

# Functional drug–target–disease network analysis of gene–phenotype connectivity for curcumin in cancer

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## Research article

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# Abstract

## Background

The anti-tumor properties of curcumin have been elucidated in many cancer types. However, a systematic functional and biological analysis related to its target proteins has yet to be documented fully. The aim of this study was to explore the underlying mechanisms of curcumin and broaden the perspective of targeted therapies.

## Methods

Direct protein targets (DPTs) of curcumin were searched in the DrugBank database. Using the STRING database, the interaction between curcumin and DPTs and indirect protein targets (IPTs) was documented. The protein–protein interaction (PPI) network of curcumin-mediated proteins was visualized using Cytoscape. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed for all curcumin-mediated proteins. Furthermore, the cancer targets were searched in the Comparative Toxicogenomics Database (CTD). The overlapping targets were studied using Kaplan–Meier analysis to evaluate cancer survival. Further genomic analysis of overlapping genes was conducted using the cBioPortal database. Lastly, CKK-8, qPCR, and WB analysis were used to validate the predicted results on HCC cells.

## Results

A total of 5 DPTs and 199 IPTs were found. These protein targets were found in 121 molecular pathways analyzed via KEGG enrichment. Based on the anti-tumor properties of curcumin, two pathways were selected, including pathways in cancer (36 genes) and hepatocellular carcinoma (HCC) pathway (22 genes). Overlapping with 505 HCC-related gene sets identified in CTD, five genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) were finally identified. High mRNA levels of TP53, RB1, and GSTM1 indicated a prolonged OS in HCC, while elevated mRNA levels of TGFB1 were correlated with poor prognosis. The viability of HepG2 cells and Hep3B cells can both be significantly reduced by curcumin at concentrations of 20 or 30  $\mu$ M after 48 or 72 h of culture. At a concentration of 20  $\mu$ M curcumin, the expression of TGFB1 and GSTP1 in Hep3B cells was reduced significantly in qPCR analysis, and reduced TGFB1 protein expression was also found in Hep3B cells.

## Conclusions

The current results provided evidence that curcumin has the potential to become an alternative chemotherapy or chemoprevention for HCC.

# Background

Curcumin, also known as diferuloylmethane, is the active ingredient of the dietary spice occurring in the rhizomes of *Curcuma longa*, a plant belonging to the ginger family (Fig. 1A). Extensive studies investigating curcumin over the past few decades revealed the health benefits of curcumin, including anticancer, anti-inflammatory, antioxidant, and hypoglycemic effects[1]. In recent years, curcumin has been increasingly recognized for its anti-tumor and cancer chemopreventive properties, especially in gastrointestinal tumors[2]. For example, curcumin has shown chemopreventive effects in animal models of colon cancer[3], stomach cancer[4], and hepatocellular carcinoma (HCC)[5]. Several phase I and II clinical trials with curcumin have been conducted for the treatment of different types of cancer[6, 7]. However, the precise molecular mechanisms underlying the anti-tumor and cancer chemopreventive activities of curcumin have yet to be elucidated.

Recent studies using data mining techniques for the analysis of specific bioinformatics domains have made great progress[8]. Accordingly, a strategy to systematically explore the rich data publicly available and the underlying connectivity between gene and phenotype mediated by curcumin should be helpful. With advances in genomics, data network analysis has been used to effectively analyze candidate genes linked to experimentally verifiable pathways via mining of web-accessible, open portal databases[9]. In addition, an in-depth analysis of the databases may uncover hidden, previously unknown relationships between drugs, targets, and cancers. These results can be used to generate authentic and rational leads for further investigations. Recently, the combination of related databases including DrugBank, STRING, cBio Cancer Genomics Portal (cBioPortal), and Comparative Toxicogenomics Database (CTD) has been utilized for drug–target–cancer network analysis[10, 11]. In this study, we employed DrugBank to broadly analyze curcumin and drug–target data to obtain related direct protein targets (DPTs). The interactions between DPTs and indirect protein targets (IPTs) were predicted with the STRING database. The readout DPTs and IPTs were further analyzed for functionality via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis with STRING. The pivotal genes were obtained via Intersection of CTD and STRING databases, and the genomic alterations were investigated using the cBioPortal database. Our findings provide information to obtain a better understanding of the anti-tumor mechanisms of curcumin and identify new targets in the treatment of HCC by curcumin.

## Methods

### Search for DPTs of curcumin

The DrugBank database (<https://www.drugbank.ca>) is a richly annotated resource that combines detailed drug data with comprehensive drug target and drug action information[12]. In this study, the latest release of DrugBank database (version 5.1.4)[13] was employed to search for the interaction between curcumin and its DPTs and generate a curcumin-target network. The word “Curcumin” was searched as a keyword under drug classification entry.

## PPI network generation

The STRING database (<http://www.string-db.org/>) provides a critical assessment and integration of protein-protein interactions, including direct (physical) as well as indirect (functional) associations[14]. Using the latest STRING database (version 11.0)[15] search function, data underlying the interaction between DPTs and IPTs were first generated for curcumin by setting a minimum required interaction score of 0.5 and a maximum number of interactors of 50. The data were integrated into a curcumin-mediated network and visualized using Cytoscape (version 3.7.1)[16], which is an open-source software platform for visualization of complex networks and their integration with any attribute[17].

## KEGG pathway enrichment analysis and overlapping HCC genes

Biochemical pathways linked to curcumin-DPT/IPT interaction were navigated via the KEGG pathway enrichment analysis tool in the STRING database. The top 15 pathways with an FDR less than  $1.00E-10$  were selected. To identify disease targets for HCC, we searched for “Hepatocellular carcinoma” in CTD (<http://www.ctdbase.org/>). CTD is a robust, publicly available database that aims to advance the understanding of environmental exposures and their impact on human health. It provides manually curated information about chemical–gene/protein interactions, and chemical–disease and gene–disease relationships[18]. Overlapping genes of HCC and cancer-related pathways and HCC targets were selected for further analysis.

## Exploring cancer genomics data linked to curcumin

The cBioPortal (<https://www.cbioportal.org/>) for Cancer Genomics provides a web resource for exploration, visualization, and analysis of multidimensional cancer genomics data[19, 20]. In this study, the screened genes from foregoing investigation were assessed in all HCC studies available in cBioPortal databases. Using the portal search function, curcumin-related genes in HCC were evaluated for genomic alteration, performing network analysis, and identifying mutual exclusivity or concurrent relationships between gene pairs of the same gene set. The study with the largest sample size was chosen to analyze the screened proteins in cBioPortal databases to further interpret the result.

## Analysis of curcumin-associated genomics datasets and HCC survival

The Kaplan–Meier method (<http://kmplot.com/>)[21] was used to evaluate the overall survival (OS) in 364 HCC samples with altered genes. The OS was defined as the time to death. A two-tailed P value of less than 0.05 was considered statistically significant.

## Cell culture

HepG2 and Hep3B cells were purchased from Cell Cook. They were authenticated using short tandem repeat matching analysis and cultured in DMEM supplemented with 10% v/v FBS and 1% v/v penicillin/streptomycin in CO<sub>2</sub> incubator at 37 °C and 95% relative humidity.

## Cell viability test

The MTT assay was used to assess cell viability. The 100  $\mu$ L cells (about 5000–10,000 cells) were transfused into the 96-well culture plate, which was then placed in the hatching house overnight for pre-culture (37 °C, 5% CO<sub>2</sub>). According to the drug concentration, all cells were divided into five groups. After pre-culture, 10  $\mu$ l of drug solution was added to every well (the drug concentrations were 0, 5, 10, 20, 30  $\mu$ M) and incubated at 37 °C. After that, 10  $\mu$ l CCK-8 solution was added to each well at 24, 48, and 72 h and incubated at 37 °C for 4 h. We determined the absorbance value of each hole at 450 nm and calculated the cell vitality value. The formula is as follows: Cell viability% = (OD in experimental group – OD in blank group)/(OD in control group – OD in blank group)  $\times$  100%.

Extraction of total RNA and reverse transcription-quantitative PCR (RT-qPCR).

After being treated and cultured, 5–10  $\times$  10<sup>6</sup> cells per group were collected, and total RNA was isolated using TRIzol® reagent according to the manufacturer's protocol. A total of 1  $\mu$ g extracted RNA was used to synthesize cDNA in a reaction using oligo (dT) primers and M-MLV reverse transcriptase according to the manufacturer's instructions. PCR amplification of mRNA was performed with a GoTaq® qPCR Master mix on an RT-qPCR instrument. The thermocycling conditions were as follows: Initial denaturation at 95 °C for 120 s; 40 cycles of 95 °C for 15 s, and a final extension at 60 °C for 30 s. The PCR products were calculated with the 2- $\Delta\Delta$ Cq method, using GAPDH as an mRNA internal control.

Western blot analysis

After being treated and cultured, the cells were harvested, and protein extractions were prepared with a modified RIPA buffer (Beyotime Institute of Biotechnology) with 0.5% SDS in the presence of a proteinase inhibitor cocktail (Beyotime Institute of Biotechnology). A bicinchoninic acid protein concentration kit was used to determine protein concentration. A total of 20  $\mu$ g protein/lane was separated using 6% or 12% SDS-PAGE. Proteins were then transferred to a PVDF membrane. The membranes were then blocked with 5% BSA in TBS(containing 0.05% Tween-20) for 2 h at room temperature and incubated overnight at 4 °C with the primary antibodies. The samples were washed three times with PBS containing 0.1% Tween-20 for 5 min each. Then the membranes were incubated with horseradish peroxidase-linked immunoglobulin G secondary antibody for 2 h at room temperature. The membranes were developed using an enhanced chemiluminescence system.

## Results

Characterization of curcumin DPTs

We queried Drugbank using curcumin as the keyword to identify the bioactivities and to determine the DPTs of curcumin and retrieve relevant information. The result showed an accession number of DB11672 and classified curcumin as a highly pleiotropic molecule with anti-tumor, antibacterial, anti-inflammatory, hypoglycemic, antioxidant, wound-healing, and antimicrobial activities. Clinical data showed that

curcumin is undergoing clinical trials for colon cancer, prostate cancer, breast cancer, and lung cancer. Subsequent screening demonstrated five DPTs in human beings. Table 1 summarizes the five DPTs of curcumin: PPARG, VDR, ABCC5, CBR1, and GSTP1. In addition, the interactions between five DPTs were analyzed by STRING and illustrated in Fig. 1B.

Table 1  
Direct protein targets of curcumin identified using DrugBank

Uniprot ID	Gene name	Targets	General function
P37231	PPARG	Peroxisome proliferator-activated receptor gamma	Zinc ion binding
P11473	VDR	Vitamin D3 receptor	Zinc ion binding
O15440	ABCC5	Multidrug resistance-associated protein 5	Organic anion transmembrane transporter activity
P16152	CBR1	Carbonyl reductase [NADPH] 1	Prostaglandin-e2 9-reductase activity
P09211	GSTP1	Glutathione S-transferase P	S-nitrosoglutathione binding

#### Characterization of curcumin IPTs and visualization of PPI network construction

Expanding our search using STRING database, we detected a total of 204 target proteins of curcumin including 199 IPTs, which were related to five DPTs (Table S1). The dataset obtained was integrated to construct a biological network using Cytoscape. As shown in Fig. 2A, 1962 PPI pairs were found in the network of curcumin-mediated proteins (Table S2). In this network, nodes represent proteins, and edges denote protein-protein associations. In addition, the node degree, which indicates the centrality of proteins, was calculated using CentiScaPe 2.2 (Table S3). As a result, 14 proteins including four DPT targets (GSTP1, VDR, CBR1, and PPARG) with a degree value  $\geq 50$  are shown in Table 2 and displayed in Fig. 2A with larger node sizes.

Table 2  
Protein targets with degree value  $\geq 50$

Gene	Node degree value	Gene name	Node degree value
EP300	75	NCOA1	52
CREBBP	72	CYP3A4	51
RXRA	68	GSTP1	50
NCOR1	60	NCOA3	50
CYP2E1	58	VDR	50
CYP1A1	54	CBR1	50
NCOR2	53	PPARG	50

### KEGG enrichment pathway analysis of proteins connected to curcumin

To identify the functional features of curcumin-mediated targets, the KEGG pathway enrichment analysis was performed using STRING. Target genes were found in 121 molecular pathways in KEGG enrichment (Table S4). As shown in Table 3 and Fig. 2B, the top 15 KEGG pathways connected to curcumin-mediated proteins included metabolism of xenobiotics by cytochrome P450 (52 genes), chemical carcinogenesis (50 genes), drug metabolism-cytochrome P450 (44 genes), retinol metabolism (32 genes), steroid hormone biosynthesis (30 genes), drug metabolism-other enzymes (31 genes), pentose and glucuronate interconversion (22 genes), ascorbate and aldarate metabolism (20 genes), metabolic pathways (65 genes), glutathione metabolism (20 genes), porphyrin and chlorophyll metabolism (19 genes), arachidonic acid metabolism (18 genes), pathways in cancer (36 genes), HCC (22 genes), and fluid shear stress and atherosclerosis (19 genes). These KEGG enrichment pathways showed functional features of curcumin gene sets and indicated that curcumin-mediated proteins were mainly associated with basal metabolism and cancer-related pathways. Based on the anti-tumor properties of curcumin reported in diverse malignant tumors, two pathways were selected, including pathways in cancer (36 genes) and HCC pathway (22 genes). This result suggested that HCC might be used as a phenotype connected to curcumin-mediated proteins. In addition, to further identify disease targets for HCC, the keyword "Carcinoma, Hepatocellular" was searched in CTD, and a total of 505 genes with either a curated association or an inferred association via a curated chemical interaction with the disease were identified (Table S5). These genes were marked with "T," which indicates a gene that is or may be a therapeutic disease target, or "M," which denotes a gene that may be a disease biomarker or involved in the etiology of a disease. Five overlapping genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) resulting from the intersections between pathways in cancer (36 genes), HCC pathway (22 genes), and targets for HCC by CTD (505 genes) were visualized using Venn diagrams and STRING (Fig. 3A and B).

Table 3. Top 15 enriched KEGG pathways identified using STRING

Pathways	gene	FDR	matching proteins
	count		
Metabolism of xenobiotics by cytochrome P450	52	5.68E-70	ADH1A, ADH1B, ADH1C, ADH5, AKR1C1, AKR7A2, ALDH3A1, CBR1, CBR3, CYP1A1, CYP1A2, CYP1B1, CYP2A13, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1, CYP2F1, CYP3A4, CYP3A5, ENSG00000270386, EPHX1, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, HPGDS, HSD11B1, MGST1, MGST2, MGST3, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Chemical carcinogenesis	50	1.80E-65	ADH1A, ADH1B, ADH1C, ADH5, AKR1C2, ALDH3A1, CBR1, CYP1A1, CYP1A2, CYP1B1, CYP2A13, CYP2A6, CYP2C18, CYP2C19, CYP2C8, CYP2C9, CYP2E1, CYP3A4, CYP3A5, ENSG00000270386, EPHX1, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, HPGDS, HSD11B1, MGST1, MGST2, MGST3, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Drug metabolism - cytochrome P450	44	1.30E-57	ADH1A, ADH1B, ADH1C, ADH5, ALDH3A1, CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP3A5, ENSG00000270386, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, HPGDS, MGST1, MGST2, MGST3, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Retinol metabolism	32	8.99E-39	ADH1A, ADH1B, ADH1C, ADH5, CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C18, CYP2C8, CYP2C9, CYP3A4, CYP3A5, ENSG00000270386, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Steroid hormone biosynthesis	30	2.17E-36	AKR1C1, AKR1C2, AKR1C3, AKR1C4, CYP1A1, CYP1A2, CYP1B1, CYP2E1, CYP3A4, CYP3A5, ENSG00000270386, HSD11B1, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Drug metabolism - other enzymes	31	3.65E-35	CYP2A6, CYP2E1, CYP3A4, ENSG00000270386, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, MGST1, MGST2, MGST3, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8,

UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7

Pentose glucuronate interconversions	and	22	3.05E- 28	AKR1A1, AKR1B1, ENSG00000270386, KL, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Ascorbate aldarate metabolism	and	20	1.50E- 26	ALDH9A1, ENSG00000270386, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Metabolic pathways		65	3.44E- 26	ADH1A, ADH1B, ADH1C, ADH5, ADPGK, AKR1A1, AKR1B1, AKR1C3, AKR1C4, ALDH3A1, ALDH9A1, CBR1, CBR3, CYP1A1, CYP1A2, CYP24A1, CYP27A1, CYP27B1, CYP2A6, CYP2B6, CYP2C18, CYP2C19, CYP2C8, CYP2C9, CYP2E1, CYP2R1, CYP3A4, CYP3A5, DHFR, DHFRL1, ENSG00000270386, GCH1, GCLC, GGT1, GSS, GSTZ1, HPGDS, HSD11B1, KL, MCEE, PCK1, PRDX6, PTGES, PTGES2, PTGES3, PTS, SPR, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Glutathione metabolism		20	1.62E- 22	GCLC, GGT1, GPX1, GPX2, GPX3, GPX4, GPX7, GPX8, GSR, GSS, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, HPGDS, MGST1, MGST2, MGST3
Porphyrin chlorophyll metabolism	and	19	3.09E- 22	ENSG00000270386, UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2A2, UGT2A3, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28, UGT2B4, UGT2B7
Arachidonic acid metabolism		18	2.35E- 18	AKR1C3, CBR1, CBR3, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2E1, GGT1, GPX1, GPX2, GPX3, GPX7, GPX8, HPGDS, PTGES, PTGES2, PTGES3
Pathways in cancer		36	1.05E- 17	CEBPA, CREBBP, EP300, FGF23, FOS, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, HDAC1, JUN, MAPK8, MGST1, MGST2, MGST3, NCOA1, NCOA3, NFKB1, NQO1, PAX8, PPARG, RARA, RARB, RASSF1, RB1, RELA, RXRA, RXRB, RXRG, SMAD3, SMAD4, TGFB1, TP53, TRAF2
HCC		22	4.14E- 16	ACTL6A, ARID1A, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, MGST1, MGST2, MGST3, NQO1, RB1, SMAD3, SMAD4, SMARCA4, SMARCC1, SMARCC2, SMARCD1, SMARCE1, TGFB1, TP53
Fluid shear stress		19	2.45E-	FOS, GSTA1, GSTM1, GSTO1, GSTO2, GSTP1, GSTT2B, JUN, MAPK8, MGST1, MGST2, MGST3, NFKB1,

FDR, false discovery rate

## Genetic alterations connected with curcumin-associated genes in HCC

To further validate the link between curcumin-associated genes and HCC, cBioPortal databases were used to explore the five genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) associated with curcumin in HCC. Seven HCC studies were included in cBioPortal[22–27], and the five selected overlapping genes were queried. The results showed that 363 (33%) of the 1135 samples in 7 studies had alterations in one or more of these genes. Alterations ranged from 0.2–29% for gene sets submitted for analysis (Fig. 4). The five genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) carried five gene pairs showing mutually exclusivity alterations, while another five gene pairs showed concurrent alterations (with TP53 and RB1 showing a statistically significant alteration, Table 4). Furthermore, Liver HCC (TCGA, Provisional) study, which carried the largest sample size, was selected individually to analyze the screened proteins in cBioPortal databases. The results showed that 156 (35%) patients/samples carried an alteration in at least one of the five genes queried using OncoPrint; the frequency of alteration in each gene is shown in Fig. 5. Most gene alterations included mutations and deletions. Most of the TP53 alterations were classified into missense mutations, truncating mutations, and deep deletions. Gene changes associated with RB1 included truncating mutations and deep deletions, while for TGFB1, GSTP1 and GSTM1, amplifications were the most common gene alterations.

Table 4  
Mutual exclusivity analysis of 5 genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) in 7 studies associated with HCC

A	B	Log2 Odds Ratio	p-Value	Tendency
TP53	RB1	0.755	0.024	Co-occurrence
RB1	TGFB1	2.131	0.108	Co-occurrence
TP53	GSTP1	1.045	0.187	Co-occurrence
TP53	TGFB1	0.564	0.421	Co-occurrence
RB1	GSTP1	<-3	0.437	Mutual exclusivity
TP53	GSTM1	1.299	0.495	Co-occurrence
RB1	GSTM1	<-3	0.861	Mutual exclusivity
TGFB1	GSTP1	<-3	0.918	Mutual exclusivity
GSTP1	GSTM1	<-3	0.979	Mutual exclusivity
TGFB1	GSTM1	<-3	0.985	Mutual exclusivity

#### Curcumin-associated genes and survival in HCC

The Kaplan–Meier analysis was used to perform survival analysis of patients with HCC based on the five selected genes (TP53, RB1, TGFB1, GSTP1, and GSTM1). A total of 364 patients were involved in this analysis for OS. As shown in Fig. 6A–E, high mRNA levels of TP53, RB1, and GSTM1 indicated increased OS in HCC, whereas elevated mRNA levels of TGFB1 were correlated with poor prognosis in the same group of patients.

#### Curcumin-induced decrease in the activity of HepG2 and Hep3B cells

The test results showed that cell viability appeared dependent on the concentration of curcumin. For Hep3B cells, curcumin (10  $\mu$ M) can significantly decrease cell viability after 24, 48, and 72 h, and 20 and 30  $\mu$ M of curcumin were found to significantly reduce cell viability after 24, 48, and 72 h (Fig. 7A). For HepG2 cells, curcumin (20 and 30  $\mu$ M) can significantly decrease the cell viability after 48 and 72 h (Fig. 7B). According to these result, we chose curcumin at a concentration of 20  $\mu$ M and 48 h as the duration of treatment for subsequent testing.

#### Curcumin regulation of TGFB1 and GSTP1 expression in Hep3B cells

The expression of TP53, Rb1, TGFB1, GSTP1, and GSTM1 was assessed using qPCR in Hep3B cells treated with 20  $\mu$ M curcumin for 48 h. The results showed that 20  $\mu$ M curcumin could regulate the expression of TGFB1 and GSTP1 in Hep3B cells, but it had no significant effect on the expression of

other genes (Fig. 7C). After treatment of Hep3B cells with 0 and 20  $\mu$ M curcumin for 48 h, the levels of TGFB1 protein were detected via WB. The results showed that 20  $\mu$ M curcumin could moderately inhibit the expression of TGFB1 in Hep3B cells (Fig. 7D, 7E).

## Discussion

Over the past several years, numerous studies have evaluated the effects of curcumin and its analogs in diverse cancers in vitro and in vivo. According to these studies, the potential use of curcumin as a chemopreventive and therapeutic agent in cancers depends on its potent antioxidant and anti-inflammatory activities as well as its ability to modulate various molecular signaling mechanisms [1, 2, 6, 7]. Nevertheless, the mechanism underlying the wide range of anti-cancer effects of curcumin remains incomplete. Current knowledge about curcumin function and mechanisms is based on conventional experiments, which have yet to be fully integrated and understood. Therefore, new analytical methods are needed to correlate curcumin with its target proteins and the observed biological effects.

Functional/activity network (FAN)[9] is a new analytical method, which elucidates the molecular mechanisms of a drug and its association with clinical outcomes in cancer by using a set of web-based tools, such as DrugBank, STRING, cBioPortal, CTD, or Cytoscape. Using this method, we conducted the functional drug–target–cancer network analysis of gene–phenotype connectivity associated with curcumin. Our study bridged curcumin with its primary or secondary targets and illustrated the underlying mechanisms of curcumin and its clinical outcomes in HCC.

Primary liver cancer is the sixth most common cancer and the second largest cause of cancer mortality in the world[28]. HCC constitutes approximately 80% of all primary liver cancer[29]. The effects of curcumin and its analogs have been a subject of investigation over the past decade in preclinical models of HCC[30], but research in this field is far from complete. A search of PubMed using “curcumin” as the keyword returned more than 13000 publications, whereas using “curcumin and hepatocellular carcinoma,” only 192 publications were retrieved to date. By using functional/activity network, more direct mechanisms may be mined with reasonable experimental feasibility to validate hypotheses explaining the effects of curcumin in HCC.

In this study, we demonstrated the feasibility of FAN analysis underlying the connectivity between curcumin and HCC. As a result, we identified a network including five genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) as targets of curcumin in HCC via a functional drug–target–cancer network analysis. Among the five genes, TP53 and RB1 are tumor suppressor genes consistent with their high transcriptional expression correlated with better OS in HCC in this study. Similar to other cancers, TP53 and RB1 were considered as the most commonly inactivated or mutated in the case of HCC (Fig. 5). Moreover, mutual exclusivity analysis revealed a tendency toward concurrence between TP53 and RB1 in our report. Previous studies indicated that curcumin may inhibit cancer growth and induce apoptosis in colon cancer cells[31], ameliorate the in vitro efficacy of carfilzomib in human multiple myeloma cells[32], promote apoptosis in non-small cell lung cancer[33], and inhibit cell growth in nasopharyngeal carcinoma mediated via TP53 signaling pathway[34]. Debata et al. reported that the sunitinib-curcumin combination

was effective in restoring the tumor suppressor activity of RB gene in renal cancer cells[35]. Furthermore, Su et al. reported that curcumin significantly enhanced p53 or markedly inhibited the RB pathway by suppressing RB phosphorylation in the signaling pathways of glioblastoma[36]. The foregoing studies demonstrated the anti-tumor activity of curcumin and prompted further investigation of curcumin in HCC.

In the case of HCC, most of the genetic alterations in TGFB1, GSTP1, and GSTM1 were amplifications (Fig. 5), which may cause increased expression. However, the prognostic significance varied in this study. The amplification of TGFB1 indicated a worse OS in HCC, while amplifications of GSTM1 and GSTP1 (not statistically significant) showed better prognosis. TGFB1 is a pleiotropic gene with a dual role in hepatocarcinogenesis: apoptosis induction in early phases, but promotion of tumorigenesis in cells with mechanisms to overcome the suppressor effects<sup>[37]</sup>. Recent studies found that the expression of TGFB1 genes was downregulated in breast cancer cells treated with curcumin[38]. Glutathione S-transferases (GSTs) are a family of phase II detoxification enzymes that catalyze the conjugation of a wide variety of endogenous and exogenous toxins. A previous study showed that the inter-individual GST variation plays a central role in reducing cell exposure to carcinogens[39]. For example, the reduced GSTP1 expression may contribute to oxidative stress in HCC[40]. Similar results were observed in breast cancer cells and curcumin-activated GSTP1 expression via antioxidant response element[41]. However, studies linking curcumin and GST genes in HCC are rare, underscoring the need for further investigation in this field.

These results were partially confirmed by *in vitro* experiments. HepG2 and Hep3B were used as the experimental objects because of their advantages of fast growth and easy passage. Our study confirmed the inhibitory effect of curcumin on liver cancer cells, and this inhibition increased with time and concentration. In the liver HCC (TCGA, Provisional) study, three genes (TGFB1, GSTP1, and GSTM1) showed amplification. For this reason, changes in these amplified genes were more easily detected using PCR. This was confirmed in our study when we found that two genes that were prone to amplification were suppressed in HCC cells treated with curcumin. The other genes, however, did not show changes in expression because the main means of mutation was not amplification mutations but truncating mutations and deep deletions. In terms of the relationship between curcumin and protein expression, only one protein change TGFB1 was detected by WB. This may be because of the complexity of post-transcriptional regulation of mRNA, which indicated that mRNA amplification may not be consistent with protein expression. We need to establish the specific molecular mechanism by which curcumin regulates hepatocellular carcinoma cells.

## Conclusions

In summary, by using Drugbank, STRING, CDC, and cBioPortal databases, we discovered the connectivity between curcumin and HCC. Curcumin has the potential to become an alternative chemotherapy or chemoprevention for HCC. The drug–target–cancer network analysis utilized in this study facilitated the testing and validation of reasonable hypotheses explaining curcumin-induced gene alterations in cancers by applying the available biological information in studies from bedside to bench. As advances in curcumin research using traditional experimental approaches continue, additional drug targets will

undoubtedly be identified, leading to improved curcumin-related genetic networks, signaling pathways, and cancer types.

## Abbreviations

DPTs

direct protein targets

IPTs

indirect protein targets

PPI

protein–protein interaction

KEGG

Kyoto Encyclopedia of Genes and Genomes

CTD

Comparative Toxicogenomics Database

HCC

hepatocellular carcinoma

cBioPortal

cBio Cancer Genomics Portal

OS

overall survival

FAN

functional/activity network

OD

optical density

## Declarations

### Ethics approval and consent to participate:

Not applicable.

### Consent for publication:

Not applicable.

### Availability of data and materials:

All of the data generated or analyzed during this study are included in this published article and its supplementary information files.

## Competing interests:

The authors declare that they have no competing interests.

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## Authors' contributions:

conceptualization, LZZ and YYZ; methodology, ZYL and JHT; data analysis, JYL; writing—original draft composition, LZZ; writing—review and editing, YYZ; supervision, YC; project administration, LZL; funding acquisition, LZZ. All authors read and approved the final manuscript.

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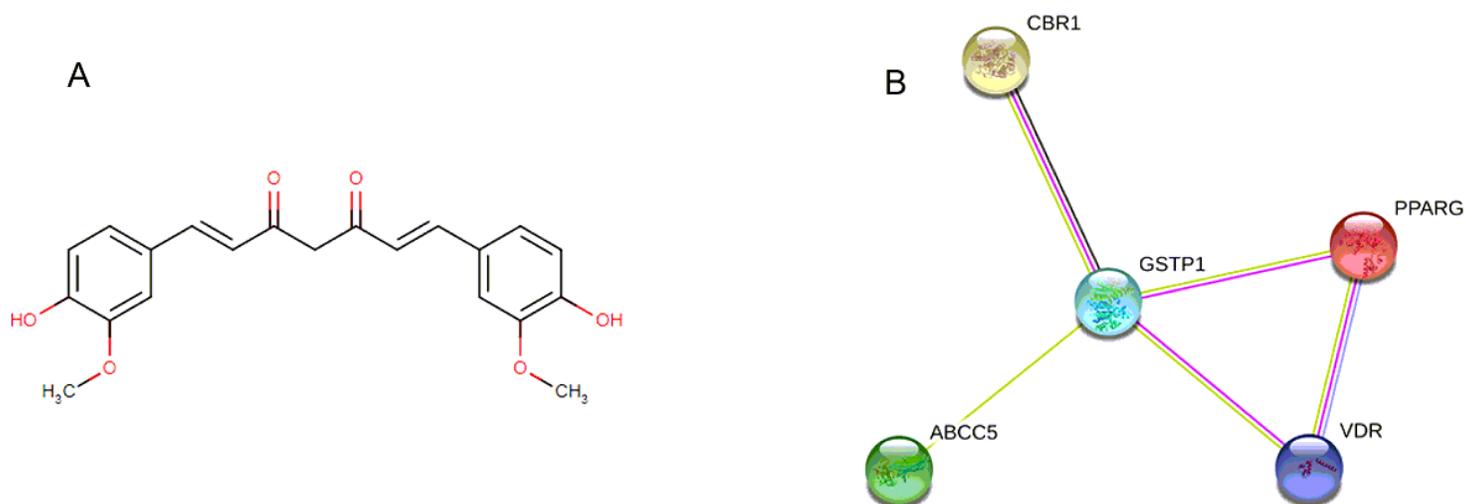
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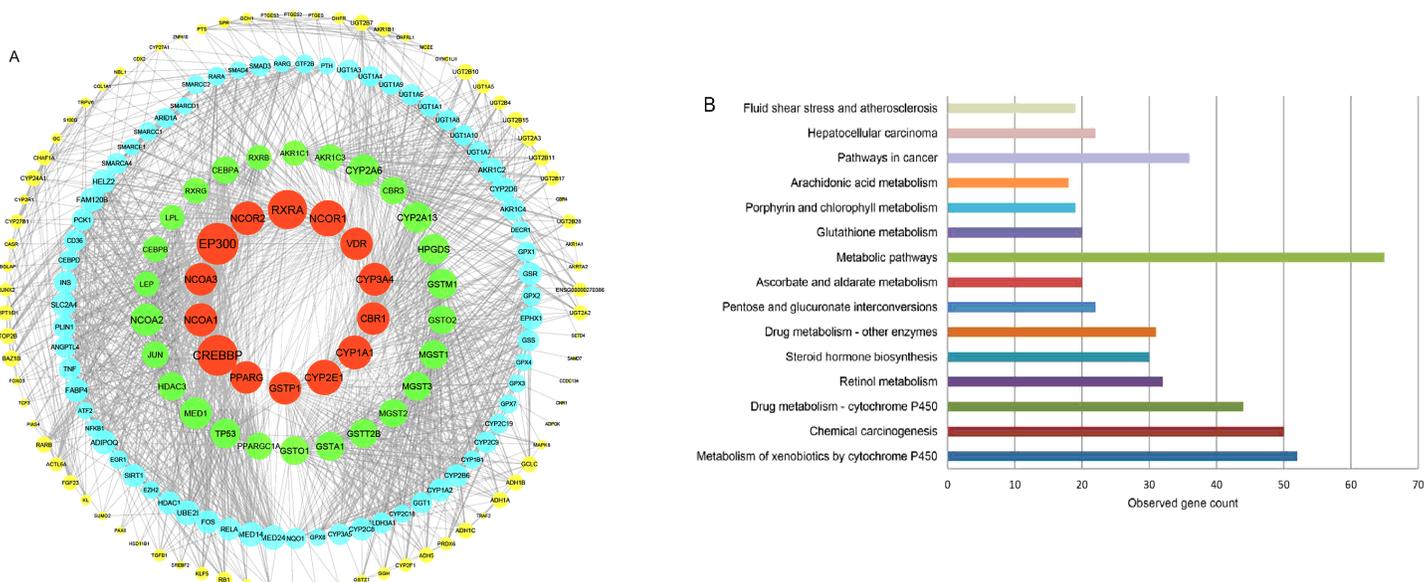
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## Figures



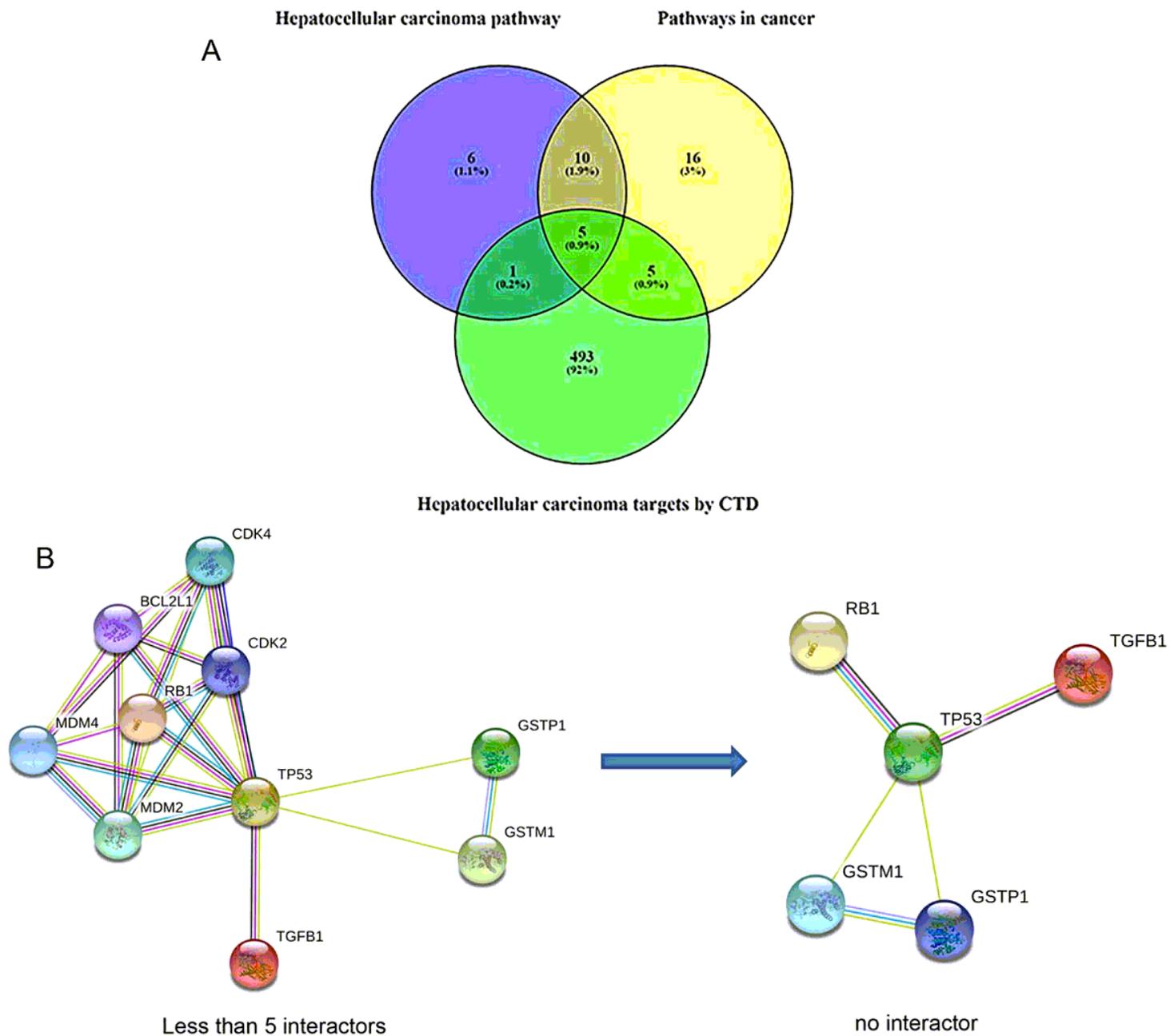
**Figure 1**

(A) The structure of curcumin. (B) The interactions of 5 DPTs of curcumin by STRING with a minimum required interaction score 0.2: purplish-red lines indicate experimentally determined values, red lines indicate gene fusions, green lines indicate textmining, blue lines indicate gene co-occurrence, and light blue lines indicate protein homology.



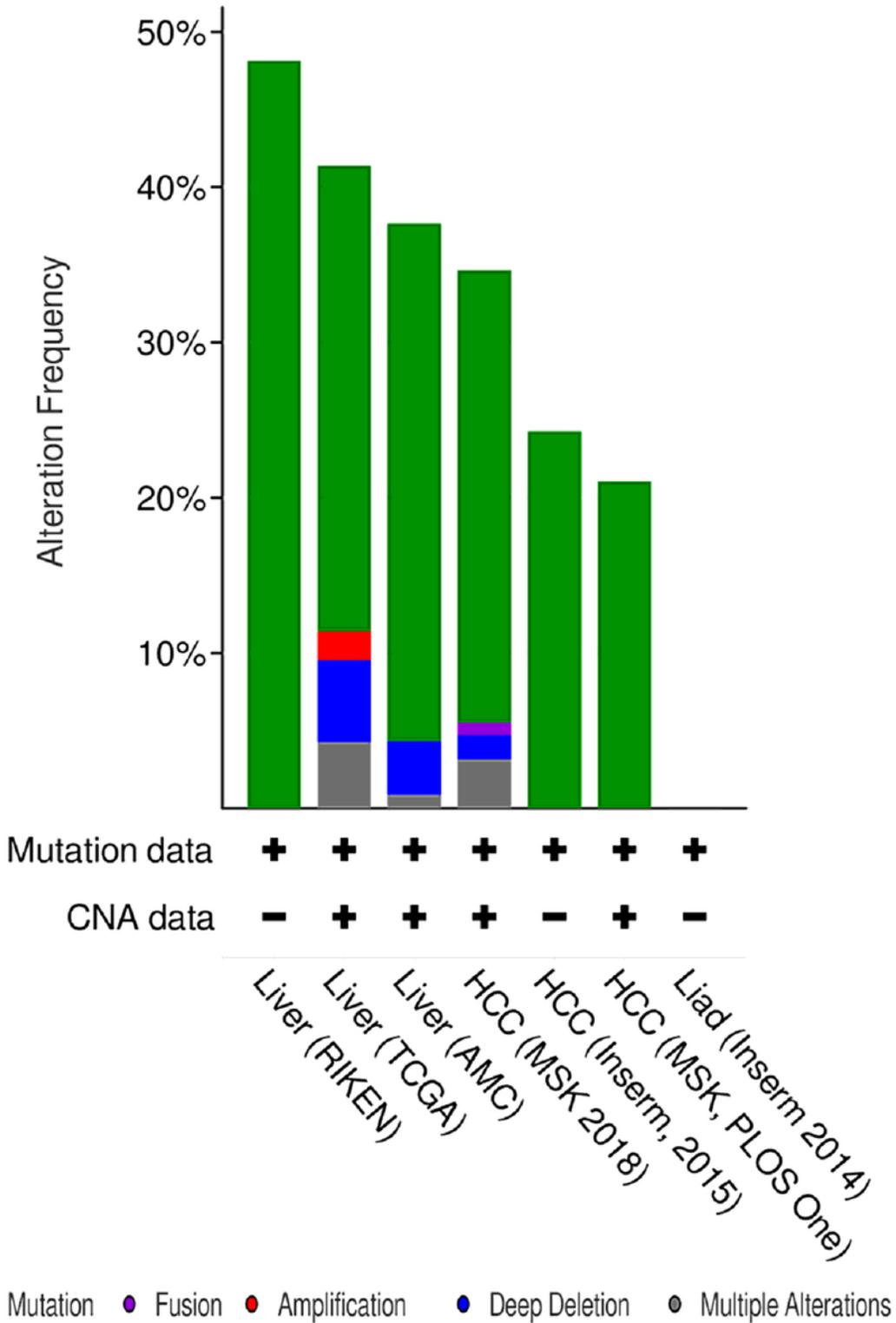
**Figure 2**

(A) PPI network of curcumin-mediated proteins analyzed by Cytoscape. Network nodes represent proteins, and edges represent protein-protein associations. High node degree values are represented by big sizes. Proteins with a node degree value  $\geq 50$  are shown in red, 30–49 in green, 15–29 in blue, and 1–14 in yellow. (B) The top 15 statistically enriched KEGG pathways and involved gene numbers.



**Figure 3**

(A) Venn diagrams for intersections of 3 gene sets. (B) A visual display of the network connected to 5 selected protein targets (TP53, RB1, TGFB1, GSTP1, and GSTM1). Neighboring proteins connected to the 5 query proteins, filtered from no more than 5 interactors to no interactor.



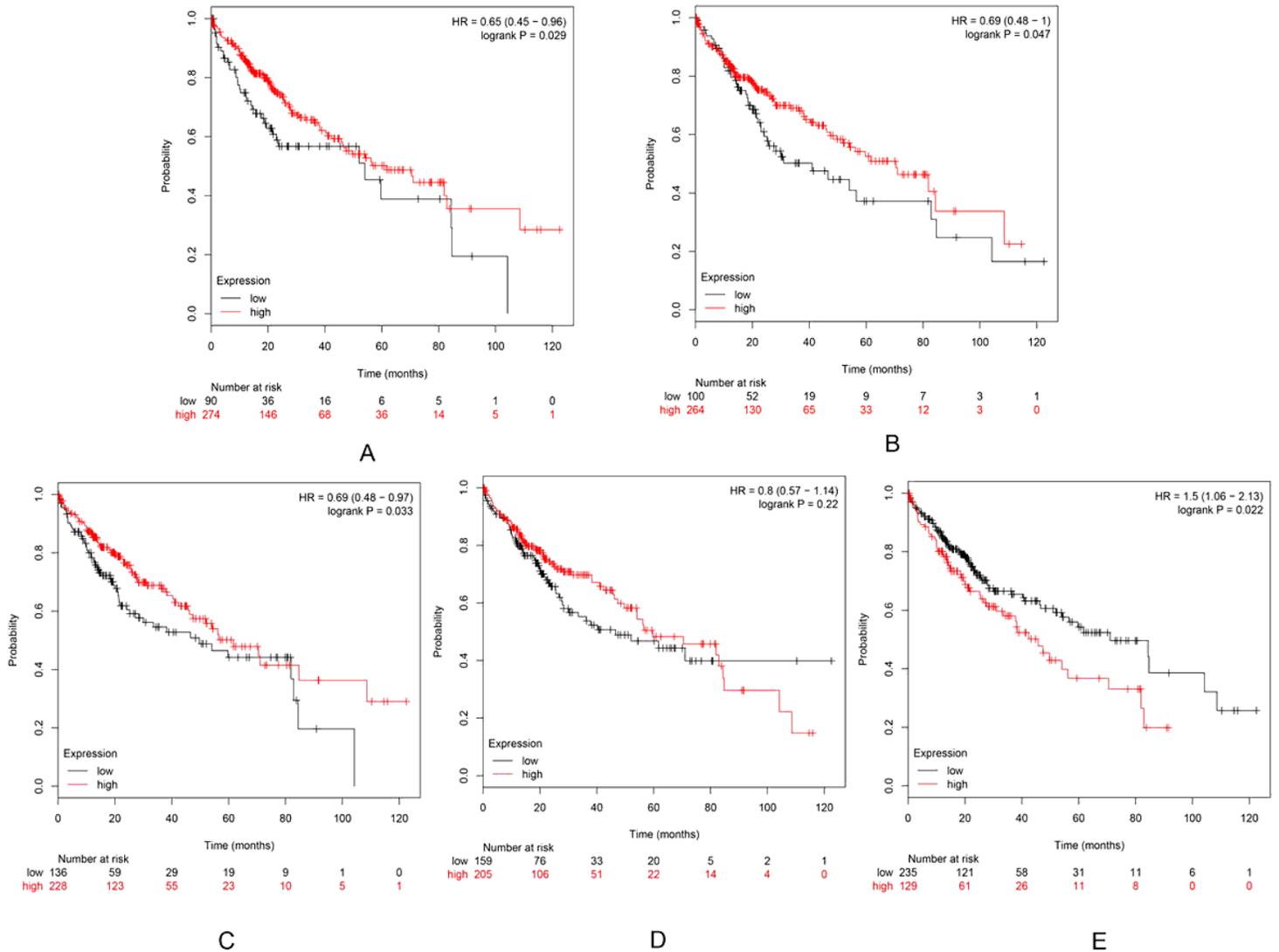
**Figure 4**

Overview of genetic alterations related to curcumin-associated genes in genomics data sets available in 7 different HCC studies in cBioPortal databases.



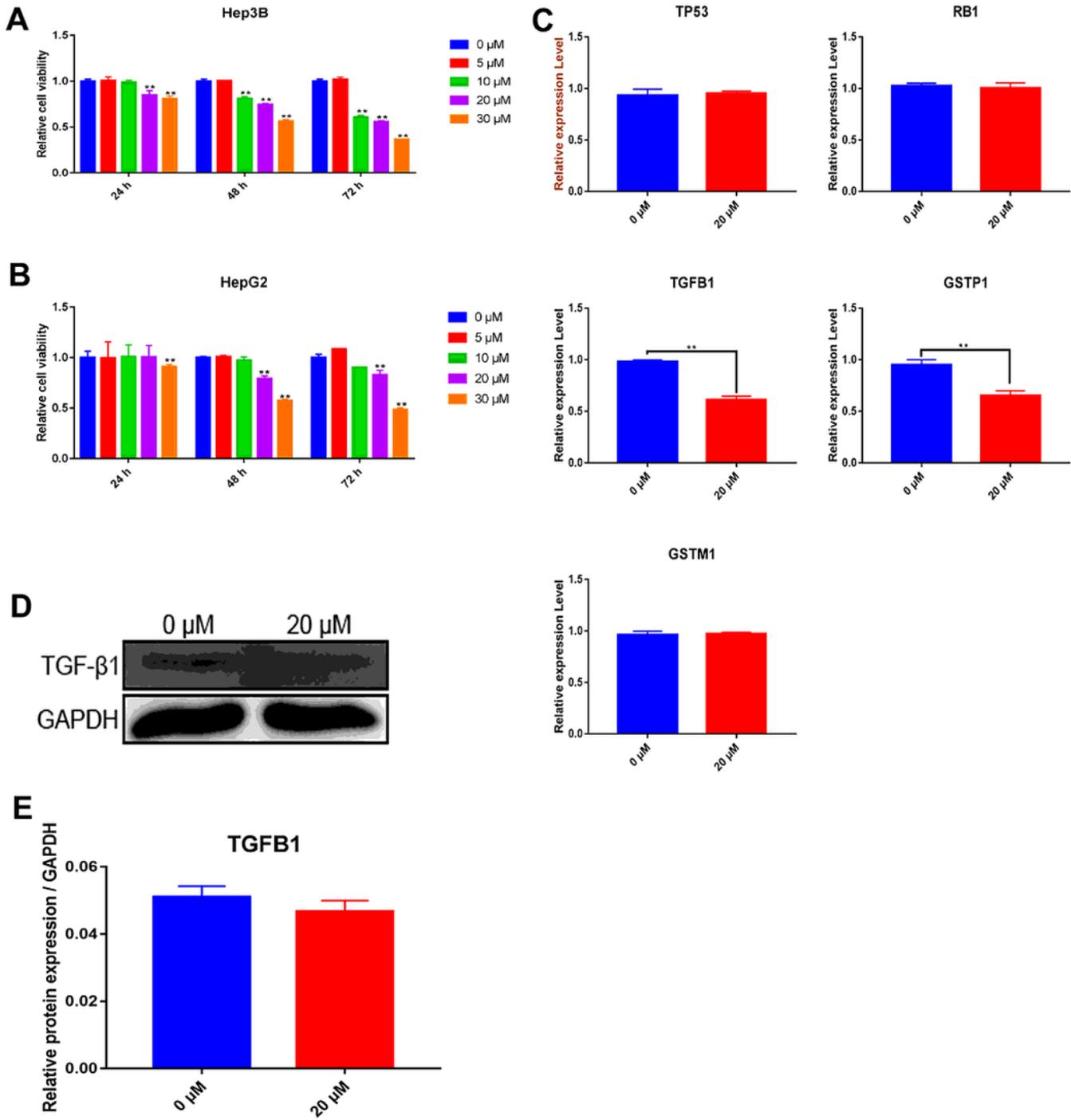
**Figure 5**

A visual heatmap of mRNA-level alterations based on 5 genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) across a HCC study (data taken from the Liver HCC (TCGA, Provisional) study) in cBioPortal databases. Each row represents a gene, and each column represents a tumor sample.



**Figure 6**

Survival analysis of 5 selected genes according to mRNA expression in HCC: (A) TP53, (B) RB1, (C) GSTM1, (D) GSTP1, and (E) TGFB1. Red lines indicate high levels of mRNA expression, while black lines indicate low levels of mRNA expression.



**Figure 7**

Validation experiment in vitro (A) Cell viability inhibition experiment of Hep3B. (B) Cell viability inhibition experiment of HepG2. (C) qPCR analysis of five overlapping genes. (D) Western blot analysis of TGFB1. (E) Quantitative analysis of TGFB1 protein expression.

## Supplementary Files

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