gene ex-

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pression data on a genomic scale, and generated large amounts of data. Gene Expression Omnibus (GEO) and Array Express Archive are two public database of microarray gene expression data, which are the generic gene expression database designed to hold data from all microarray platforms[11,12]. Recently, there were several studies researched on gene expression of HCC by microarray[13,14]. Also, pathway analysis has become a powerful tool for gaining insight into the underlying biology of differentially expressed genes and proteins. It was reported that the main pathogenic mechanism of HCC linked with alterations in several important cellular signaling pathways[8]. An integrated systematic assessment of the pathways in which these genes interconnect may lead to a more precise set of alterations that may serve as key biomarkers or drug targets for clinical interrogation[15]. Protein-protein interaction (PPI) network has a small number of highly connected protein nodes which has known as hub genes (proteins) that play a key role in maintaining the network structure. Using PPI network, nine novel hub genes associated with liver metastasis of gastric cancer were identified[16]. The utilization of these bioinformatics methods provided the secondary use of existing public microarray data to predict the molecular pathogenesis and biomarkers for cancer, including HCC[17,18].

Despite the rich transcriptome data, unveiling disease mechanism has remained a major challenge. The overlap is very low for the most significantly dys-regulated genes across multiple studies. Inconsistent results have been presented due to multiple sources of problems, including small sample size, measurement error, and different statistical methods[19].

The systems biology approach views the biological system as a whole in order to study the effects of disease and global interactions with the environment, which facilitates understanding of biological processes and disease[20]. To better understand the complex pathology associated with HCC and identify molecular networks involved in the disease, we took a systems biology approach to investigated gene signatures between patients with hepatic carcinoma and healthy people, included meta-analysis using a new model measure the genome-wide relative significance (GWRS) and genome-wide global significance (GWGS), Gene Ontology (GO) enrichment analysis, pathway analysis and hub genes identified by PPI network. This analysis revealed the up- and down-regulated gene signatures, associated bioprocess, signaling pathway and interaction between genes (proteins), which provided informations for understanding the underlying mechanisms of HCC.

Materials and Methods

Data collection

The gene expression datasets of normal people and HCC patients (GEO access number: GSE6222[21], GSE41804[14], GSE51401[22]) were got from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/). A total of 117 samples (54 cases and 63 controls) were collected. These data were collected from one type of platform: Affymetrix Human Genome U133 Plus 2.0 Array. The following information was also extracted from each identified study: GEO accession number, sample type, number of cases and controls, and gene expression data.

The Integrated Analysis of Gene Signatures of Multiple Microarray Data

A novel model, which measured the GWRS and GWGS of gene expression, was used for identified gene signatures in multiple microarray data[23]. The degree of differential expression of genes in each single microarray database was measured by GWRS. The GWGS of a gene was measured based on its corresponding GWRS across multiple microarray datasets.

The detailed method had been described in previous study[23]. The number of datasets was denoted by n, the number of unique genes across n datasets was denoted by m. The GWRS of the i-th gene in the j-th dataset was measured by:

$s_{ij} = -2\log(\frac{r_{ij}}{m})$, where r_{ij}, i = 1–m, j = 1–n, was the rank number of the i-th gene in the j-th study. The GWGS of the gene was measured by: = sri = $\sum_{j=1}^{n} \omega_j s_{ij}$, where w_j represented the relative weight of the j-th dataset. When a probe-set was mapped to multiple genes, allgenes were given the expression of the probe-set. We used the maxim-based method to deal with the situations which multiple probe-sets associated to a gene. The gene was removed if it was absent for one dataset. The degree of differential expression of genes were measured by fold-change. We assigned a rank number for each gene according to their degree of differential expression. The top 100 up-regulated genes and top 100 down-regulated genes were selected for further analysis.

Functional and Pathway Enrichment Analysis

To investigate the functions of these gene signatures, we performed GO enrichment analysis based on Gene Ontology database (http://www.geneontology.org/). To further assess the signaling pathway of the gene signatures, we performed a pathway analysis based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Our top genes were applied to this database in order to investigate the biochemistry pathways that might be involved in the occurrence and development of HCC. The two analyses were performed used DAVID[24] (http://david.abcc.ncifcrf.gov/tools.jsp). The significant categories were identified by EASE score. The threshold of EASE score < 0.01 and the minimum number of genes for the corresponding term > 2 were considered significant for a category.

PPI Network Construction

Proteins seldom accomplish their functions independently, it is important to know the interactions of these proteins by researching larger functional groups of proteins[25]. The PPI network provide a valuable framework for better understanding of the functional organization of the proteome. A PPI network can be modeled as an undirected graph, where vertices represent proteins and edges represent interactions between proteins[26]. In this network, proteins with very high degree (highly connected) interact with several other proteins, suggesting a central regulatory role. They are likely to be regulatory "hubs"[27]. To research the interaction among those genes and reveal the hub nodes in the regulation network, we constructed the PPI network. The PPI data were downloaded from STRING database. Then the gene signatures were imported into the interaction network, and the interactions were screened with both end nodes having gene signatures. The PPI networks were constructed by using Cytoscape 2.1 software. The nodes that degree ≥ 1 were reserved in the PPI network. Genes with degree > 31.56 were considered as hub genes (proteins).

Results

The Integrated Analysis of Gene Signatures in Multiple Studies

The GWRS and GWGS model used in our study was considered as a new more robust model for meta-analysis[23]. First, when GWRS was calculated, we measured the degree of differential expression by fold-change based algorithm, which was proved more suitable than other statistical test such as *t*-test (*p*-value) and SAM. A gene with large fold-changes was ranked highly. The GWGS of a gene was measured by s^r. A gene with a large s^r value was considered to be significant across multiple individual studies. By using the intersection of the microarray datasets, a total of 5151 gene signatures were screened including 2920 up-regulated and 2231 down-regulated genes, and the top 100 up-regulated gene signatures and top 100 down-regulated gene signatures, listed in Tables I and II respectively, were selected from the three microarray datasets for further research.

Functional and Pathway Enrichment Analysis

By GO analysis, we identified 99 significant enrichments of these top gene signatures, which were classified in 3 GO categories, including biological processes (BP, 64), molecular functions (MF, 15) and cellular components (CC, 20). In BP, those genes significantly participated in nuclear division ($p = 5.83\times10^{-20}$), mitosis ($p = 5.83\times10^{-20}$) and M phase of mitotic cell cycle ($p = 9.39\times10^{-20}$). The most significant terms of MF and CC were carbohydrate binding ($p = 4.52\times10^{-5}$) and spindle ($p = 1.72\times10^{-12}$) respectively. The top 10 GO terms of BP, MF and CC based count were shown in Figure 1.

Pathway analysis based on KEGG database showed that these genes significantly enriched in 8 terms (Table III). The most significant terms were cell cycle ($p = 1.33\times10^{-8}$), oocyte meiosis ($p = 1.41\times10^{-4}$),drug metabolism ($p = 2.15\times10^{-4}$) and p53 signaling pathway ($p = 3.57\times10^{-4}$). Among the 8 terms, the cell cycle pathway was the most significant term, which also enriched more genes than other terms.

Structure Interaction Network of the Gene Signatures

Using Cytoscape 2.1, the interaction network with 130 nodes and 2049 edges was finally identified (Figure 2). A gene whose degree larger than the threshold value (degree = 31.56) was considered as a hub gene. In this work, forty-three genes in PPI network were selected as the hub genes (Table IV), which might play important roles in the biological processes of HCC. The gene BIRC5 showed the highest degree (degree = 128) in the network, followed by

Table I. The top 100 up-regulated genes identified from the microarray datasets. The number was sorted by the average of rank.

NO.	Genes	NO.	Genes	NO.	Genes	NO.	Genes
1	SPINK1	26	KIF4A	51	PRR11	76	PSPH
2	TOP2A	27	PBK	52	CCDC34	77	CENPU
3	ASPM	28	GINS1	53	SSX1	78	UBE2C
4	GPC3	29	CENPW	54	RACGAP1	79	ACSL4
5	ANLN	30	CLGN	55	KIF18B	80	RGS5
6	SULT1C2	31	CAP2	56	MMP12	81	CDKN2C
7	CCNB1	32	PRC1	57	ST8SIA6-AS1	82	REG3A
8	PEG10	33	CD109	58	CCNE2	83	MCM2
9	CDKN3	34	NUF2	59	UBE2T	84	RBM24
10	ECT2	35	NQO1	60	KIF20A	85	MDK
11	CENPF	36	KIAA0101	61	CASC5	86	IQGAP3
12	BIRC5	37	TPX2	62	DTL	87	LOC344887
13	HMMR	38	ENAH	63	CCNA2	88	ZWINT
14	RRM2	39	COL15A1	64	XK	89	RAD51AP1
15	AKR1B10	40	NDC80	65	COCH	90	CHML
16	NEK2	41	MELK	66	NCAPG	91	CDCA5
17	CDK1	42	SFN	67	GJC1	92	TTK
18	IGF2BP3	43	CDC20	68	AFP	93	SORT1
19	AURKA	44	BUB1B	69	CEP55	94	BCAT1
20	CRNDE	45	ROBO1	70	TRIM16	95	IGSF3
21	NUSAP1	46	CTHRC1	71	MAGEA3	96	BUB1
22	FLVCR1	47	MAD2L1	72	MKI67	97	PRIM1
23	CCNB2	48	PLCB1	73	SERPINI1	98	RRAGD
24	ZIC2	49	FAM83D	74	CDCA3	99	P4HA2
25	PTTG1	50	DLGAP5	75	MAGEA6	100	MZT1

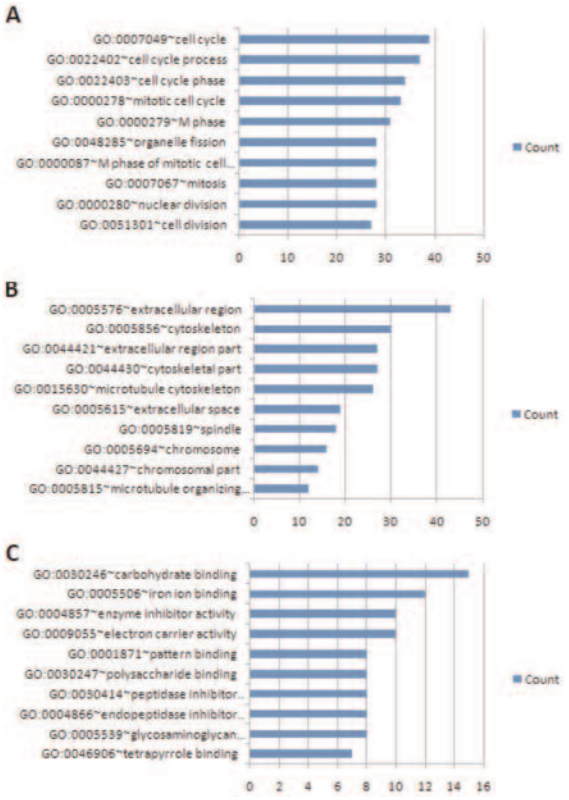

Figure 1. Summary of the top 10 GO terms of biological processes ***(A)***, cellular components ***(B)*** and molecular functions ***(C)***. The count represents the number of genes that enriched in the term.

CDC20 (degree = 123), CCNB1 (degree = 122), BUB1B (degree = 117), MAD2L1 (degree = 115) and CDK1 (degree = 114). A gene whose degree larger than the threshold value (degree = 31.56) is considered as a hub gene.

Across Analysis of PPI and Pathway Analysis

In this study, KEGG pathway analysis had identified 8 significant terms, including cell cycle, oocyte meiosis, drug metabolism, p53 signaling pathway, linoleic acid metabolism, retinol metabolism, metabolism of xenobiotics by cytochrome P450 and progesterone-mediated oocyte maturation. To further discuss whether genes in corresponding pathway can be mapped on the hubs PPI network, we analysed the genes extracted in each pathway respectively. Twelve of all 14 genes were mapped on the hubs PPI network in cell cycle pathway, and the ratios were 9/9, 5/7, 6/6 in oocyte meiosis, p53 signaling pathway and progesterone-mediated oocyte maturation pathway respectively (Figure 3). There were no genes mapped on the hubs network in drug metabolism, linoleic acid metabolism, retinol metabolism and metabolism of xenobiotics by cytochrome P450 pathway (Figure 4). Thus, across the PPI and pathway

Table II. The top 100 down-regulated genes identified from the microarray datasets. The number was sorted by the average of rank.

NO.	Genes	NO.	Genes	NO.	Genes	NO.	Genes
1	FCN2	26	ADH4	51	THBS1	76	FOSB
2	FCN3	27	TIMD4	52	IGJ	77	SKAP1
3	CLEC1B	28	IL13RA2	53	CLDN10	78	RELN
4	CRHBP	29	TMEM27	54	GLS2	79	ITGA9
5	MARCO	30	CD5L	55	HHIP	80	CRISPLD2
6	OIT3	31	ESR1	56	ITLN1	81	MS4A6A
7	CLEC4M	32	THRSP	57	MOGAT2	82	C11orf96
8	LINC01093	33	HAO2	58	CFTR	83	BMPER
9	CLEC4G	34	MAN1C1	59	PTPRB	84	TSPAN7
10	C9	35	DNASE1L3	60	ECM1	85	SDPR
11	CXCL14	36	STEAP4	61	NPY1R	86	FOS
12	TACSTD2	37	LINC00844	62	CHST4	87	CYP8B1
13	C7	38	SLC22A1	63	LCAT	88	HBB
14	LIFR	39	SLC25A47	64	TFPI2	89	CNDP1
15	CYP1A2	40	LY6E	65	F9	90	CYP2A6
16	PLAC8	41	AKR1D1	66	CYP3A4	91	SERPINB9
17	CETP	42	GPR182	67	HPD	92	MRO
18	GPM6A	43	VIPR1	68	RSPO3	93	IDO2
19	CYP2E1	44	IL33	69	ADH1B	94	TUBE1
20	LYVE1	45	CD1D	70	RNF125	95	TSPYL5
21	SRPX	46	SPP2	71	CYP2C8	96	ACSM3
22	DCN	47	SOCS2	72	INMT	97	KBTBD11
23	CFP	48	SLC4A4	73	GLYAT	98	NRG1
24	CXCL12	49	HAMP	74	CPEB3	99	EXPH5
25	STAB2	50	FEZ1	75	FAM65C	100	S100A12

analysis, the hub genes were remarkable enriched in pathways such as cell cycle, oocyte meiosis, p53 signaling pathway and progesterone-mediated oocyte maturation pathway.

Discussion

Identifying biomarkers in complex diseases such as HCC will contribute us to understanding the pathogenesis and diagnosing disease. In this work, we presented the gene signatures by a new model; then, analyzed them by functional enrichment analysis, pathway enrichment analysis and PPI network. We found several significant pathways (cell cycle, oocyte meiosis, p53 signaling pathway and progesterone-mediated oocyte maturation) and 43 hub genes by PPI network and those pathways and genes may be useful diagnostic approaches for HCC. Across the pathway analysis and PPI network, we found that these significant dysregulated pathway involved

Table III. The pathway analysis based on KEGG showed genes significantly enriched in 8 terms.

Term	*p* value	Count	Genes
Cell cycle	1.33E-08	14	CDK1, TTK, CDC20, PTTG1, SFN, MCM2, CCNB1, CCNE2, MAD2L1, CCNB2, CDKN2C, BUB1, BUB1B, CCNA2
Oocyte meiosis	1.41E-04	9	CCNE2, CCNB1, CDK1, CCNB2, MAD2L1, BUB1, CDC20, AURKA, PTTG1
Drug metabolism	2.15E-04	7	CYP3A4, ADH4, CYP2C8, ADH1B, CYP2A6, CYP2E1, CYP1A2
p53 signaling pathway	3.57E-04	7	CCNE2, CCNB1, CDK1, CCNB2, RRM2, SFN, THBS1
Linoleic acid metabolism	5.85E-04	5	CYP3A4, AKR1B10, CYP2C8, CYP2E1, CYP1A2
Retinol metabolism	9.12E-04	6	CYP3A4, ADH4, CYP2C8, ADH1B, CYP2A6, CYP1A2
Metabolism of xenobiotics by cytochrome P450	0.001476	6	CYP3A4, ADH4, CYP2C8, ADH1B, CYP2E1, CYP1A2
Progesterone-mediated oocyte maturation	0.007085	6	CCNB1, CDK1, CCNB2, MAD2L1, BUB1, CCNA2

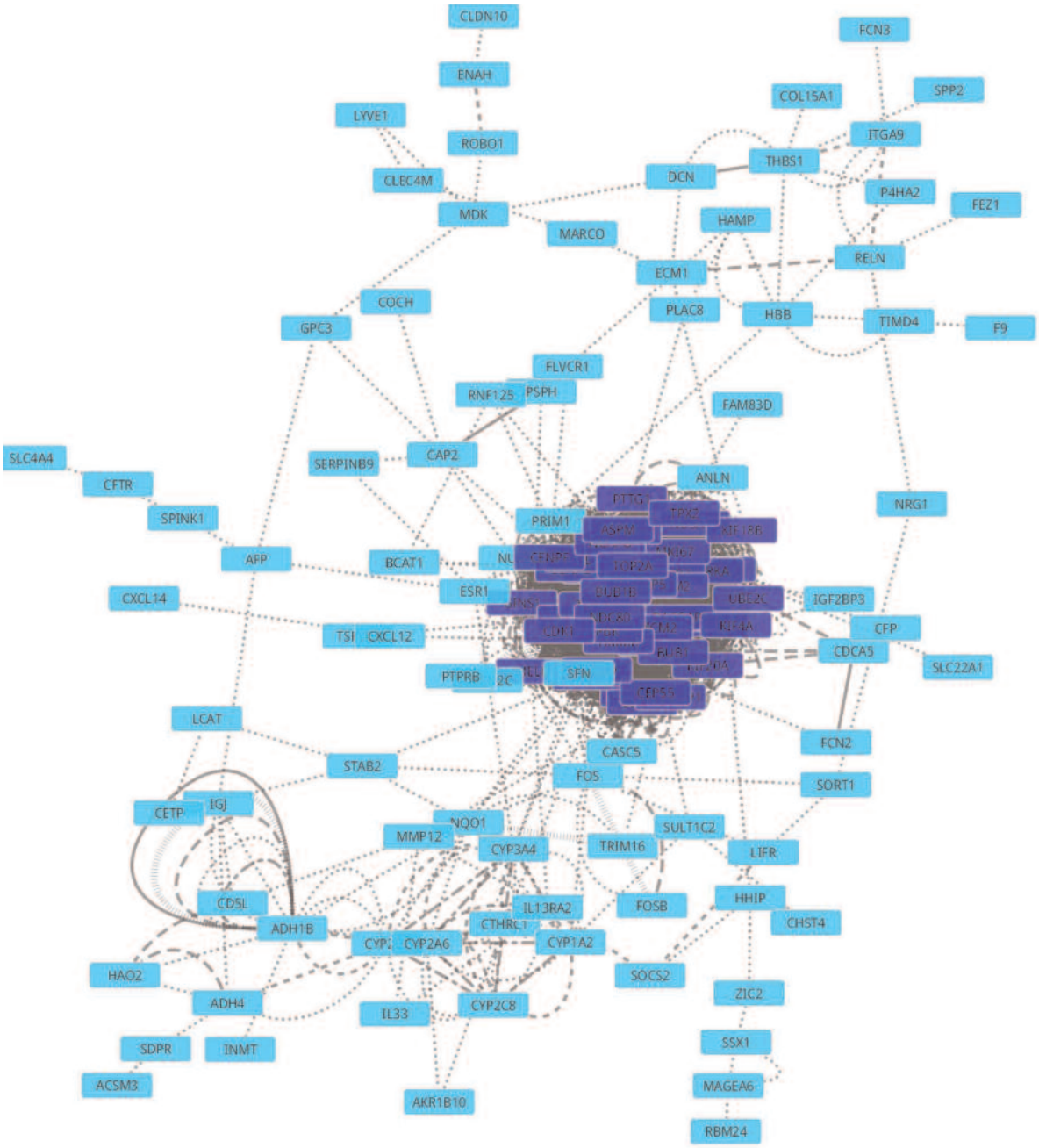

Figure 2. The PPI network of the top 100 up- and down-regulated genes. The node stands for the protein (gene), edge stands for the interaction of proteins (genes). The network includes 130 nodes and 2049 edges. A gene whose degree larger than the threshold value (degree = 31.56) is considered as a hub gene. Forty-three nodes with degree greater than the threshold value are filled with dark blue.

hallmark cancer genes, including CCNE2, CCNB1, CCNB2 and CDK1 which were identified in these significant pathways.

The pathways included cell cycle, oocyte meiosis, p53 signaling pathway and progesterone-mediated oocyte maturation, which can be mapped on the hubs network, were enriched mainly by up-regulated gene signatures, except for THBS1 in p53 signaling pathway which was down-regulated in HCC. P53 as the tumor suppressor factor had been shown to initiate DNA repair, cell-cycle arrest and, importantly, apoptosis, and to respond to many types of cancer therapy[28-31]. The dysregulation of p53 function occurred frequently

Table IV. The forty-three hub genes that degree greater than the threshold value (degree = 31.56) in PPI network.

Genes	Degree	Genes	Degree
BIRC5	128	CENPF	83
CDC20	123	RACGAP1	83
CCNB1	122	DLGAP5	82
BUB1B	117	AURKA	81
MAD2L1	115	ASPM	80
CDK1	114	MKI67	80
CCNB2	111	RAD51AP1	78
CCNA2	111	CEP55	77
PRC1	110	CDKN3	76
BUB1	108	NCAPG	73
NDC80	106	UBE2C	71
TOP2A	106	ECT2	69
KIF20A	97	ZWINT	67
HMMR	96	DTL	66
RRM2	90	CDCA3	66
TPX2	90	TTK	63
KIF4A	90	GINS1	60
MCM2	89	MELK	60
PBK	86	PTTG1	59
NEK2	85	KIF18B	42
NUSAP1	84	CCNE2	41
KIAA0101	83		

in human malignancies[32]. Edamoto et al[33] indicated that the p53 signaling pathway was dysregulated in one-third of HCC. The roles of these up-regulated genes such as CCNE2, CCNB1, CCNB2 and CDK1 are encoding the proteins related to cell cycle, their over-expression can lead to uncontrolled cell growth. In general levels, p53 and these genes associated cell cycle were negatively correlated[34], thus the up-regulation of these genes made p53, the tumor suppressor gene, inactivity. THBS1, as an anti-angiogenic and p53-regulated gene, is known to repress tumor progression[35,36]. Miao et al[37] showed that THBS1 had an antitumor activity as it can prevent the implantation of the melanoma cell line in C57BL/6 mice. Mouillesseaux et al[38] reported that the disruption of ribosomal biogenesis induced a THBS1-mediated anti-angiogenic pathway. Unsurprisingly, THBS1 was down-regulated in our work, which was consistent with these previous study.

The pathways included drug metabolism, linoleic acid metabolism, retinol metabolism and

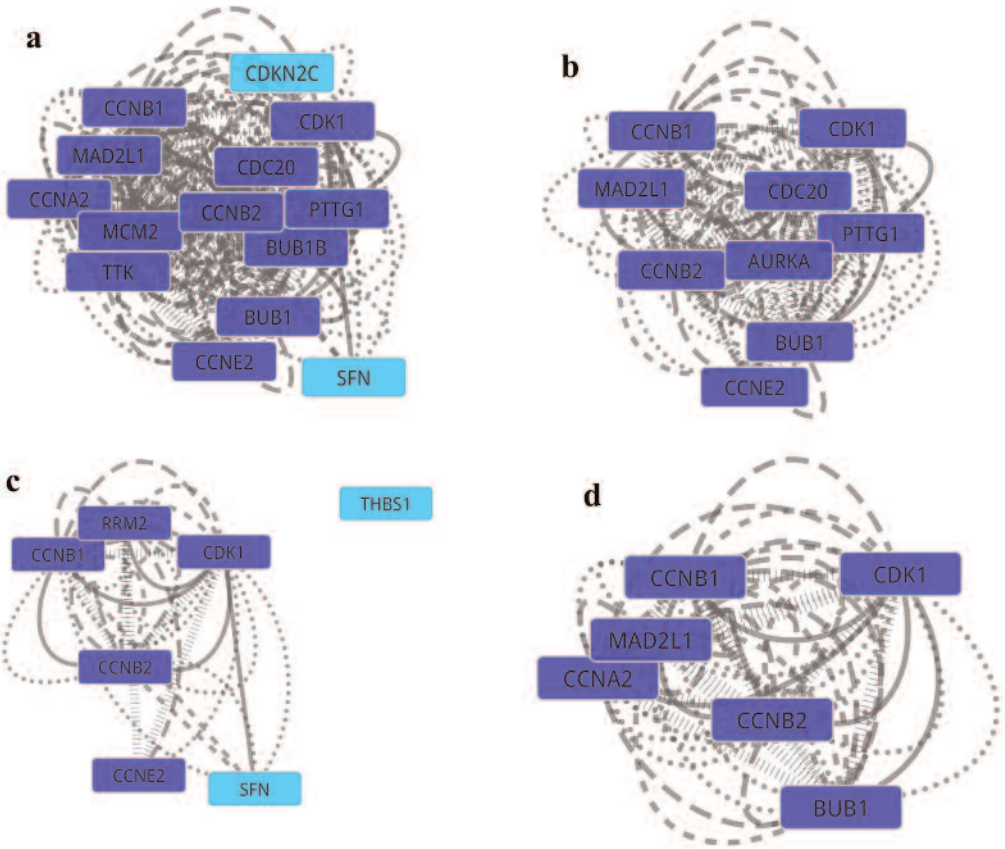

Figure 3. The sub-network of genes in cell cycle, oocyte meiosis, p53 signaling pathway and progesterone-mediated oocyte maturation pathway. The dark blue nodes stand for the hub genes.

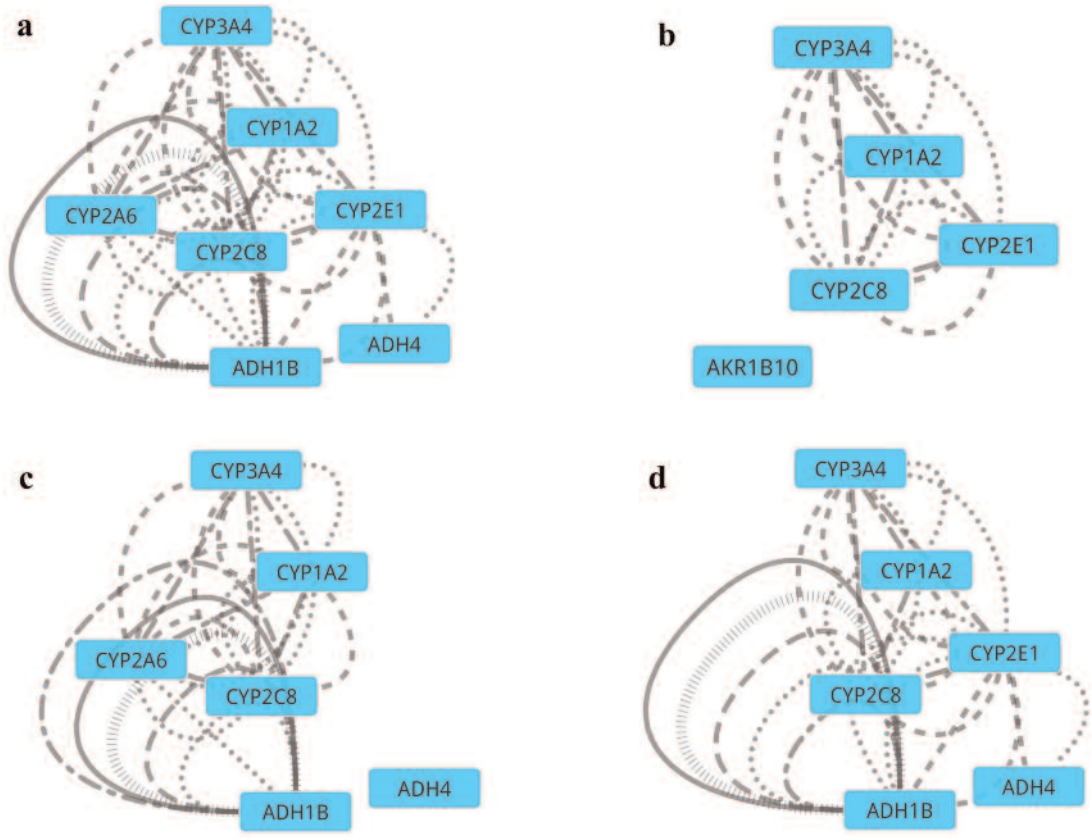

Figure 4. The sub-network of genes in drug metabolism, linoleic acid metabolism, retinol metabolism and metabolism of xenobiotics by cytochrome P450 pathway. No genes in these pathways mapped on the hubs network.

metabolism of xenobiotics by cytochrome P450, which can't be mapped on the hubs network, were enriched mainly by down-regulated gene signatures, except for AKR1B10 in linoleic acid metabolism which was up-regulated in HCC. AKR1B10 encodes member B10 of the aldo-keto reductase family 1. It was reported that the overexpression of AKR1B10 was highly correlated with non-small cell lung carcinomas[39,40]. Schmitz et al[41] found that its expression was associated with less aggressive hepatocellular carcinoma. AKR1B10, up-regulated in HCC of this study, might play an role in liver carcinogenesis.

In our study, the gene BIRC5 showed the highest degree (degree = 128) in the network. BIRC5 gene was located at chromosome 17q in the region that was frequently gained in high risk neuroblastoma[42]. It belonged to the family of genes known as inhibitors of apoptosis. It was found overexpression in neuroblastoma, and head and neck squamous cell carcinomas[42,43]. The mechanisms of up-regulating of BIRC5 in cancer were poorly understood[44]. Curcumin could down-regulated BIRC5 gene expression in human pancreatic cancer cell lines[45].

There were other genes with high degree in PPI network, such as CDC20, CCNB1, BUB1B, MAD2L1 and CDK1. CDC20 was an essential regulator of cell division in human, and had been shown to interact with BUB1B and MAD2L1[46,47]. BUB1B was a spindle-assembly checkpoint gene, which was essential in the mitotic checkpoint during normal mitosis progression[48,49]. CDK1 belonged to CDK family, and it was thought the only essential cell cycle CDK[50]. These might suggested that gene expression was not regulated independently, but by the complex interactions of many genes and factors.

Conclusions

In this work, we presented several hub genes related to HCC. The bioprocess and signaling pathways associated with them were presented systematically. Many of these genes were few reported

with HCC. Those genes might play important roles in HCC, and more research should focus on them.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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