Potential biomarkers of HCC based on gene expression and DNA methylation profiles

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Abstract. The aim of the present study was to identify potential biomarkers of hepatocellular carcinoma (HCC). Three gene expression profiles of GSE95698, GSE49515 and GSE76427 and a DNA methylation profile of GSE73003 were downloaded from the Gene Expression Omnibus (GEO) database, each comprising data regarding HCC and control tissue samples. The differentially expressed genes (DEGs) between the HCC group and the control group were identified using the limma software package. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the overlapping DEGs. The PPI network of the overlapping DEGs was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins. A total of 41 DEGs were identified in HCC the group compared with control group. The overlapping DEGs were enriched in 11 GO terms and 3 KEGG pathways. A total of 6,349 DMSs were identified, and 6 of the differentially expressed genes were also differentially methylated [Denticleless protein homolog (*DTL*), Dual specificity phosphatase 1 (*DUSP1*), Eomesodermin, Endothelial cell specific molecule 1, Nuclear factor κ-light-chain gene enhancer of activated B cells inhibitor, α (NFKBIA) and suppressor of cytokine signaling 2 (*SOCS2*)]. The present study suggested that *DTL*, *DUSP1*, *NFKBIA* and *SOCS2* may be potential biomarkers of HCC, and the tumor protein 'p53 signaling', 'forkhead box O1' signaling and 'metabolic' pathways may serve roles in the pathogenesis of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults, the fifth most common type of cancer and the third leading cause of cancer-associated mortality worldwide (1). HCC is closely associated with chronic viral hepatitis infection and exposure to toxins, including alcohol and aflatoxin (2). Other diseases can markedly increase the risk of HCC, including chronic liver inflammation, hemochromatosis and α1-antitrypsin deficiency (3,4). Chronic infections of hepatitis B and/or C contribute to the development of HCC by repeatedly causing the immune system to attack the liver cells, and consumption of large amounts of alcohol can have a similar effect (5). The prevalence of aflatoxin and hepatitis B has caused relatively high rates of HCC in a number of developing countries (6,7). Aflatoxin is produced by certain Aspergillus fungus species, and is a carcinogen known to contribute to the carcinogenesis of HCC (6). Tumors can develop following a mutation to cellular machinery, causing the cell to replicate at a higher rate and/or the avoidance of apoptosis (8). Numerous genes have been reported to serve roles in HCC carcinogenesis, which may be exploitable for the development of effective prevention and treatment regimens for HCC (9,10). Downregulated expression of human PMS1 homolog 2, mismatch repair system component (*hPMS2*) provides a growth advantage and stimulates proliferation of HCC cells, and it is believed that hPMS2 is involved at an early stage of HCC (11). Knockdown of *DLC1* has been demonstrated to aid *MYC* in the induction of hepatoblast transformation *in vitro*, and in the development of HCC *in vivo* (12). Another study demonstrated that the 'cell cycle' pathway and cell division cycle 25a may be crucial in the pathogenesis and progression of HCC (13). In addition, hypermethylation of DNA has been implicated as an early event in hepatocarcinogenesis (14). A mate-analysis study provided empirical evidence that abnormal suppressor of cytokine signaling 1 (*SOCS1*) promoter-methylation may contribute to the pathogenesis of HCC (15). Retinol metabolism genes and serine hydroxymethyltransferase 1 have also been indicated to be epigenetically regulated through promoter DNA methylation in alcohol-associated HCC (16). However, our incomplete understanding of the molecular and cellular mechanisms that drive HCC limits the available therapeutic

options. In the present study, three gene expression profiles and a DNA methylation profile were jointly analyzed in order to identify biomarkers of HCC.

Materials and methods

Gene expression and DNA methylation profiles. The gene expression profiles, GSE95698, GSE49515 (17) and GSE76427, and DNA methylation prolife, GSE73003, were downloaded from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/). A total of 3 human HCC tissue samples (HCC group) and their matching paracancerous tissue samples (control group) were selected from GSE95698, and the genes were detected using the Agilent-039494 SurePrint G3 Human GE V2 8x60 K Microarray 039381 platform. Data regarding 20 peripheral blood samples from 10 HCC patients (HCC group) and 10 healthy people (control group) were selected from the GSE49515 profile, and the genes were detected with Affymetrix Human Genome U133 Plus 2.0 Array. A total of 115 HCC tissue samples (HCC group) and 52 paracancerous tissue samples (control group) were selected from GSE76427, which were detected using an Illumina HumanHT-12 V4.0 expression beadchip. A total of 20 HCC tissue samples (HCC group) and 20 non-tumor tissue samples (control group) were selected from GSE73003, which were detected using an Illumina HumanMethylation27 BeadChip (Human Methylation27_270596_V1.2).

Data processing and differential analysis. For the gene expression profiles of GSE95698, GSE49515 and GSE76427, the raw data were obtained and normalized using the preprocess Core function package (version 3.5; http://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html). If multiple probes corresponded to the same gene, the average expression of these probes was used as the expression value of the gene. Subsequently, the differentially expressed genes (DEGs) in the HCC group compared with control group for the 3 sets of gene expression profiles were identified using the limma software package (version 3.18.13; http://www.bioconductor.org/packages/2.13/bioc/html/limma.html). $P<0.05$ and $|\log(\text{fold-change})|>0.5$ were used as the DEG threshold criteria. Thus, 3 sets of DEGs were obtained and the overlapping DEGs were selected for further study. For the DNA methylation profile of GSE73003, the preprocess Core function package (version 3.5) was used for normalization, and the differentially methylated sites (DMSs) were identified with the β-distribution test and t-test using a threshold of $P<0.05$. Overlaps between the overlapping DEGs and the differentially methylated sites were further studied.

Functional- and pathway-enrichment analyses of the overlapping DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.8; https://david.ncifcrf.gov/) is a widely used web-based tool for functional and pathway enrichment analyses (18). In the present study, it was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the overlapping DEGs. The GO terms and KEGG pathways with $P<0.05$ were selected.

Construction of the protein-protein interaction (PPI) network. Search Tool for the Retrieval of Interacting Genes (STRING; http://string-db.org) is a biological database and web resource used to identify known and predicted PPIs (19). In the present study, the PPI network of the overlapping DEGs was constructed using STRING (version 10.0) and visualized using Cytoscape software (version 3.5.1; http://www.cytoscape.org/download.php). Significant nodes were selected using a threshold score of >500 (obtained via STRING).

Patients. A total of 5 HCC patients (3 male and 2 female; age range, 32-65 years old; mean age 52.5 years old) were accepted in the Second People's Hospital of Tianjin (Tianjin, China) between November 2016 and May 2017. Tumor and paracancerous tissue specimens were collected. All patients signed informed consent prior to enrollment in the study, and all procedures were conducted under the ethical approval of the Second People's Hospital of Tianjin and the Chinese national research committee.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR and methylation-specific PCR (MSP) were performed to detect the mRNA expression level and methylation status of Denticleless protein homolog (*DTL*), Dual specificity phosphatase 1 (*DUSP1*), Nuclear factor k-light-chain gene enhancer of activated B cells inhibitor, a (*NFKBIA)* and *SOCS2*. The total RNA was extracted using TRIzol (Invitrogen; Scientific, Inc., Waltham, MA, USA). The PrimeScript® 1st Stand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China), the SYBR® Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan) and the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to conduct RT-PCR, according to the manufacturer's instructions. The reaction conditions of reverse transcription were as follows: 30˚C for 10 min, 42˚C for 60 min, and 95˚C for 5 min. The EZ-DNA Methylation-Gold kit™ (Zymo Research Corp., Irvine, CA, USA) was used to conduct MSP, according to the manufacturer's instructions. All primers were designed and synthesized by Takara Biomedical Technology Co., Ltd. (Beijing, China), and their sequences are listed in Table I. β-actin was used as an internal control. The thermocycling conditions were as follows: 95˚C for 5 min; followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec, and 72˚C for 35 sec; and a final 5 min at 72˚C extension. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression value of the target gene (20).

Statistical analysis. SPSS (version 17; SPSS Inc., Chicago, IL, USA) was used for all statistical analyses, and data are expressed as the mean ± standard deviation. Student's t-test was used to compare 2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DEGs and DMSs. A total of 1,402 (562 upregulated and 840 downregulated), 2,049 (859 upregulated and 1,190 downregulated) and 2,159 (1,339 upregulated and 820 downregulated)

Table I. Primer sequences.

Gene	Primer sequences (5'-3')	Size (bp)
DTL (mRNA)		490
Forward	CCTCTGTCCGATCCTCCAAA	
Reverse	AAAGATTTTCAGTCCCGCGG	
DTL (methyl)		152
Forward	TTTTTTGTTCGATTTTTTAAAGACG	
Reverse	TAATAACCCTACAACTCTACCCGAA	
DUSP1 (mRNA)		490
Forward	AGAATGTTCCTGACTCGGCA	
Reverse	AAGCAAAATCCAATCCCGGG	
DUSP1 (methyl)		142
Forward	AGGCGAAAATATATAAGTTAAGCGA	
Reverse	AATACCGAATCAAAAACATTCTACG	
NFKBIA (mRNA)		560
Forward	AGCGATGGGGTCTCACTATG	
Reverse	TCCAACAGCTTAGGTCAGGG	
NFKBIA (methyl)		147
Forward	ATTTTAGTTTTTTAAGTAGGTGCGA	
Reverse	ATAAAACAAAAAAATCACTTACGTT	
SOCS2 (mRNA)		630
Forward	GCTTGGGGTTAAATGGTGCA	
Reverse	AAGGGATGGGGCTCTTTCTC	
SOCS2 (methyl)		181
Forward	GTATTGATTTTAAGGAAGGACGC	
Reverse	CCTACGAAAATAACTCCTCCG	
β-actin (mRNA)		308
Forward	CATCTCTTGCTCGAAGTCCA	
Reverse	ATCATGTTTGAGACCTTCAACA	
β-actin (methyl)		110
Forward	ATTATTATTGGTAATGAGCGGTTTC	
Reverse	TTCATAATAAAATTAAATATAATTTCGTA	

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; methyl, methylation; DTL, denticleless protein homolog; DUSP1, dual specificity phosphatase 1; NFKBIA, nuclear factor of k light chain gene enhancer in B cells inhibitor, α; SOCS2, suppressor of cytokine signaling 2.

DEGs were separately identified in the HCC group compared with control group in GSE95698, GSE49515 and GSE76427, respectively. A total of 41 overlaps were identified among all 3 sets of DEGs (Fig. 1; Table II). Among these, 8 genes were upregulated simultaneously [*CCNB1*, *CEP55*, *DTL*, Endothelial cell specific molecule 1 (*ESM1*), *NEU1*, *RRM2*, *UHRF1* and *VPS72*], and 14 genes were downregulated simultaneously (*CCL21*, *CYP1A1*, *DAO*, *DUSP1*, *GABARAPL1*, *GADD45B*, *HSD11B1*, *NFIL3*, *NFKBIA*, *NR4A2*, *NSUN6*, *RHOB*, *SLC27A2* and *TRIB1*) in the 3 sets of DEGs.

For GSE73003, a total of 6,349 (2,854 upregulated and 3,495 downregulated) DMSs were identified in the HCC group compared with the control group, and the top 40 most-significant DMSs are listed in Table III. A total of 12 genes were among the 41 overlapping DEGs and the differentially methylated genes, and they are presented in Fig. 2. The expressions of *DTL*, *DUSP1*, eomesodermin (*EOMES*), *ESM1*, *NFKBIA* and *SOCS2* were negatively with their methylation levels.

Enriched GO terms and KEGG pathways. The overlapping DEGs were enriched in 11 GO terms and 3 KEGG pathways, listed in Tables IV and V, respectively.

The PPI network. The PPI network of the 41 overlapping DEGs, including 66 interaction pairs, is presented in Fig. 3. Furthermore, the PPI network of nodes with scores >500 is presented in Fig. 4, including *DTL*, *DUSP1*, *NFKBIA* and *SOCS2*. *DTL* was differentially expressed (upregulated) and differentially methylated (hypomethylated). *DUSP1*, *NFKBIA* and *SOCS2* were differentially expressed (downregulated) and differentially methylated (upregulated). The results of RT-qPCR

Table II. Overlapping differentially expressed genes in the hepatocellular carcinoma group compared with the control group in GSE95698, GSE49515 and GSE76427.

A, GSE49515

Genes	Mean lgFC	P-value
CCNB1	0.80	2.09×10^{-3}
CEP55	1.14	1.25×10^{-3}
DTL	0.83	9.88×10^{-3}
ESM1	0.53	4.88×10^{-3}
NEU1	0.78	2.31×10^{-7}
RRM2	0.56	2.41×10^{-2}
UHRF1	1.04	7.11×10^{-4}
VPS72	0.65	7.69×10^{-5}
CCL21	-0.60	8.37×10^{-5}
CYP1A1	-0.57	1.40×10^{-4}
DAO	-3.93	1.53×10^{-8}
DUSP1	-1.11	1.64×10^{-5}
GABARAPL1	-0.73	3.31×10^{-5}
GADD45B	-0.66	9.85×10^{-7}
HSD11B1	-0.90	6.32×10^{-3}
NFIL3	-1.66	1.04×10^{-6}
NFKBIA	-0.78	4.47×10^{-3}
NR4A2	-2.5	2.49×10^{-6}
NSUN6	-0.6	4.80×10^{-3}
RHOB	-0.88	1.13×10^{-5}
SLC27A2	-1.43	1.49×10^{-4}
TRIB1	-0.53	1.26×10^{-3}
ABHD6	1.02	1.20×10^{-7}
AKR7A3	0.92	1.65×10^{-5}
ALDH8A1	0.85	1.12×10^{-2}
ANG	1.51	1.13×10^{-5}
C11orf24	0.58	2.66×10^{-4}
CLEC1B	2.09	3.86×10^{-3}
CYP2J2	0.52	1.12×10^{-2}
EOMES	0.64	4.07×10^{-2}
GHR	1.62	1.67×10^{-5}
GZMK	0.67	4.42×10^{-3}
ITPRIPL2	0.55	1.58×10^{-3}
PDK4	0.61	4.69×10^{-3}
RARRES3	1.98	1.48×10^{-4}
SESTD1	-0.54	1.81×10^{-4}
SOCS2	0.85	1.26×10^{-5}
ST3GAL6	0.56	5.16×10^{-3}
TMEM45A	1.28	3.71×10^{-2}
UGP2	0.86	8.84×10^{-6}
XAF1	0.81	1.55×10^{-2}

B, GSE76427

Genes	Mean lgFC	P-value
CCNB1	0.62	1.77×10^{-13}
CEP55	0.75	3.33×10^{-12}
DTL	0.60	3.92×10^{-10}

Table II. Continued.

B, GSE76427

Genes	Mean lgFC	P-value
ESM1	1.13	8.87×10^{-13}
NEU1	0.71	5.27×10^{-11}
RRM2	0.51	1.55×10^{-13}
UHRF1	0.59	1.34×10^{-12}
VPS72	0.56	3.47×10^{-17}
CCL21	-0.81	1.01×10^{-4}
CYP1A1	-0.72	1.69×10^{-2}
DAO	-0.56	7.49×10^{-6}
DUSP1	-0.77	1.65×10^{-5}
GABARAPL1	-1.27	3.43×10^{-17}
GADD45B	-1.40	2.45×10^{-15}
HSD11B1	-0.59	1.83×10^{-2}
NFIL3	-0.53	1.39×10^{-6}
NFKBIA	-0.63	4.06×10^{-11}
NR4A2	-0.51	9.59×10^{-4}
NSUN6	-0.89	1.34×10^{-17}
RHOB	-1.09	1.25×10^{-10}
SLC27A2	-0.83	3.90×10^{-7}
TRIB1	-0.99	1.79×10^{-12}
ABHD6	-0.87	3.63×10^{-16}
AKR7A3	-1.04	1.15×10^{-8}
ALDH8A1	-1.12	2.44×10^{-16}
ANG	-0.70	4.79×10^{-6}
C11orf24	-0.74	3.27×10^{-12}
CLEC1B	-3.49	2.37×10^{-32}
CYP2J2	-0.88	3.49×10^{-10}
EOMES	-0.68	1.94×10^{-7}
GHR	-2.04	5.96×10^{-22}
GZMK	-0.58	7.91×10^{-4}
ITPRIPL2	-0.55	5.94×10^{-6}
PDK4	-0.97	5.40×10^{-7}
RARRES3	-0.55	4.11×10^{-5}
SESTD1	0.78	4.46×10^{-18}
SOCS2	-1.42	6.75×10^{-16}
ST3GAL6	-1.31	4.97×10^{-17}
TMEM45A	-0.88	1.55×10^{-5}
UGP2	-0.74	1.02×10^{-13}
XAF1	-0.89	2.72×10^{-8}

C, GSE95698

Genes	Mean lgFC	P-value
CCNB1	2.29	1.92×10^{-2}
CEP55	2.00	2.17×10^{-2}
DTL	3.32	1.93×10^{-3}
ESM1	6.12	1.75×10^{-3}
NEU1	1.18	2.25×10^{-2}
RRM2	2.23	4.49×10^{-2}
UHRF1	2.63	1.79×10^{-2}
VPS72	1.16	2.32×10^{-2}

Table II. Continued.

C, GSE95698

Genes	Mean lgFC	P-value
CCL21	-2.05	$4.34x10^{-2}$
CYP1A1	-5.69	$6.24x10^{-5}$
DAO	-4.92	$4.83x10^{-2}$
DUSP1	-1.66	$1.55x10^{-2}$
GABARAPL1	-1.64	$1.07x10^{-2}$
GADD45B	-2.17	$2.79x10^{-3}$
HSD11B1	-3.30	$1.10x10^{-2}$
NFIL3	-1.04	$3.03x10^{-2}$
NFKBIA	-0.90	$4.79x10^{-2}$
NR4A2	-2.76	$9.34x10^{-3}$
NSUN6	-1.85	$2.99x10^{-2}$
RHOB	-1.94	$1.13x10^{-2}$
SLC27A2	-3.15	$4.95x10^{-2}$
TRIB1	-1.88	$1.74x10^{-2}$
ABHD6	-2.22	$9.50x10^{-3}$
AKR7A3	-2.81	$2.15x10^{-2}$
ALDH8A1	-3.97	$3.41x10^{-2}$
ANG	-2.44	$3.99x10^{-2}$
C11orf24	-1.00	$4.95x10^{-2}$
CLEC1B	-7.55	$6.77x10^{-5}$
CYP2J2	-3.32	$2.29x10^{-2}$
EOMES	-3.02	$6.40x10^{-3}$
GHR	-4.4	$2.30x10^{-2}$
GZMK	-2.42	$2.89x10^{-2}$
ITPRIPL2	0.99	$3.91x10^{-2}$
PDK4	-2.39	$4.18x10^{-2}$
RARRES3	-1.76	$9.60x10^{-3}$
SESTD1	1.88	$3.61x10^{-2}$
SOCS2	-2.21	$2.07x10^{-2}$
ST3GAL6	-2.76	$2.32x10^{-3}$
TMEM45A	-2.25	$1.37x10^{-2}$
UGP2	-1.42	$3.12x10^{-2}$
XAF1	-1.51	$1.79x10^{-2}$

FC, fold change.

Table III. The top 40 most significant differentially methylated sites in the hepatocellular carcinoma group compared with the control group.

Gene	Regulation	P-value	Db-value
LYPD3	Up	$1.13x10^{-11}$	0.26
FLJ21159	Up	$1.70x10^{-11}$	0.26
ZNF154	Up	$2.94x10^{-11}$	0.52
ZNF540	Up	$7.96x10^{-11}$	0.33
GPR25	Up	$8.68x10^{-11}$	0.20
MS4A3	Down	$9.61x10^{-11}$	-0.23
LDHB	Up	$1.62x10^{-10}$	0.44
ALOX12	Up	$1.65x10^{-10}$	0.12
PRKG2	Down	$1.99x10^{-10}$	-0.31
FLJ25773	Down	$2.10x10^{-10}$	-0.24
PKDREJ	Up	$2.14x10^{-10}$	0.17
TBC1D1	Up	$2.47x10^{-10}$	0.13
SLC39A12	Down	$2.94x10^{-10}$	-0.30
SERHL	Up	$2.98x10^{-10}$	0.26
C6orf206	Up	$3.19x10^{-10}$	0.32
C11orf2	Up	$3.22x10^{-10}$	0.06
CCDC37	Up	$3.39x10^{-10}$	0.32
LILRA1	Down	$3.79x10^{-10}$	-0.21
OR51B4	Down	$4.22x10^{-10}$	-0.27
HDAC1	Down	$4.30x10^{-10}$	-0.05
XLF	Down	$5.08x10^{-10}$	-0.04
KCTD4	Up	$5.57x10^{-10}$	0.24
INA	Up	$5.58x10^{-10}$	0.34
ABHD9	Up	$6.00x10^{-10}$	0.42
AQP6	Down	$6.06x10^{-10}$	-0.07
ANKRD33	Up	$6.90x10^{-10}$	0.19
TSPYL5	Up	$9.71x10^{-10}$	0.26
RPS6KC1	Down	$1.19x10^{-9}$	-0.07
FLJ13149	Down	$1.33x10^{-9}$	-0.06
FCAR	Down	$1.38x10^{-9}$	-0.22
CD1B	Down	$1.40x10^{-9}$	-0.23
KLK2	Down	$1.50x10^{-9}$	-0.21
CDH18	Down	$1.50x10^{-9}$	-0.34
BOLL	Up	$1.67x10^{-9}$	0.24
SGNE1	Up	$1.67x10^{-9}$	0.25
CYP11B1	Down	$1.75x10^{-9}$	-0.38
S100A8	Down	$1.92x10^{-9}$	-0.21
MAPKAP1	Down	$2.01x10^{-9}$	-0.06
QTRT1	Down	$2.21x10^{-9}$	-0.09
KLK9	Down	$2.32x10^{-9}$	-0.20
FLJ46481	Down	$2.35x10^{-9}$	-0.24

and MSP are presented in Fig. 5. The mRNA expression of *DTL* was significantly increased in HCC tissue compared with paracancerous samples, and the mRNA expression of *DUSP1*, *NFKBIA* and *SOCS2* was significantly decreased (P<0.05). Furthermore, the opposite effect on the methylation levels of these genes was observed (P<0.05). These results were consistent with those of the bioinformatics differential expression analysis.

Discussion

Recent advances have significantly improved our understanding of the molecular pathogenesis of HCC and its complex genetic landscape (21-23). The integration of multiple profiling data may provide additional insight into the molecular mechanisms of HCC. In the present study, 41 genes were simultaneously differentially expressed in 3 expression profiles, and they were enriched in 3 KEGG pathways: 'p53 signaling', 'Foxo signaling' and 'metabolic' pathways (Table V). The 'p53 signaling' pathway influences a myriad of diverse cellular processes, and p53 has been suggested

to be activated in >50% human cancer types (24). The 'p53 signaling' pathway has been associated cancer occurrence and in mediating the response to cancer therapies (25). Kirstein and Vogel (26) summarized the pathological mechanisms of HCC carcinogenesis and progression. It was demonstrated that the most frequently identified mutations in HCC led to inactivation of p53. Cisplatin has been effectively used in the treatment of HCC, and p53 signaling is a potential target of cisplatin treatment (27). Paired box 5 (PAX5) has been suggested to be a functional tumor suppressor involved in HCC through direct regulation of the p53 signaling pathway, and Ras association domain family member 10 has been demonstrated to suppress human HCC growth by activating p53 signaling (28,29). The tyrosine kinase inhibitor, genistein, has been demonstrated to induce apoptosis and cell cycle arrest in HepG2 cells by activating the p53 signaling pathway (30). The forkhead box O1 (Foxo) signaling pathway participates in the regulation of multiple biological processes, including cellular responses to oxidative stress, cellular apoptosis and cell proliferation (31-33). It was reported that juglanthraquinone C could induce the apoptosis of HCC cells by activating the Foxo signaling pathway (34). PS341 (Bortezomib) was the first proteasome-inhibitor drug to be approved in clinical treatment for multiple myeloma, functioning by mediating targeted therapy against HCC through the Foxo3 signaling pathway (35). A previous study reported that Foxo-inhibition resulted in the myeloid maturation and death of acute myeloid leukemia cells. Leukemic cells resistant to Foxo-inhibition responded to JUN N-terminal kinase inhibition (36). Metabolic pathways mainly comprise metabolism of substance and energy, involved in a variety growth and development processes, and the occurrence and development of disease. Lu *et al* (37) hypothesized that blocking the cholesterol metabolic pathway may have potential therapeutic applications for patients with HCC. Björnsson (38) explored the central metabolic pathway in HCC using a differential rank conservation algorithm, revealing the involvement of 'fatty acid metabolism'. Therefore, 'p53 signaling', 'Foxo signaling' and 'metabolic' pathways may serve roles in the pathogenesis of HCC, and may guide future research into the development of novel HCC therapies.

Combination analysis of the 41 overlapping DEGs revealed that the expression and methylation of 6 genes was downregulated in HCC (*DTL*, *DUSP1*, *EOMES*, *ESM1*, *NFKBIA* and *SOCS2*). In order to further investigate the association between these genes, a PPI network was constructed. *DTL*, *DUSP1*, *NFKBIA* and *SOCS2* were nodes a score >500 (Fig. 4). Denticleless protein homolog (DTL) is a cell cycle-regulated nuclear and centrosome protein a protein (39). It was confirmed to be a critical target of miR-215, and *DTL*-knockdown by siRNA resulted in enhanced G2-arrest, p53 and p21 induction, and reduced cell proliferation of osteosarcoma and colon cancer cells (39). A previous study reported that overexpression of *DTL* promoted proliferation of tumor cells and was associated with a malignant outcome in esophageal squamous cell carcinoma (40). Another study reported that gastric carcinoma patients with *DTL*-overexpressing tumors had a relatively poor overall survival rate compared with those not exhibiting *DTL* expression (P=0.0498) and disease-free survival rate (P=0.0324) (41). DUSP1 has been demonstrated to be involved in cell cycle inhibition, apoptosis and

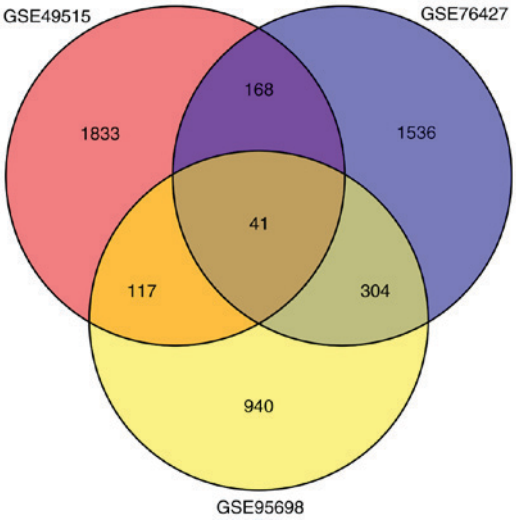

Figure 1. The Venn diagram illustrating the 3 sets of differentially expressed genes.

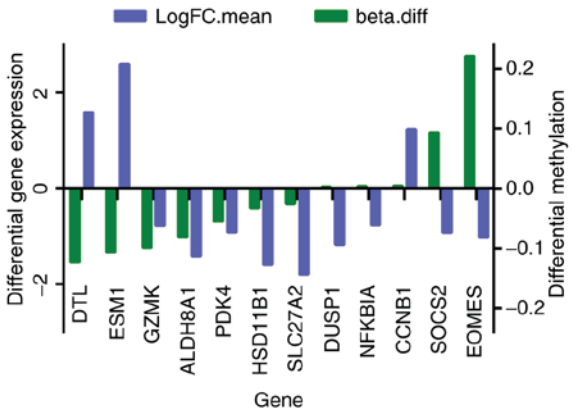

Figure 2. In total, 12 overlapping genes were differentially expressed and methylated in the HCC group compared with control. Beta.diff, fold-change in gene methylation level; LogFC.mean, fold-change in gene differential expression; HCC, hepatocellular carcinoma; DTL, denticleless E3 ubiquitin protein ligase; ESM1, endothelial cell specific molecule 1; GZMK, granzyme K; ALDH8A1, aldehyde dehydrogenase 8 family, member A1; PDK4, pyruvate dehydrogenase kinase 4; HSD11B1, hydroxysteroid 11-beta dehydrogenase 1; SLC27A2, solute carrier family 27 member 2; DUSP1, dual specificity phosphatase 1; NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, α; CCNB1, cyclin B1; SOCS2, suppressor of cytokine signaling 2; EOMES, eomesodermin.

senescence (42). A South Korean study revealed that *DUSP1* functioned as a tumor suppressor during hepatocarcinogenesis, and that the *DUSP1* expression was associated with the activation of p53 (43). Hao *et al* (44) revealed that disruption of a positive regulatory loop between DUSP1 and p53 promoted HCC development and progression. Wei *et al* (45) found that *miR-101* inhibited macrophage-induced growth of HCC tumors by regulating tumor growth factor-β secretion via targeting *DUSP1*. NFKBIA inhibits NF-κB by forming a heterodimer with NF-κB, and preventing its translocation to the nucleus (46). A previous study investigation revealed the distribution frequency of the *NFKBIA* genotype and haplotype polymorphisms between HCC and control specimens (47). It has been reported that the expression of *NFKBIA*

Table IV. Enriched GO terms of the overlapping differentially expressed genes.

Category	Term	Number of enriched genes	P-value
BP	GO:0044255: Cellular lipid metabolic process	8	<0.01
BP	GO:0008610: Lipid biosynthetic process	5	0.01
BP	GO:0071310: Cellular response to organic substance	8	0.03
BP	GO:1901701: Cellular response to oxygen-containing compound	5	0.04
BP	GO:0043436: Oxoacid metabolic process	5	0.04
BP	GO:0031668: Cellular response to extracellular stimulus	3	0.04
BP	GO:0044242: Cellular lipid catabolic process	3	0.04
BP	GO:0006082: Organic acid metabolic process	5	0.05
CC	GO:0043231: Intracellular membrane-bounded organelle	23	0.05
MF	GO:0016712: Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	2	<0.01
MF	GO:0020037: Heme binding	2	0.03

GO, gene ontology; BP, biological process; CC, cellular components; MF, molecular function.

Table V. Enriched Kyoto Encyclopedia of Genes and Genomes pathways of the overlapping differentially expressed genes.

Term	Number of enriched genes	P-value	Genes
cfa04115: P53 signaling pathway	3	0.012539936	CCNB1, RRM2, GADD45B
cfa04068: Foxo signaling pathway	3	0.046310836	CCNB1, GABARAPL1, GADD45B
cfa01100: Metabolic pathways	7	0.081506099	CYP2J2, CYP1A1, RRM2, ST3GAL6, HSD11B1, DAO, UGP2

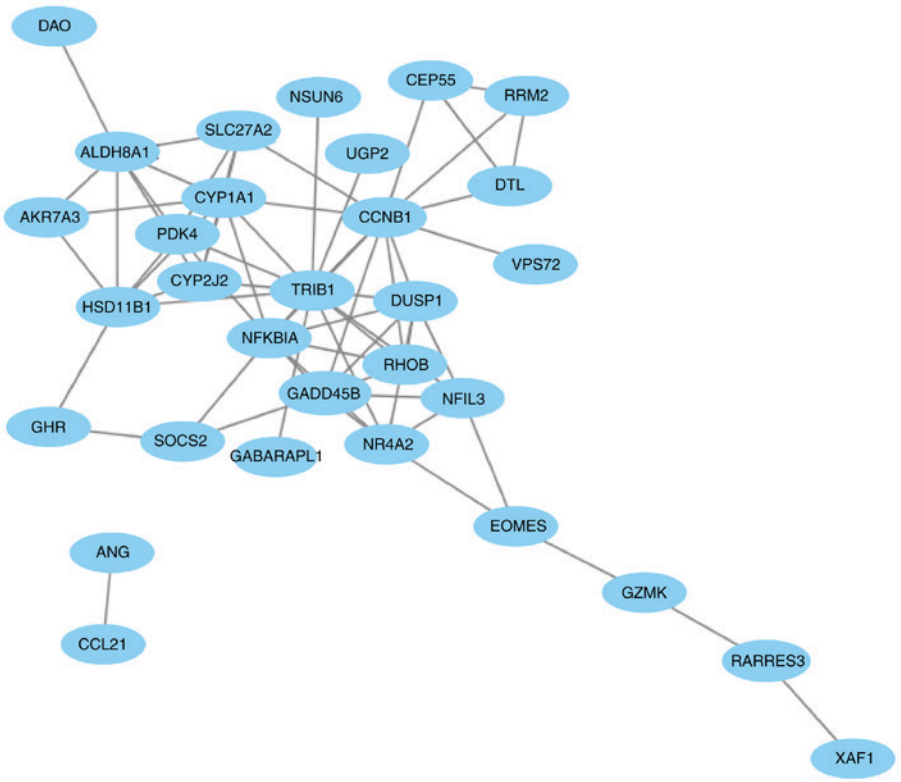

Figure 3. The protein-protein interaction network of the 41 overlapping differentially expressed genes.

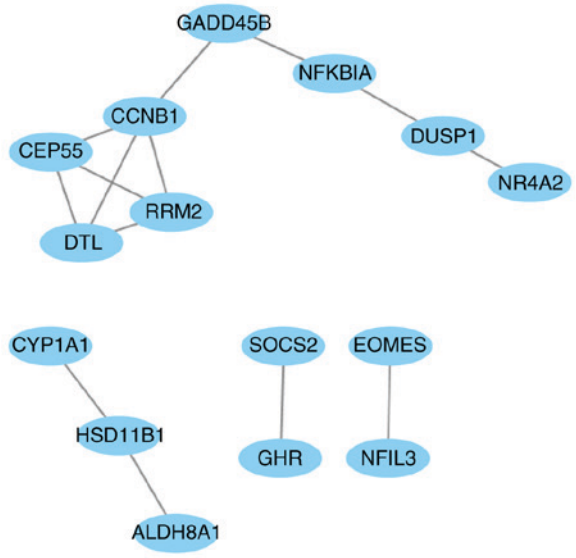

Figure 4. The protein-protein interaction network based on nodes scoring >500 in Search Tool for the Retrieval of Interacting Genes (version 10).

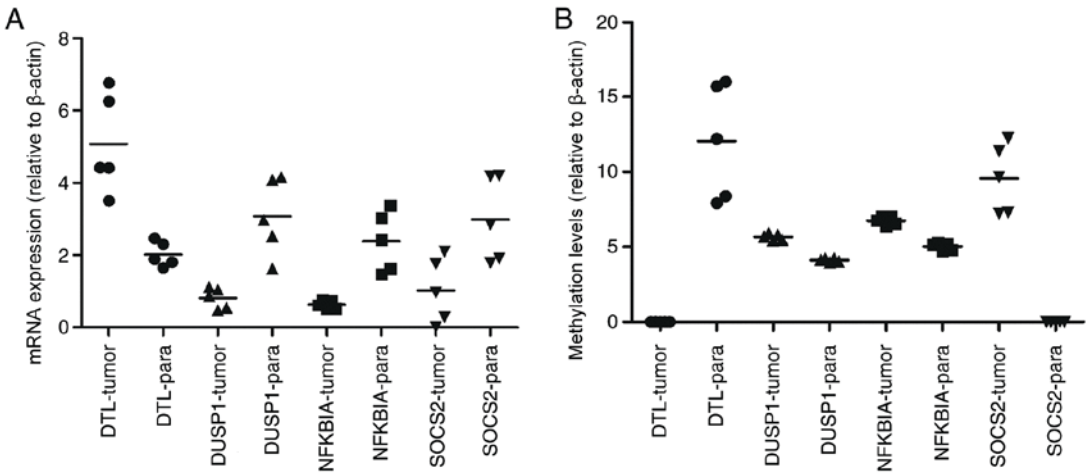

Figure 5. Clinical verification of mRNA expression and methylation levels of *DTL, DUSP1, NFKBIA* and *SOCS2* in HCC tumor and paracancerous samples. (A) Comparison of tumor vs. paracancerous mRNA expression levels: DTL, P=0.0007; DUSP1, P=0.0268; NFKBIA, P=0.0008, and SOCS2, P=0.0091. (B) Comparison of tumor vs. paracancerous methylation levels: DTL, P<0.0001; DUSP1, P<0.0001; NFKBIA, P<0.0001, and SOCS2, P<0.0001. -tumor, measured in tumor samples, -para measured in paracancerous samples.

is decreased in liver cancer tissue compared with control tissue, and that negative *NFKBIA* expression predicted poor prognosis in patients with primary HCC (48). A nested case-control study in Shanghai (China) suggested that genetic variants of *NFKB1* influenced liver cancer-susceptibility in the Chinese population (49). *SOCS2* has been reported to inhibit tumor metastasis (50). The expression of *SOCS2* has also been demonstrated to be markedly reduced in HCC and associated with aggressive tumor progression and poor prognosis in patients with HCC (51). Although *ESM1* and *EOMES* were differentially expressed and methylated in the HCC group compared with control group, the differences were non-significant (P=0.061, 0.053, respectively) and they were minimally involved in the PPI networks. Therefore, we hypothesize that *ESM1* and *EOMES* do not serve important roles in the pathogenesis.

In summary, *DTL*, *DUSP1*, *NFKBIA* and *SOCS2* were closely associated with HCC, and may provide useful insight for developing the treatment and prognosis of HCC. In the present study, it was confirmed that *DTL* expression was upregulated in HCC, and *DUSP1*, *NFKBIA* and *SOCS2* were downregulated, and the opposite effects on their methylation levels were observed (Fig. 5). We hypothesize that *DTL*, *DUSP1*, *NFKBIA* and *SOCS2* may be involved in HCC carcinogenesis. In conclusion, the present study identified potential biomarkers of HCC, including *DTL*, *DUSP1*, *NFKBIA* and *SOCS2*. Furthermore, the 'p53 signaling', 'Foxo signaling' and 'metabolic' pathways may be involved in the carcinogenesis of HCC, and may provide insight into the development of novel HCC therapies. Future work will involve detection of these potential biomarkers in large clinical samples by immunohistochemistry (IHC).

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

All authors had access to the data and were responsible for the concept and design of the study, data analysis/interpretation and critical revision and approval of the article. CM and WJ were responsible for data collection. WJ was responsible for drafting the article. CM and XS were responsible for statistical analyses.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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