Functional drug–target–disease network analysis of gene–phenotype connectivity for curcumin in hepatocellular carcinoma

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Background: The anti-tumor properties of curcumin have been demonstrated for many types of cancer. However, a systematic functional and biological analysis of its target proteins has yet to be fully documented. 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 interactions between curcumin and DPTs and indirect protein targets (IPTs) weres documented. The protein–protein interaction (PPI) network of curcumin-mediated proteins was visualized using Cytoscape. 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, MTT, quantitative polymerase chain reaction (qPCR), and western blot (WB) analysis were used to validate the predicted results on hepatocellular carcinoma (HCC) cells. Results: A total of five 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 HCC (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 overall survival (OS) in HCC, whereas elevated mRNA levels of TGFB1 were correlated with poor prognosis. The viability of both HepG2 cells and Hep3B cells was significantly reduced by curcumin at concentrations of 20 or 30 μM after 48 or 72h of culture. At a concentration of 20 μM curcumin cultured for 48h, 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.

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Abstract

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Methods: Direct protein targets (DPTs) of curcumin were searched in the DrugBank database. Using the STRING database, the interactions between curcumin and DPTs and indirect protein targets (IPTs) weres documented. The protein–protein interaction (PPI) network of curcumin-mediated proteins was visualized using Cytoscape. 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, MTT, quantitative polymerase chain reaction (qPCR), and western blot (WB) analysis were used to validate the predicted results on hepatocellular carcinoma (HCC) cells.

Results: A total of five 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 HCC (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 overall survival (OS) in HCC, whereas elevated mRNA levels of TGFB1 were correlated with poor prognosis. The viability of both HepG2 cells and Hep3B cells was significantly reduced by curcumin at concentrations of 20 or 30 μM after 48 or 72h of culture. At a concentration of 20 μM curcumin cultured for 48h, 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.

Introduction

Primary liver cancer is the sixth most common cancer and the second largest cause of cancer mortality in the world (Ferlay et al. 2015). Hepatocellular carcinoma (HCC) constitutes approximately 80% of all primary liver cancer (McGlynn et al. 2015). The main causes of HCC are hepatitis B and C virus infection, alcoholic liver disease, non-alcoholic fatty liver diseases related to obesity and/or diabetes, aflatoxin B1 contamination of food, and less frequently, genetic predisposition. The incidence of HCC is increasing worldwide, mostly due to the spread of chronic infections with hepatitis B and C viruses. Most of these risk factors cause persistent inflammation in the liver, leading to the formation of liver cirrhosis and then HCC.

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 (Figure 2A). Extensive studies investigating curcumin over the past few decades revealed the health benefits of curcumin, including anticancer, anti-inflammatory, antioxidant, and hypoglycemic effects (Prasad et al. 2014). In recent years, curcumin has been increasingly recognized for its anti-tumor and chemopreventive properties, especially in gastrointestinal tumors (Huminiecki et al. 2017). For example, curcumin has shown chemopreventive effects in animal models of colon cancer (Kawamori et al. 1999), stomach cancer (Huang et al. 1994), and HCC (Chuang et al. 2000). Curcumin has been reported to decrease cell growth and induce apoptosis mainly through the inhibition of nuclear factor kappa-B (NFκB) (Ghasemi et al. 2019). Recent reports also indicate the curcumin could decrease tumor cell viability by inhibiting the activity of ATP synthase in isolated mitochondrial membranes which leads to a dramatic drop of ATP and a reduction of oxygen consumption (Bianchi et al. 2018). In addition, DYRK2 has been identified as a direct target of curcumin. By diminishes DYRK2-mediated 26S proteasome phosphorylation in cells, curcumin leading to reduced proteasome activity and inhibited cell proliferation (Banerjee et al. 2018). Several phase I and II clinical trials with curcumin have been conducted for the treatment of different types of cancer (Gupta et al. 2013; Hatcher et al. 2008).

Recent studies using data mining techniques for the analysis of specific bioinformatics domains have made great progress (Lan et al. 2018). 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 (Hsieh et al. 2016). In addition, an in-depth analysis of the databases may uncover, 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(Fu et al. 2019; Zhang et al. 2018). In this study, DrugBank was employed to broadly analyze curcumin and drug-target data to obtain related direct protein targets (DPTs). Interactions between DPTs and indirect protein targets (IPTs) were

119   predicted with the STRING database. The readout DPTs and IPTs were further analyzed for
120   functionality via Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment
121   analysis with STRING. The pivotal genes were obtained via intersections of the CTD and STRING
122   databases, and the genomic alterations were investigated using the cBioPortal database. Our
123   findings provide information to obtain a better understanding of the anti-tumor mechanisms of
124   curcumin and identify new targets in the treatment of HCC by curcumin.
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126

127   ## Materials and Methods
128
129   Search for DPTs of curcumin
130   The DrugBank database (https://www.drugbank.ca) is a richly annotated resource that combines
131   detailed drug data with comprehensive drug target and drug action information (Wishart et al.
132   2008). In this study, the latest release of DrugBank database (version 5.1.4) (Wishart et al. 2018)
133   was employed to search for the interactions between curcumin and its DPTs and generate a
134   curcumin-target network. The word "Curcumin" was searched as a keyword under drug
135   classification entry.
136
137   PPI network generation
138   The STRING database (http://www.string-db.org/) provides a critical assessment and integration
139   of protein-protein interactions, including direct (physical) as well as indirect (functional)
140   associations (Szklarczyk et al. 2015). Using the latest STRING database (version 11.0)(Szklarczyk
141   et al. 2019) search function, data underlying the interaction between DPTs and IPTs were first
142   generated for curcumin by setting a minimum required interaction score of 0.5 and a maximum
143   number of interactors of 50. The data were integrated into a curcumin-mediated network and
144   visualized using Cytoscape (version 3.7.1) (Su et al. 2014), which is an open-source software
145   platform for the visualization of complex networks and their integration with any attribute
146   (Shannon et al. 2003).
147
148   KEGG pathway enrichment analysis and overlapping HCC genes
149   Biochemical pathways linked to curcuminDPT/IPT interactions were navigated via the KEGG
150   pathway enrichment analysis tool in the STRING database. The top 15 pathways with a False
151   Discovery Rate (FDR) of less than 1.00E-10 were selected. To identify disease targets for
152   HCC,"Hepatocellular carcinoma" was searched for in CTD (http://www.ctdbase.org/). CTD is a
153   robust, publicly available database that aims to advance the understanding of environmental
154   exposures and their impacts on human health. It provides manually curated information about
155   chemical–gene/protein interactions, and chemical–disease and gene-disease relationships (Davis
156   et al. 2019). Overlapping genes of HCC and cancer-related pathways and HCC targets were
157   selected for further analysis.
158

159 Exploring cancer genomics data linked to curcumin
160 The cBioPortal (https://www.cbioportal.org/) for Cancer Genomics provides a web resource for
161 exploration, visualization, and analysis of multidimensional cancer genomics data (Cerami et al.
162 2012; Gao et al. 2013). In this study, the screened genes from the foregoing investigation were
163 assessed in all HCC studies available in cBioPortal databases. Using the portal search function,
164 curcumin-related genes in HCC were evaluated for genomic alteration, network analysis was
165 performed, and mutual exclusivity or concurrent relationships between gene pairs of the same gene
166 set were identified. The study with the largest sample size was chosen to analyze the screened
167 proteins in cBioPortal databases to further interpret the results.
168
169 Analysis of curcumin-associated genomics datasets and HCC survival
170 The Kaplan–Meier method (http://kmplot.com/)(Nagy et al. 2018) was used to evaluate the overall
171 survival (OS) in 364 HCC samples with altered genes. OS was defined as the time to death. A two-
172 tailed P value of less than 0.05 was considered statistically significant.
173
174 Cell culture
175 HepG2 and Hep3B cells were purchased from Cell Cook (http://www.cellcook.com/). They were
176 authenticated using short tandem repeat matching analysis and cultured in DMEM supplemented
177 with 10% v/v FBS and 1% v/v penicillin/streptomycin in a CO_2 incubator at 37°C and 95% relative
178 humidity.
179
180 Cell viability test
181 The MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide) assay was used to
182 assess cell viability. First, 100 μL of cells (about 5000–10,000 cells) was transfused into a 96-well
183 culture plate, which was then placed in a hatching house overnight for pre-culture (37°C, 5% CO_2).
184 According to the drug concentration, all cells were divided into five groups. After pre-culture, 10
185 μL of drug solution was added to every well (the drug concentrations were 0, 5, 10, 20, 30 μM)
186 and incubated at 37°C. After that, 10 μL MTT solution was added to each well at 24, 48, and 72 h
187 and incubated at 37°C for 4h. The absorbance value of each hole at 450nm was determined, and
188 the cell vitality value was calculated. The cell vitality formula is as follows: Cell viability% = (OD
189 in experimental group – OD in blank group)/(OD in control group – OD in blank group) × 100%.
190
191 Extraction of total RNA and reverse transcription-quantitative PCR (RT-qPCR)
192 Cells of diffetent group were collected after being treated and cultured, and total RNA was
193 extracted using TRIzol® reagent. Oligo (dT) primers and M-MLV reverse transcriptase were used
194 to synthesize cDNA under the instruction of protocol. PCR amplification was performed on an
195 RT-qPCR instrument with a GoTaq® qPCR Master mix. The relative gene expression of each
196 sample was determined with the 2-ΔΔCq method.
197
198 Western blot analysis

199 Protein extractions were prepared with a modified RIPA buffer (Beyotime Institute of
200 Biotechnology) with 0.5% SDS. Proteinase inhibitor cocktail (Beyotime Institute of
201 Biotechnology) were involved in the whole process. Protein samples was separated using
202 SDS-PAGE, and then transferred to a PVDF membrane. The membranes were blocked with 5%
203 BSA in TBS (containing 0.05% Tween-20) and incubated overnight with the primary antibodies.
204 After the membranes were washed, the membranes were incubated with horseradish peroxidase-
205 linked immunoglobulin G secondary antibody (Ms, Jackson ImmunoResearch) for 2h. The
206 membranes were developed using an enhanced chemiluminescence system (Thermo Scientific
207 Pierce).
208
209 **Results**
210
211 Characterization of curcumin DPTs
212 A flow chart of study steps was present in Figure 1. Drugbank was queried using curcumin as the
213 keyword to identify the bioactivities and to determine the DPTs of curcumin and retrieve relevant
214 information. The result showed an accession number of DB11672 and classified curcumin as a
215 highly pleiotropic molecule with anti-tumor, antibacterial, anti-inflammatory, hypoglycemic,
216 antioxidant, wound-healing, and antimicrobial activities (Gupta et al. 2013). Clinical data showed
217 that curcumin is undergoing clinical trials for colon cancer (NCT02724202), prostate cancer
218 (NCT03769766), breast cancer (NCT03980509), and lung cancer (NCT02321293). Subsequent
219 screening demonstrated five DPTs in human beings. Table 1 summarizes the five DPTs of
220 curcumin: PPARG, VDR, ABCC5, CBR1, and GSTP1. In addition, the interactions between five
221 DPTs were analyzed by STRING and illustrated in Figure 2B.
222
223 Characterization of curcumin IPTs and visualization of PPI network construction
224 Expanding the search using STRING database, a total of 204 target proteins of curcumin were
225 detected, including 199 IPTs, which were related to five DPTs (Table S1). The dataset obtained
226 was integrated to construct a biological network using Cytoscape. As shown in Figure 2C, 1962
227 PPI pairs were found in the network of curcumin-mediated proteins (Table S2). In this network,
228 nodes represent proteins, and edges denote protein-protein associations. In addition, the node
229 degree, which indicates the centrality of proteins, was calculated using CentiScaPe 2.2 (Table S3).
230 As a result, 14 proteins including four DPT targets (GSTP1, VDR, CBR1, and PPARG) with a
231 degree value ≥ 50 are shown in Table 2 and displayed in Figure 2C with larger node sizes.
232
233 KEGG enrichment pathway analysis of proteins connected to curcumin
234 To identify the functional features of curcumin-mediated targets, KEGG pathway enrichment
235 analysis was performed using STRING. Target genes were found in 121 molecular pathways in
236 KEGG enrichment (Table S4). As shown in Table 3 and Figure 2D, the top 15 KEGG pathways
237 connected to curcumin-mediated proteins included metabolism of xenobiotics by cytochrome
238 P450 (52 genes), chemical carcinogenesis (50 genes), drug metabolism-cytochrome P450 (44

239   genes), retinol metabolism (32 genes), steroid hormone biosynthesis (30 genes), drug metabolism-
240   other enzymes (31 genes), pentose and glucuronate interconversion (22 genes), ascorbate and
241   aldarate metabolism (20 genes), metabolic pathways (65 genes), glutathione metabolism (20
242   genes), porphyrin and chlorophyll metabolism (19 genes), arachidonic acid metabolism (18 genes),
243   pathways in cancer (36 genes), HCC (22 genes), and fluid shear stress and atherosclerosis (19
244   genes). These KEGG enrichment pathways showed functional features of curcumin gene sets and
245   indicated that curcumin-mediated proteins were mainly associated with basal metabolism and
246   cancer-related pathways. Based on the anti-tumor properties of curcumin reported in diverse
247   malignant tumors, two pathways were selected, including pathways in cancer (36 genes) and HCC
248   (22 genes). This result suggested that HCC might be used as a phenotype connected to curcumin-
249   mediated proteins. In addition, to further identify disease targets for HCC, the phrase "Carcinoma,
250   Hepatocellular" was searched in CTD, and a total of 505 genes with either a curated association
251   or an inferred association via a curated chemical interaction with the disease were identified (Table
252   S5). These genes were marked with "T," which indicates a gene that is or may be a therapeutic
253   disease target, or "M," which denotes a gene that may be a disease biomarker or involved in the
254   etiology of a disease. Five overlapping genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) resulting
255   from the intersections between pathways in cancer (36 genes), HCC pathway (22 genes), and
256   targets for HCC by CTD (505 genes) were visualized using Venn diagrams and STRING (Figure
257   2E and 2F).

258

259   Genetic alterations connected with curcumin-associated genes in HCC
260   To further validate the link between curcumin-associated genes and HCC, cBioPortal databases
261   were used to explore the five genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) associated with
262   curcumin in HCC. Seven HCC studies were included in cBioPortal (Ahn et al. 2014; Fujimoto et
263   al. 2012; Harding et al. 2019; Pilati et al. 2014; Schulze et al. 2015; Zheng et al. 2018), and the
264   five selected overlapping genes were queried. The results showed that 363 (33%) of the 1135
265   samples in seven studies had alterations in one or more of these genes. Alterations ranged from
266   0.2% to 29% for gene sets submitted for analysis (Figure 3A). The five genes (TP53, RB1, TGFB1,
267   GSTP1 and GSTM1) carried five gene pairs showing mutual exclusivity alterations, and another
268   five gene pairs showed concurrent alterations (with TP53 and RB1 showing a statistically
269   significant alteration, Table 4). Furthermore, a liver HCC study (TCGA, Provisional), which
270   carried the largest sample size, was selected to analyze the screened proteins in cBioPortal
271   databases. The results showed that 156 (35%) patients/samples carried an alteration in at least one
272   of the five genes queried using OncoPrint; the frequency of alteration in each gene is shown in
273   Figure 2G. Most gene alterations included mutations and deletions. Most of the TP53 alterations
274   were classified into missense mutations, truncating mutations, and deep deletions. Gene changes
275   associated with RB1 included truncating mutations and deep deletions, whereas for TGFB1,
276   GSTP1 and GSTM1 amplifications were the most common gene alterations.

277

278   Curcumin-associated genes and survival in HCC

279   Kaplan–Meier analysis was used to perform survival analysis of patients with HCC based on the
280   five selected genes (TP53, RB1, TGFB1, GSTP1 and GSTM1). A total of 364 patients were
281   involved in this analysis for OS (Menyhárt et al. 2018). As shown in Figure 3B–F, high mRNA
282   levels of TP53, RB1, and GSTM1 indicated increased OS in HCC, whereas elevated mRNA levels
283   of TGFB1 were correlated with poor prognosis in the same group of patients.
284
285   Curcumin-induced decrease in the activity of HepG2 and Hep3B cells
286   The test results showed that cell viability appeared dependent on the concentration of curcumin.
287   For Hep3B cells, curcumin (10 μM) significantly decreased cell viability after 48 and 72 h, and 20
288   and 30 μM of curcumin were found to significantly reduce cell viability after 24, 48, and 72 h
289   (Figure 4A). For HepG2 cells, curcumin (20 and 30 μM) significantly decreased the cell viability
290   after 48 and 72 h (Figure 4B). According to these results, the concentration of curcumin was chosen
291   to be 20 μM and the duration of treatment was chosen to be 48h for subsequent testing.
292
293   Curcumin regulation of TGFB1 and GSTP1 expression in Hep3B cells
294   The expression of TP53, RB1, TGFB1, GSTP1, and GSTM1 was assessed using qPCR in Hep3B
295   cells treated with 20 μM curcumin for 48 h. The results showed that 20 μM curcumin regulated
296   the expression of TGFB1 and GSTP1 in Hep3B cells, but it had no significant effect on the
297   expression of other genes (Figure 4C and 4D). After treatment of Hep3B cells with 0 and 20 μM
298   curcumin for 48 h, the levels of TGFB1 and GSTP1 protein were detected via western blot.
299   Because the protein background expression of GSTP1 was low, the expression of GSTP1 at the
300   protein level failed to be detected. The results showed that 20 μM curcumin did not regulate the
301   expression of TGFB1 in HepG2 cells (Figure 4E, 4F), but moderately inhibited the expression of
302   TGFB1 in Hep3B cells (Figure 4G, 4H).
303

304   **Discussion**
305
306   Over the past several years, numerous studies have evaluated the effects of curcumin and its
307   analogs in diverse cancers *in vitro* and *in vivo*. According to these studies, the potential use of
308   curcumin as a chemopreventive and therapeutic agent in cancers depends on its potent antioxidant
309   and anti-inflammatory activities as well as its ability to modulate various molecular signaling
310   mechanisms (Gupta et al. 2013; Hatcher et al. 2008; Huminiecki et al. 2017; Prasad et al. 2014).
311   Nevertheless, the mechanism underlying the wide range of anti-cancer effects of curcumin remains
312   incomplete. Current knowledge about curcumin's functions and mechanisms is based on
313   conventional experiments, which have yet to be fully integrated and understood. Therefore, new
314   analytical methods are needed to correlate curcumin with its target proteins and the observed
315   biological effects. Functional/activity network (FAN)(Hsieh et al. 2016) is a new analytical
316   method, which elucidates the molecular mechanisms of a drug and its association with clinical
317   outcomes in cancer by using a set of web-based tools, such as DrugBank, STRING, cBioPortal,
318   CTD, or Cytoscape. Using this method, a functional drug–target–cancer network analysis of gene–

319 phenotype connectivity associated with curcumin was conducted in this study. This study bridged
320 curcumin with its primary or secondary targets and illustrated the underlying mechanisms of
321 curcumin and its clinical outcomes in HCC.
322 The effects of curcumin and its analogs have been a subject of investigation over the past decade
323 in preclinical models of HCC (Darvesh et al. 2012), but research in this field is far from complete.
324 A search of PubMed using "curcumin" as the keyword returned more than 13000 publications,
325 whereas the phrase "curcumin and hepatocellular carcinoma" returned only 192 publications. By
326 using FAN, more direct mechanisms may be mined with reasonable experimental feasibility to
327 validate hypotheses explaining the effects of curcumin in HCC.
328 In this study, the feasibility of FAN analysis for identifying the underlying connectivity between
329 curcumin and HCC was demonstrated.A network including five genes (TP53, RB1, TGFB1,
330 GSTP1, and GSTM1) as targets of curcumin in HCC   was identified via a functional drug–target–
331 cancer network analysis. Among the five genes, TP53 and RB1 are tumor suppressor genes, which
332 is consistent with the finding in this study that their high transcriptional expression is correlated
333 with better OS in HCC. Similar to other cancers, TP53 and RB1 are the most commonly inactivated
334 or mutated genes in the case of HCC. Moreover, mutual exclusivity analysis revealed a tendency
335 toward concurrence between TP53 and RB1. Previous studies indicated that curcumin may inhibit
336 cancer growth and induce apoptosis in colon cancer cells (Dasiram et al. 2017), ameliorate the *in*
337 *vitro* efficacy of carfilzomib in human multiple myeloma cells (Allegra et al. 2018), promote
338 apoptosis in non-small cell lung cancer (Ye et al. 2015), and inhibit cell growth in nasopharyngeal
339 carcinoma mediated via the TP53 signaling pathway(Wu et al. 2014). Debata et al. reported that
340 the sunitinib-curcumin combination was effective in restoring the tumor suppressor activity of the
341 RB gene in renal cancer cells (Debata et al. 2013). Furthermore, Su et al. reported that curcumin
342 significantly enhanced p53 or markedly inhibited the RB pathway by suppressing RB
343 phosphorylation in the signaling pathways of glioblastoma (Su et al. 2010). These studies
344 demonstrated the anti-tumor activity of curcumin and prompted further investigation of curcumin
345 in HCC.
346 In the case of HCC, most of the genetic alterations in TGFB1, GSTP1, and GSTM1 were
347 amplifications, which may cause increased expression. However, the prognostic significance
348 varied in this study. The amplification of TGFB1 indicated a worse OS in HCC, whereas
349 amplifications of GSTM1 and GSTP1 (not statistically significant) indicated better prognosis.
350 TGFB1 is a pleiotropic gene with a dual role in hepatocarcinogenesis: apoptosis induction in early
351 phases, but promotion of tumorigenesis in cells with mechanisms to overcome the suppressor
352 effects (Moreno-Caceres et al. 2017). Recent studies found that the expression of TGFB1 genes
353 were downregulated in breast cancer cells treated with curcumin (Calaf & Roy 2017). Glutathione
354 S-transferases (GSTs) are a family of phase II detoxification enzymes that catalyze the conjugation
355 of a wide variety of endogenous and exogenous toxins. A previous study showed that inter-
356 individual GST variation plays a central role in reducing cell exposure to carcinogens (Di Pietro
357 et al. 2010). For example, reduced GSTP1 expression may contribute to oxidative stress in HCC
358 (Li et al. 2013). Similar results were observed in breast cancer cells and curcumin-activated GSTP1

359 expression via antioxidant response element (Nishinaka et al. 2007). However, studies linking
360 curcumin and GST genes in HCC are rare, underscoring the need for further investigation in this
361 field.
362 These results were partially confirmed by *in vitro* experiments. HepG2 and Hep3B were used as
363 the experimental objects because of their advantages of fast growth and easy passage. The present
364 study confirmed the inhibitory effect of curcumin on liver cancer cells, and this inhibition
365 increased with time and concentration. In the liver HCC study (TCGA, Provisional), three genes
366 (TGFB1, GSTP1, and GSTM1) showed amplification. For this reason, changes in these amplified
367 genes were more easily detected using PCR. This was confirmed in the present study when it was
368 found that two genes prone to amplification were suppressed in HCC cells treated with curcumin.
369 The other genes, however, did not show changes in expression because the main means of mutation
370 was not amplification mutations but truncating mutations and deep deletions. In terms of the
371 relationship between curcumin and protein expression, only one protein change TGFB1 was
372 detected by western blot. This may be because of the complexity of post-transcriptional regulation
373 of mRNA, meaning that mRNA amplification may not be consistent with protein expression. The
374 results of this study showed that TGFB1 expression was inconsistent at mRNA and protein levels
375 after curcumin treatment, possibly because TGFB1 may be regulated by intracellular activators. In
376 addition, although mRNA concentration is widely used as a surrogate for protein abundance,
377 studies comparing mRNA and protein expression on a global scale indicate that mRNA levels only
378 partly correlate with the corresponding protein concentrations. It has been estimated that protein
379 concentrations are determined by the corresponding mRNA concentrations by only 20%–40% (Nie
380 et al. 2006; Tian et al. 2004).
381 Furthermore, studies have proved that TGFB1 has a dual role in the development and progression
382 of HCC. In the previous analysis, elevated mRNA levels of TGFB1 were correlated with poor
383 prognosis in HCC patients. This may be because TGFB1 promoted the growth, invasion, and
384 metastasis of HCC cells through the epithelial-mesenchymal transition (Bierie & Moses 2006;
385 Massagué 2008; Matsuura et al.). Regarding HCC cell levels, TGFB1 was a potent growth
386 inhibitor and induces apoptosis in these cells, so it is regarded as a tumor-suppressive cytokine
387 (Senturk et al. 2010).Therefore, further studies are needed to validate and establish the specific
388 molecular mechanisms by which curcumin regulates HCC cells.
389
390
391

392 **Conclusions**

393
394 In summary, by using DrugBank, STRING, CDC, and cBioPortal databases, the connectivity
395 between curcumin and HCC was discovered. Curcumin has the potential to become an alternative
396 chemotherapy or chemoprevention treatment for HCC. The drug–target–cancer network analysis
397 utilized in this study facilitated the testing and validation of reasonable hypotheses explaining
398 curcumin-induced gene alterations in cancers by applying the available biological information in

399 studies from bedside to bench. As advances in curcumin research using traditional experimental
400 approaches continue, additional drug targets will undoubtedly be identified, leading to improved
401 curcumin-related genetic networks, signaling pathways, and cancer types.
402
403

404 **List of abbreviations**
405
406 DPTs: direct protein targets
407 IPTs: indirect protein targets
408 PPI: protein–protein interaction
409 KEGG: Kyoto Encyclopedia of Genes and Genomes
410 CTD: Comparative Toxicogenomics Database
411 HCC: hepatocellular carcinoma
412 cBioPortal: cBio Cancer Genomics Portal
413 OS: overall survival
414 FAN: functional/activity network
415 OD: optical density
416 MTT: (3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide)
417

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419

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Figure 1

Figure 1

Workflow chart of study.
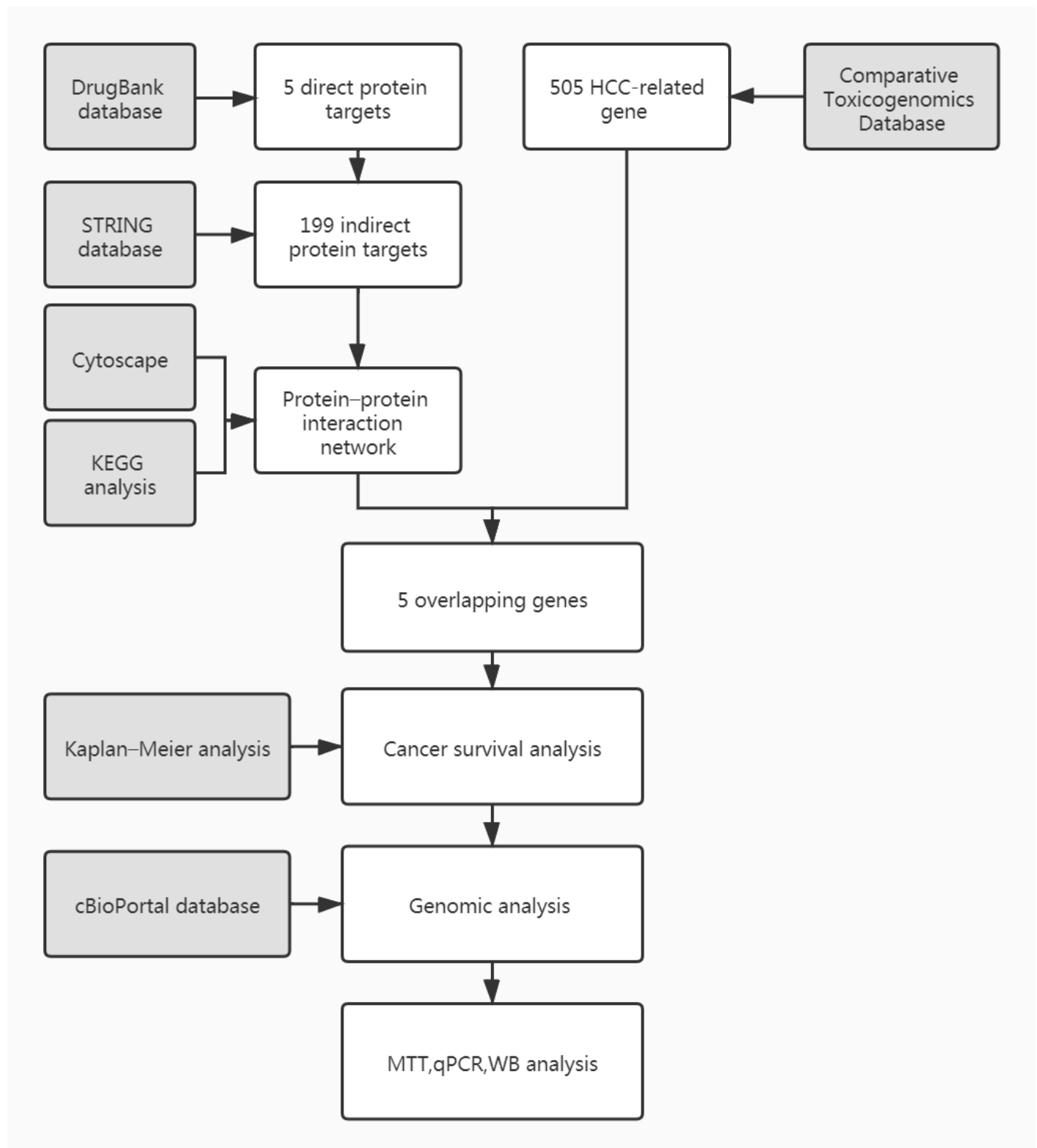
Figure 2

Figure 2

(A) The structure of curcumin. (B) The interactions of 5 DPTs of curcumin by STRING with a minimum required interaction score 0.2.(C) 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. (D) The top 15 statistically enriched KEGG pathways and involved gene numbers.(E) Venn diagrams for intersections of 3 gene sets. (F) 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.(G) Overview of genetic alterations related to curcumin-associated genes in genomics data sets available in 7 different HCC studies in cBioPortal databases.

A

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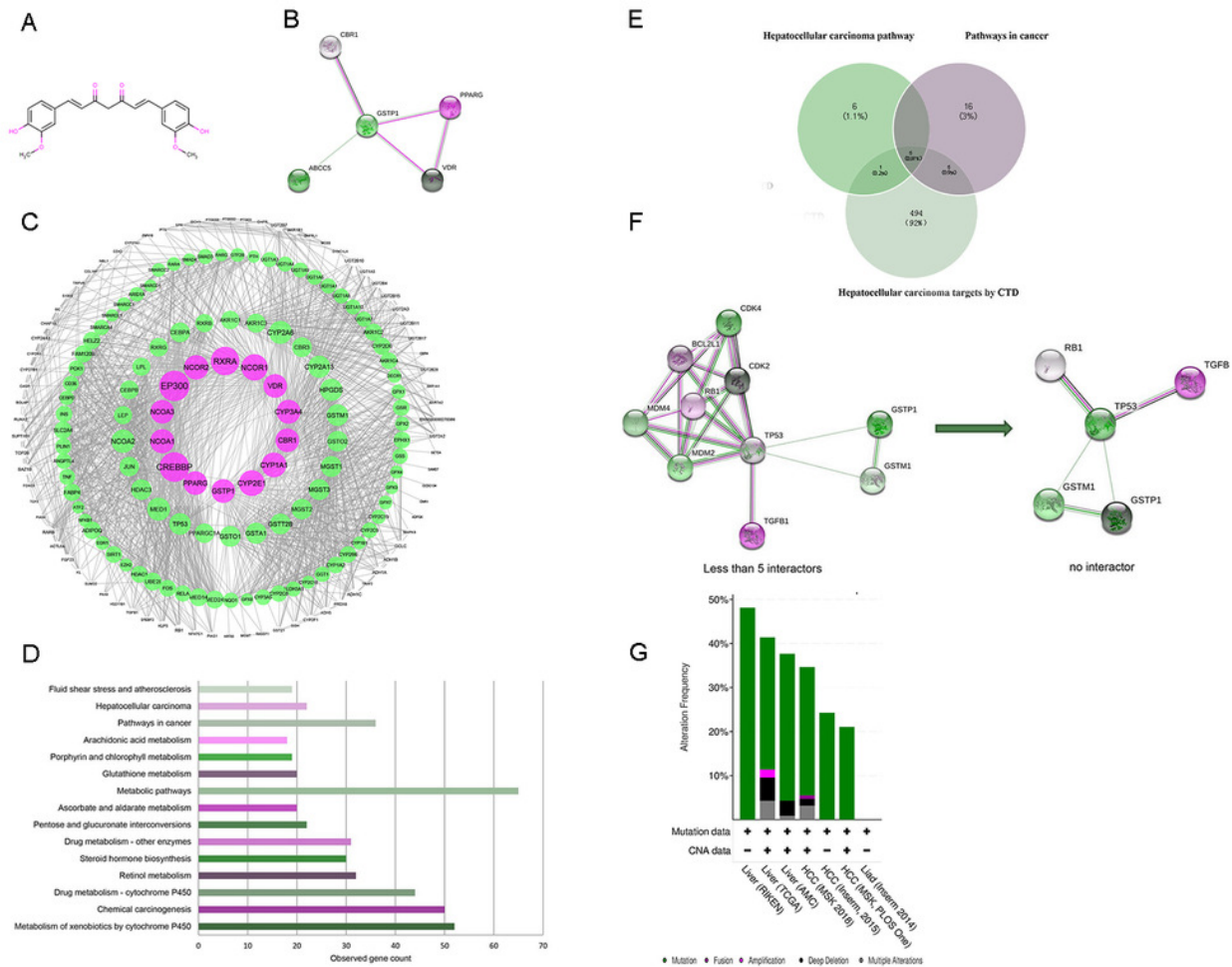
Figure 3

Figure 3

(A)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.Survival analysis of 5 selected genes according to mRNA expression in HCC: (B) TP53, (C) RB1, (D) GSTM1, (E) GSTP1, and (F) TGFB1. Magenta lines indicate high levels of mRNA expression, while black lines indicate low levels of mRNA expression.
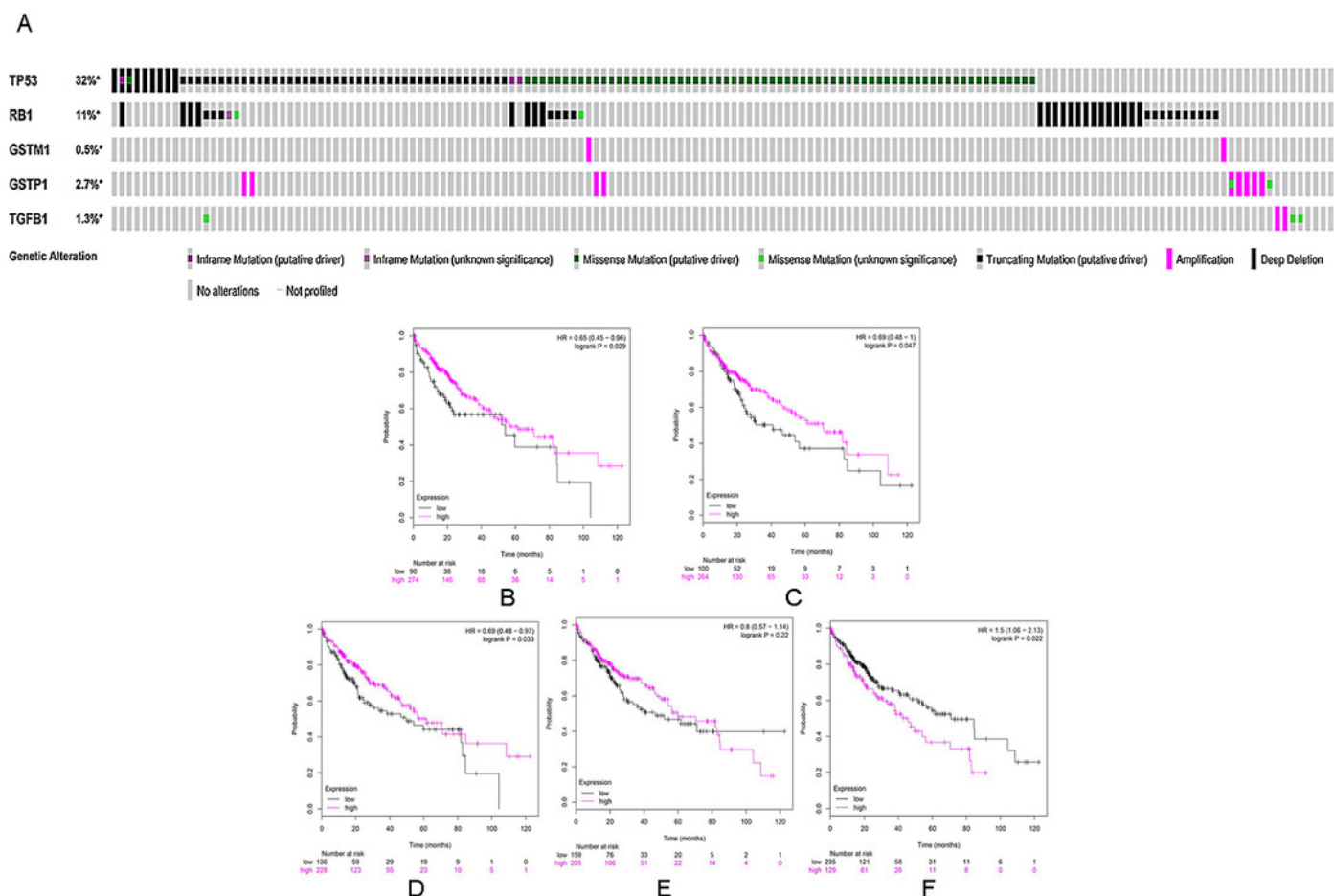
Figure 4

Figure 4

Validation experiment in vitro (A) Cell viability inhibition experiment of HepG2. (B) Cell viability inhibition experiment of Hep3B. (C) qPCR analysis of five overlapping genes in HepG2. (D) qPCR analysis of five overlapping genes in Hep3B.(E)Western blot analysis of TGFB1 in HepG2. (F) Quantitative analysis of TGFB1 protein expression in HepG2.(E)Western blot analysis of TGFB1 in Hep3B. (F) Quantitative analysis of TGFB1 protein expression in Hep3B.
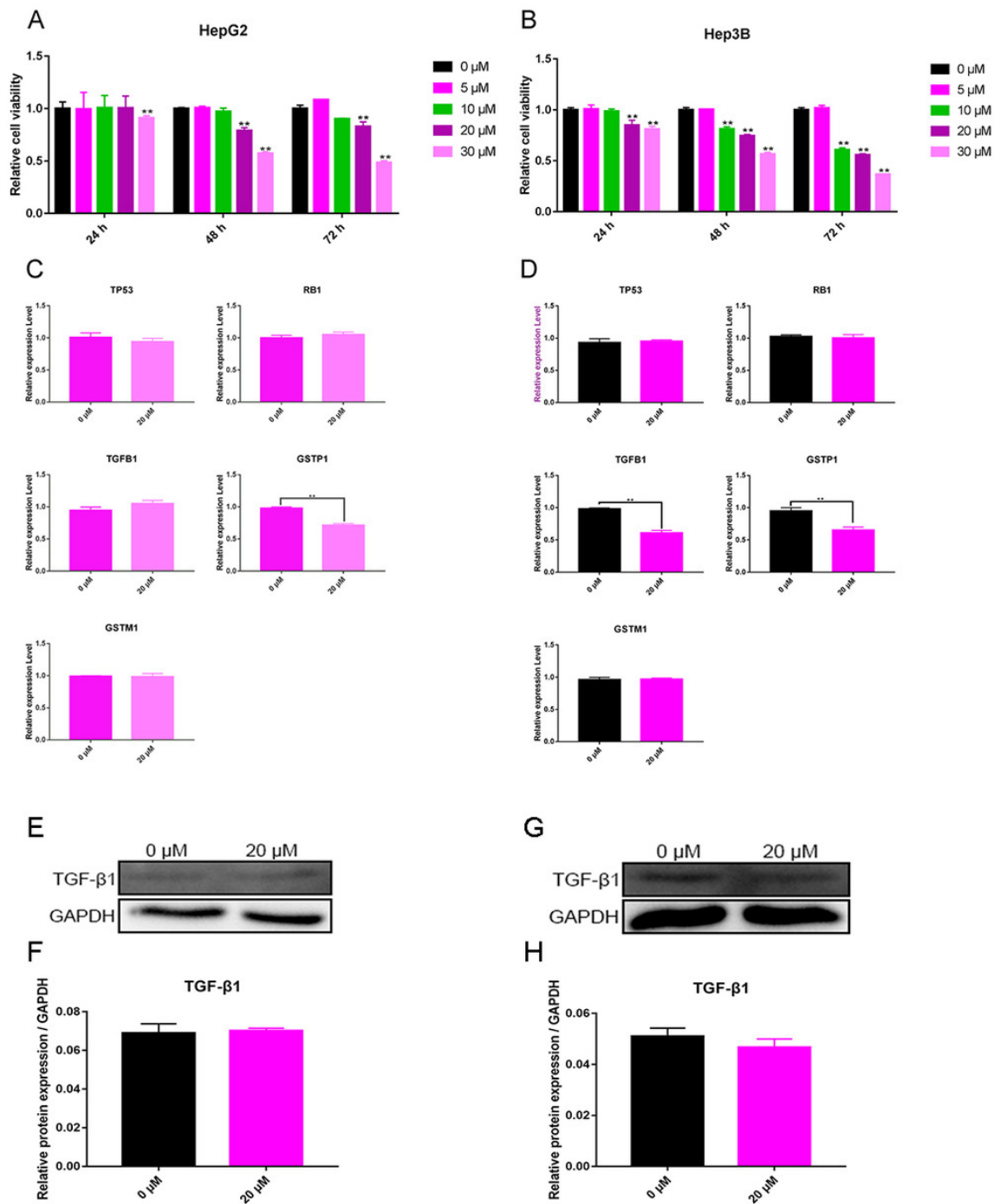
Table 1

Direct protein targets of curcumin identified using DrugBank

1 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

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Table 2

Protein targets with degree value ≥50

1 Table 2. Protein targets with degree value ≥50

Gene	Node degree value	Gene	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

Table 3

Top 15 enriched KEGG pathways identified using STRING

1 Table 3. Top 15 enriched KEGG pathways identified using STRING

Pathways	gene count	FDR	matching proteins
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,UGT2B 15,UGT2B17,UGT2B28,UGT2B4,UGT2B7
Drug metabolism - cytochrome P450	44	1.30E-57	ADH1A,ADH1B,ADH1C,ADH5,ALDH3A1,CYP 1A2,CYP2A6,CYP2B6,CYP2C19,CYP2C8,CYP2 C9,CYP2D6,CYP2E1,CYP3A4,CYP3A5,ENSG0 0000270386,GSTA1,GSTM1,GSTO1,GSTO2,GS TP1,GSTT2B,HPGDS,MGST1,MGST2,MGST3, UGT1A1,UGT1A10,UGT1A3,UGT1A4,UGT1A5 ,UGT1A6,UGT1A7,UGT1A8,UGT1A9,UGT2A2, UGT2A3,UGT2B10,UGT2B11,UGT2B15,UGT2 B17,UGT2B28,UGT2B4,UGT2B7
Retinol metabolism	32	8.99E-39	ADH1A,ADH1B,ADH1C,ADH5,CYP1A1,CYP1 A2,CYP2A6,CYP2B6,CYP2C18,CYP2C8,CYP2 C9,CYP3A4,CYP3A5,ENSG00000270386,UGT1 A1,UGT1A10,UGT1A3,UGT1A4,UGT1A5,UGT 1A6,UGT1A7,UGT1A8,UGT1A9,UGT2A2,UGT 2A3,UGT2B10,UGT2B11,UGT2B15,UGT2B17, UGT2B28,UGT2B4,UGT2B7
Steroid	30	2.17E-36	AKR1C1,AKR1C2,AKR1C3,AKR1C4,CYP1A1,

hormone biosynthesis			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 and glucuronate interconversions	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 and aldarate	20	1.50E-26	ALDH9A1,ENSG00000270386,UGT1A1,UGT1A10,UGT1A3,UGT1A4,UGT1A5,UGT1A6,UGT1

metabolism			A7,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 and chlorophyll metabolism	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	19	2.45E-14	FOS,GSTA1,GSTM1,GSTO1,GSTO2,GSTP1,GS

stress	TT2B,JUN,MAPK8,MGST1,MGST2,MGST3,NF
andatherosclero	KB1,NQO1,PIAS4,RELA,SUMO2,TNF,TP53
sis	

2 FDR, false discovery rate

Table 4<small>(on next page)</small>

Table 4

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

1 Table 4. Mutual exclusivity analysis of 5 genes (TP53, RB1, TGFB1, GSTP1, and GSTM1) in 7

2 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