Systematic Elucidation of the Mechanism of Action of Curcumin Against Colorectal Cancer via Network Pharmacology Approach

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Research Article

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Abstract

Background: Curcumin is a potential drug for the treatment of colorectal cancer (CRC). Its mechanism of action has not been elucidated.

Aim: To investigate the mechanism of action of curcumin in the treatment of CRC via network pharmacology, molecular docking and experimental verification.

Methods: The targets of curcumin and CRC were obtained from the public databases. The component-targets network of curcumin in the treatment of CRC was constructed by Cytoscape v3.7.2. Through protein-protein interaction (PPI), the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG), important targets and signaling pathways related to CRC treatment were identified. Finally, the results were verified by molecular docking and in vitro experiments.

Results: A total of 30 potential targets of curcumin for CRC treatment were collectedThe core targets included AKT1, EGFR and STAT3 were identified. GO function enrichment analysis showed 140 items, and KEGG pathway enrichment analysis showed 61 signaling pathways, that were related to the regulation of protein kinase activity, negative regulation of apoptosis process, cancer signaling pathway and PI3K-Akt signali-ng pathway. In vitro experimental verification showed that curcumin could promote the apoptosis of CRC cells, and the key proteins of these signaling pathways were differentially expressed.

Conclusion: This study explored the targets and pathways of curcumin in the treatment of colorectal cancer. In vitro experiments showed that curcumin has a therapeutic effect against CRC by inhibiting PI3K-Akt signaling pathway. Our results will lay a foundation for subsequent clinical research and drug development.

Introduction

Colorectal cancer (CRC) is a complex heterogeneous disease involving multiple genes and epigenetic factors(Duan et al. 2020). According to a recent report, there are 147950 new CRC cases that resulted in 53200 deaths in the United States in 2020(Lamichhane et al. 2020). The incidence of CRC is the top three in all tumors and the mortality is the second, which seriously endangered human health(Bray et al. 2018; Wong et al. 2019). At present, surgery, chemotherapy, radiotherapy, targeted therapy and immunotherapy are the mainstream treatments for CRC. However, long-term use of these therapies will lead to serious side effects, including nausea and vomiting, oral ulcer, diarrhea, bone marrow suppression and immunosuppression. Drug resistance is commonly occurred in CRC(Tolba 2020; Kong et al. 2020). For patients with advanced CRC, there is no effective treatment (Jiao et al. 2020). The side effects and drug resistance of chemotherapy will adversely affect the quality of life, treatment process, treatment results and treatment costs of patients (Rejhova et al. 2018). Therefore, exploring new drugs and targets for the treatment of CRC has attracted more and more attention.

With the development of traditional Chinese medicine, monomeric active ingredients intraditional Chinese medicine have become a focus of recent research. For example,curcumin, matrine, paclitaxel, et al. have been reported for the prevention and treatmentof CRC. In recent years, more and more studies have shown that curcumin has great potential in the treatment of CRC (Moradi-Marjaneh et al. 2018; Weng and Goel 2020; Selvam et al. 2019; Zhang et al. 2019a; Murono et al. 2020). *CurcumaLonga* L., commonly known as turmeric, is a rhizomatous herb of the ginger (*Zingibera-ceae*) family. It can promote blood and qi circulation and relieve pain. Curcumin is a lipophilic polyphenol compound extracted from the *Zingiberaceae* family ,which has the effects of lowering blood glucose, anti-cancer, anti-inflammation and anti-aging(Kotha and Luthria 2019). The 3D structure of curcumin is shown in Figure 1. Lynne m Howells et al. confirmed that curcumin is a safe and well tolerated adjuvant chemotherapy drug for FOLFOX chemotherapy of metastatic CRC(Howells et al. 2019). In a clinical trial, curcumin has been proved to promote the transformation of Treg cells to Th1 cells, and enhance the production of interferon-γ, supporting the anti-tumor effect of curcumin in CRC(Xu et al. 2017). The clinical efficacy of curcumin in the treatment of CRC has been recognized. Subsequent in vivo experimentsconfirmed that curcumin can reduce inflammation and CRC formation in mouse models(Seiwert et al. 2021). Up-regulation of miR-200c and down-regulation of EPM5 can inhibit EMT in CRC to prevent or delay the progression of CRC(Wang et al. 2020). It can also block G2/M and G1 cycles to inhibit cell growth and induce apoptosis of colorectal cancer cells (Pricci et al. 2020). These research results showed that curcumin has many potential effects and has definite therapeutic effects on CRC. However, most of the previous studies focused on some signaling pathways and related targets, and they did not comprehensively and systematically explain the mechanism of action of curcumin in the prevention and treatment of CRC, that limit the promotion and secondary development of curcumin.

With the development of bioinformatics, network pharmacology can systematically and comprehensively reveal the relationship between the active components of traditional Chinese medicine and its potential mechanism of action. Network pharmacology has become an efficientmethod for the study of traditional Chinese medicine(Luo et al. 2020). This study aims to explore the potential targets and molecular mechanisms of curcumin in the treatment of CRC by network pharmacology and molecular docking analysis. Firstly, we screened the molecular targetsof curcumin and pathological targets of CRC by databases. Then, the enrichment analysis was carried out according to the Gene Ontology (GO) and the Kyoto Encyclopediaof Genes and Genomes (KEGG). The multidimensional network of ‘ drug-target-pathway-disease ’ was constructed by Cytoscape v3.7.2. Finally, the interaction between curcumin and targets was verified by molecular docking analysis and in vitro experiments, and the biological mechanism of curcumin in the treatment of CRC was explained.The summary of this study is shown in the flow chart of Figure 2.

Materials And Methods

Data preparation

Drug-Likeness Prediction

Lipinski’s rule of five (RO5) is an empirical rule for screening potential oral drugs byevaluating the properties of drugs, including molecular weight (MW), octanol-water partition coefficient (XLogP3), polar surface area, number of rotatable bonds, hydrogen bond acceptor count, and hydrogen bond donor count (Yang et al. 2020). To explore the drug-likeness properties of curcumin, we searched the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/) with ' curcumin ' as the keyword, obtained the SMILES format of curcumin, and then uploaded it to the SwissADME website (http://www.swissadme.ch/) to find relevant parameters.

Collection of CRC-related Targets

CRC-related targets were downloaded from five public database sources, including Genecards database (https://www.genecards.org), OMIM database (http://www.omim.org/), TTD database (http://db.idrblab.net/ttd/), Uniport database (https://www.uniprot.org/) and Drugbank database (https://go.drugbank.com/). Species was selected as ' homo sapiens ' and the keyword as ' colorectal cancer '. Then, targets in the pathogenesis of CRC were collected.

Collection of Curcumin-related Targets

PubChem database was used to obtain the SMILES format of curcumin, which was imported into Swiss target prediction database（http://www.swisstargetprediction.ch/）. 'Homo sapiens ' was selected and ' probability > 0 ' was used as the screening condition for target prediction. Finally, the standard gene names were collected by the UniProt platform.

Common Target Screening and Network Construction of Curcumin and CRC

The curcumin-related targets and CRC-related targets were analyzed by Jvenn online platform (http://jvenn.toulouse.inra.fr/app/index.html) to obtain intersection targets and draw the Wayne diagram. The composition-targets network figure was constructed by Cytoscape v3.7.2.

Construction of Protein-Protein Interaction Network (PPI)

The intersection targets were imported into the String platform(https://www.string-db.org/). Then, the interaction relationship between the targets was obtained and saved as the TSV format file. The file was imported into Cytoscape v3.7.2 to get the network diagram. To identify the central nodes and key proteins in the PPI network, the topology parameters were calculated by NetworkAnalyzer, and the degree of centrality (betweenness,closeness, and subgraph) was determined by the CytoNCA.

GO Function and KEGG Pathway Enrichment Analysis

The common targets of curcumin and CRC obtained by the above screening was imported into the DAVID database (https://david.ncifcrf.gov/). The species was set to be "Homo Sapiens". With $P < 0.05$ as the statistical difference screening condition, the potential targets of curcumin on CRC was evaluated. The biological function and pathways of the targets were analyzed. Histograms and bubble charts are

produced through the Bioinformatics cloud platform (http://www.bioinformatics.com.cn/, an online platform for data analysis and visualization). Then, the targets-pathways network was constructed by Cytoscape v3.7.2.

Molecular Docking Analysis

Molecular docking is a validation method, which simulates the binding of receptorsand ligands by computer and predict their affinity. The mol2 file of curcumin was downloaded from TCMSP database (https://tcmspw.com/tcmsp.php). The AutoDock Tools 1.5.6 software was imported and saved in *pdbqt* format. The 3D structures of key target proteins were downloaded from the PDB database (https://www.rcsb.org), and the water molecules and inactive ligands were removed by PyMOL software. The protein was imported into AutoDock Tools 1.5.6 softwarefor hydrogenation and charge treatment, and the output was *pdbqt* format. Finally, AutoDock VINA software was used to simulate the molecular docking of the receptor and its ligand, and the optimal binding conformation was obtained. The docking results were visualized by Discovery Studio Visualizer and PyMOL. Molecular docking was used to verify the binding ability of curcumin and the targets, with Matrine as the control. Matrine has a good therapeutic effect on CRC, and its anti-tumor activity has been verified in a variety of tumors, including CRC (Cheng et al. 2020; Gu et al. 2020; Ren et al. 2014).

Experimental Verification

Cell Culture

HCT116 human colon cancer cell line, purchased from Genechem Co., Ltd. (Shanghai,China), was cultured in RPMI1640 medium containing 10% fetal bovine serum and in5% CO2 incubator at 37 ℃. The morphology and number of cells were observed andrecorded. The cells were passaged when they grew to about 90% confluence.

Flow Cytometry Analysis for Cell Apoptosis

HCT116 cells were seeded in 6-well plates with 4×10^5 cells/well for 24 hours, and treated with different concentrations of curcumin (0, 12.5, 25, and 50 μmol/L) for 24 hours. The curcumin (CAS number: 458-37-7, Purity ≥ 95%) was purchased from Univ Co., Ltd. (Shanghai,China). The cells were resuspended with 500 μL 1 × binding buffer and incubated with5 μL annexin V-FITC in the dark for 15 min at room temperature. Then, 5 μL PI was added. Finally, cell apoptosis was detected by flow cytometry.

Western Blotting Assay

The total protein was extracted using the radioimmunoprecipitation buffer. Proteins were separated via sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After sealing for 3 hours in BSA blockingsolution at room temperature, the membrane was washed three times with TBST and incubated with the primary antibody at 4 ℃ overnight. After washing the membrane with TBST three times, the membrane was incubated with the horseradish peroxidase-

conjugated secondary antibody for 2 hours at room temperature. The Enhanced Chemiluminescence Detection Kit was used to detect and visualize protein bands. We used Image J software to quantify the protein bands and GAPDH was used as an internal parameter to calculate the relative protein expression.

Data Analysis

Spss20.0 software was used for data analysis. All data are expressed as the mean ± standard deviation (SD). Paired t-test was used for comparison between groups, and $P < 0.05$ showed that the difference was statistically significant.

Results

Druglikeness Analysis of Curcumin

The SMILES format of curcumin, (COC1=C(C=CC(=C1)C=CC(=O)CC(=O)C=CC2=CC(=C(C=C2)O)OC)O), was imported into swissame website to obtain relevant parameters.According to the Lipinski's rule of five, a drug-like compound should have a molecular weight of less than 500 g/mol, a polar surface area (PSA) of less than or equal to 140 Â, a computed octanol/water partition coefficient (XLogP3-AA) of less than 5, less than 10 rotatable bonds (RB), no more than 10 hydrogen bond acceptor (HBA), and no more than 5 hydrogen bond donors (HBD) (Chen et al. 2019). It can be seen from the obtained parameters that the properties of curcumin comply with the RO5, indicating that it has good drug-like properties (Table 1).

Composition-Targets Network

The related targets of curcumin were searched, 104 targets were obtained after removing the duplication, and 1911 CRC-related targets were obtained after removing the duplication. Next, 30 common targets were screened out, that were considered potential targets of curcumin in the treatment of CRC (Figure 3A). The composition-targets network was constructed by Cytoscape v3.7.2 (Figure 3B).

Construction of Protein-Protein Interaction Network (PPI)

We uploaded 30 common targets to the STRING database to determine their functional relationships and interactions. Then the protein interactions with the default confidence level of 0.4 were imported into Cytoscape v3.7.2 to generate a protein-protein interaction (PPI) network, which consisted of 26 nodes and 90 edges, as shown in Figure 4.

To identify the pivot nodes and essential proteins in the PPI network, the topology parameters of the node degree were calculated by the network analyzer, and the three centralities (betweenness, closeness and subgraph) were determined through the CytoNC as shown in Table 2.

GO and KEGG Pathway Enrichment Analysis

The GO and KEGG enrichment analysis were performed via David platform. The GO enrichment analysis is composed of biological process (BP), cellular component (CC) and molecular function (MF). A total of

140 items, BP: 93, CC:14, MF: 33, were obtained by GO functional analysis. The results showed that the effects of curcumin were related to protein kinase activity, ATP binding, negative regulation of apoptotic process and protein serine/threonine kinase activity,et al., as shown in Figure 5A.

In the enrichment analysis of KEGG pathway, 61 enrichment results were obtained. A total of 20 typical pathways were selected to make the visualizedbubble diagram after excluding irrelevant pathways (Figure 5B). The results showed that these pathways were mainly related to pathways in cancer, PI3K and Akt signaling pathway, FOXO signaling pathway, et al. Six targets (AKT1, RAF1, BRAF, EGFR, IKBKB, and STAT3) in the first 20 pathways participated in a high frequency (≥9 times), indicating that they played important roles in CRC. Ten representative signaling pathways are selected to construct a "pathways-targets" network, as shown in Figure 5C.

Molecular Docking

Curcumin is docked with three important targets AKT1, STAT3 and EGFR. These targets are selected not only because they are the key nodes of PPI network, but also they play important roles in KEGG enrichment pathways. The binding energies of AKT1, STAT3 and EGFR with curcumin were -9.9 kcal/mol, -8.7 kcal/mol, -8.5 kcal/mol, respectively. The binding energies of matrine to AKT1,STAT3 and EGFR were -7.8 kcal/mol, -8.7 kcal/mol, -7.6 kcal/mol, respectively(Table 3). It can be seen that curcumin has a strong binding force with key targets. The binding of curcumin with AKT1 is mainly through the hydrogen bonding with amino acid residues ASN53 and GLN79, hydrophobic interaction with TRP80, and π bonding with LEU210, LEU264, LYS268, VAL270, ILE84. The binding of curcumin with EGFR is mainly through the hydrophobic interaction with amino acid residues VAL762, PHE856, ALA743,MET790, CYS775, and π-bond interaction with LEU844. The binding of curcumin with STAT3 is mainly through the hydrogen bonding of amino acid residues ASP1021, ASN1008, ARG1007, GLU957, GLY962, hydrophobic interaction with LEU881, VAL889, ALA906 and π bonding with LEU1010 (Figure 6).

Experimental Verification

Curcumin Promoted Apoptosis of CRC Cells

The effect of curcumin on the apoptosis of HCT116 cells was evaluated throughflow cytometric analysis. After treatment with 0, 12.5, 25 and 50μmol/L of curcumin for 24 h, CRC cells were stained with Annexin V-FITC and PI to determine the degree of apoptosis. The results showed that the percentage of apoptotic cells increased significantly in a dose-dependent manner after curcumin treatment of HCT116 cells, indicating that curcumin induced apoptosis of HCT116 cells (Figure 7).

Validation of Targets

We further verified these targets were involved in curcumin-induced apoptosis in CRC cells by Western blotting. As shown in Figure 8, AKT1 protein levels decreased significantly in a dose-dependent manner. The difference was statistically significant (p<0.05). The results showed that AKT1 was an important target of curcumin in the treatment of CRC.

Discussion

CRC has become the second leading cause of cancer-related death in the world.The high degree of malignancy, rapid development, poor prognosis and chemoradiotherapy resistance of CRC often lead to the failure of treatment. Traditional Chinese medicine plays an active role in theprevention and treatment of diseases with its unique theory and efficacy (Yu et al. 2019; Zhang et al. 2019b). Plant monomers occupya certain position in the treatment of cancers, and there is an increasing interests in phytochemicals extracted from medicinal plants (Yin et al. 2018; Zang et al. 2017). For example,paclitaxel, curcumin and berberine are extracted from natural plants and have been proven to be monomers with good anti-tumor activity. They have been listed as effective drugs for the treatment of tumors (Bernabeu et al. 2017; Zhang et al. 2019c). Previous studies have shown that curcumin is a promisingcandidate for the treatment of CRC (Howells et al. 2019; Xu et al. 2017; Seiwert et al. 2021; Wang et al. 2020; Pricci et al. 2020). However, the regulatory mechanism of curcumin in CRC treatment has not been systematically elucidated. Based on the "drug-target-pathway-disease" network (Lu et al. 2020), in this study, we explored the mechanism of action of curcumin in the treatment of CRC.

By analyzing the PPI network and KEGG enrichment results, we predicted that AKT1, EGFR and STAT3 were the core targets of curcumin in the treatment of CRC. Molecular docking analysis showed that curcumin had good affinity for these three targets, and AKT1 had the highest binding degree. GO enrichment results showed that the therapeutic effect of curcumin was closely related to the regulation of protein kinase activity. Meanwhile, KEGG enrichment results suggested that PI3K-Akt signaling pathway played an important role, which indicated that this signaling pathway was the key link of curcumin in the treatment of CRC. AKT1, a member of the Akt family, is a serine/threonine protein kinase. Its abnormal expression plays an important role in the occurrence and developmentof a variety of malignant tumors (Huang et al. 2019; Bertucci et al. 2019; Alwhaibi et al. 2019; Carpten et al. 2007). PI3K-Akt is considered to be a key regulator of cell proliferation, angiogenesis, migration and invasion, that are related to the occurrence, development and metastasis of tumors (Heron-Milhavet et al. 2011; Xiang et al. 2016). Our in vitro experiments confirmed that curcumin could induce apoptosis of CRC cells by inhibiting PI3K-Akt signaling pathway, and also confirmed the reliability of network pharmacology. Pei Yongbin et al. proved that curcumin can inhibit the growth of colorectal cancer in mice, and the mechanism may be related to curcumin in inhibiting PI3K/Akt signaling pathway, which was in consistent with the results of our study (Pei et al. 2021).

Moreover, studies have shown that under pathological conditions, epidermal growth factor receptor (EGFR) is the driving force of tumorigenesis and it is considered as a biomarker oftumor drug resistance (Sigismund et al. 2018). EGFR is a valuable therapeutictarget for CRC, and EGFR inhibitors are effective drugs for the treatment of metastatic CRC (Martinelli et al. 2020), suggesting that curcumin may be used to treat metastatic CRC by inhibiting EGFR. STAT3 is involved in the microenvironment of tumorigenesis by secreting a large number of pro-inflammatory cytokines, that make the treatment of CRC difficult. STAT3 also plays an important role in the development of CRC(Yang et al. 2019), which is in consistent with our study. Curcumin may regulate the tumor microenvironment, coordinate the balance between host

and symbiont, so as to achieve the homeostasis of internal environment, maintain the body balance, and play the role of prevention and treatment of CRC.

In conclusion, in this study, through the combination of network pharmacology, molecular docking, and in vitro experiments, we verified that curcumin had a therapeutic effect against CRC by inhibiting PI3K-Akt signaling pathway. The mechanism of action of curcumin is binding to AKT1, STAT3 and EGFR by hydrogen bond, hydrophobic effect and π-cation bond. This study provides a rational for further clinical research and new drug development using curcumin against CRC.

Declarations

Ethical Approval

Not applicable

Consent to Participate

All authors contributed to the article.

Consent to Publish

All authors approved the submitted version.

Authors Contributions

XYH and YMX contributed to study design, data interpretation, experimental results and manuscript preparation. XLF and CG contributed to manuscript editing, revised the manuscript for important intellectual content, and approved the final version. XQL and YL contributed to data acquisition and analysis. The authors declare that all data were generated in-house and that no paper mill was used.

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Competing Interests

The authors declare that there are no competing interests.

Availability of data and materials

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

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Tables

TABLE 1 Molecular properties of curcumin.

Property	Value
Molecular weight	368.4g/mol
Bioavailability Score	0.55
XlogP3-AA	3.2
Hydrogen Bond Donor Count	2
Hydrogen Bond Acceptor Count	6
Rotatable Bond Count	8
PSA	93.1
Molar Refractivity	102.80

TABLE 2 Topological parameters of the targets.

Targets	Degree	Subgragh	Betweenness	Closeness
AKT1	18	1576.9823	142.27713	0.78125
EGFR	15	1044.5238	123.55815	0.714286
STAT3	14	1046.328	76.63744	0.657895
EP300	12	1024.9966	26.278643	0.625
CDK4	10	789.8613	11.725108	0.595238
MMP9	10	728.5876	40.98225	0.609756
CHEK1	10	700.0684	27.078138	0.581395
GSK3B	9	602.72296	11.776262	0.568182
AURKA	9	560.9203	23.439177	0.555556
RPS6KB1	7	513.06287	1.7409091	0.555556
TOP2A	7	294.21695	9.762843	0.531915
EPHA2	7	255.23312	55.70498	0.555556
NFE2L2	5	301.88513	0.33333334	0.510204
TOP1	5	241.85707	0.5833333	0.510204
TLR9	5	241.24434	1.7222222	0.520833
HDAC4	5	220.83974	3.2777777	0.510204
RAF1	4	152.114	1.0666667	0.5
IKBKB	4	145.47293	1.7599567	0.5
EPHA3	4	43.854324	7.5857143	0.438597
EPHB2	4	37.902145	4.2099566	0.416667
BRAF	3	85.04361	0	0.480769
HDAC7	3	74.83233	0.5	0.471698
ADRBK1	3	63.870117	48	0.490196
MELK	3	56.340717	0	0.396825
EPHA7	3	14.385622	0	0.373134
MAOA	1	2.3126676	0	0.333333

TABLE 3 Molecular docking binding energies of core targets and compounds(kcal·mol^{-1}).

Compound	AKT1	STAT3	EGFR
Curcumin	-9.9	-8.7	-8.5
Matrine	-7.8	-8.7	-7.6

Figures

Figure 1

The 3D structure of curcumin.

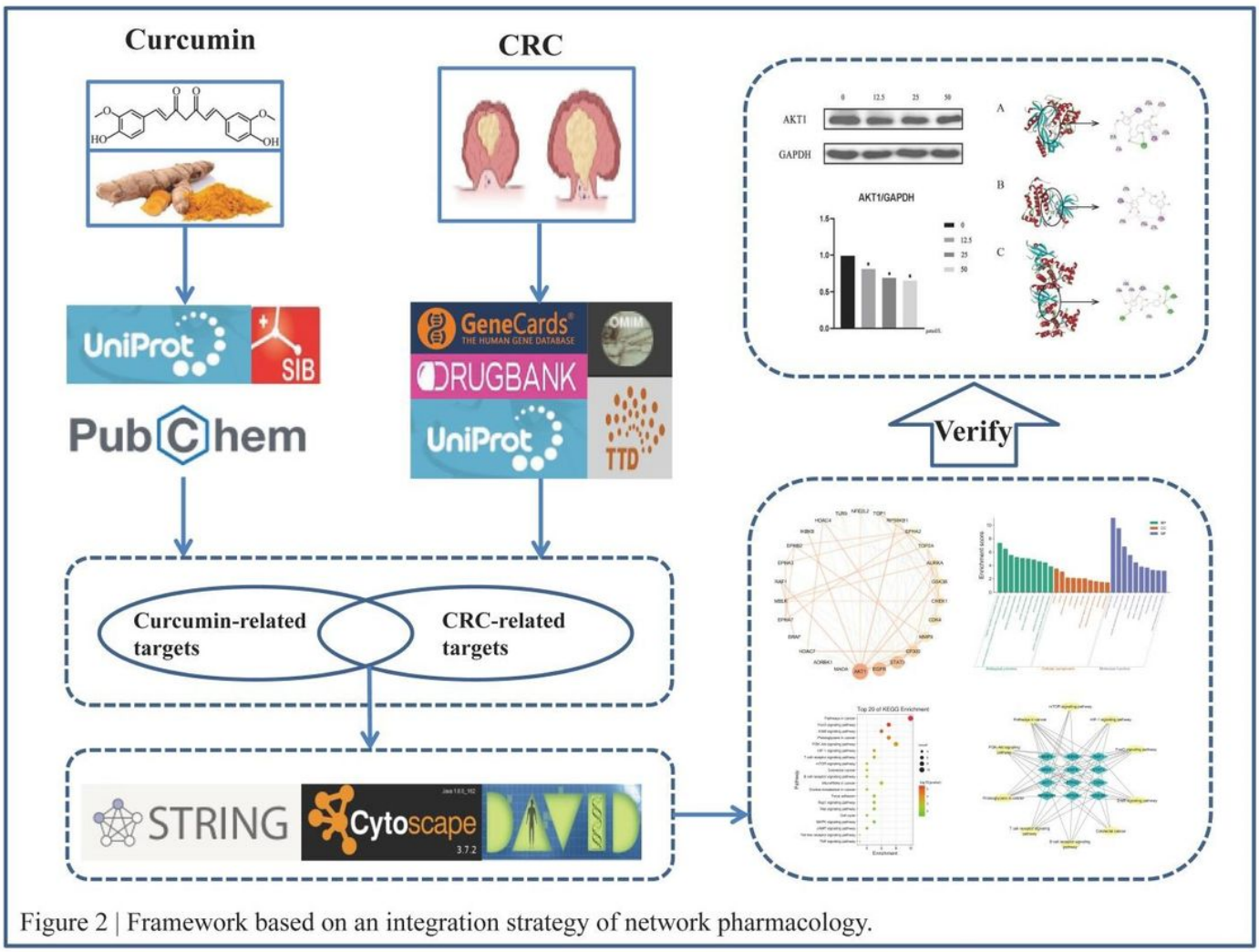

Figure 2 | Framework based on an integration strategy of network pharmacology.

Figure 2

Study framework based on an integration strategy of network pharmacology.

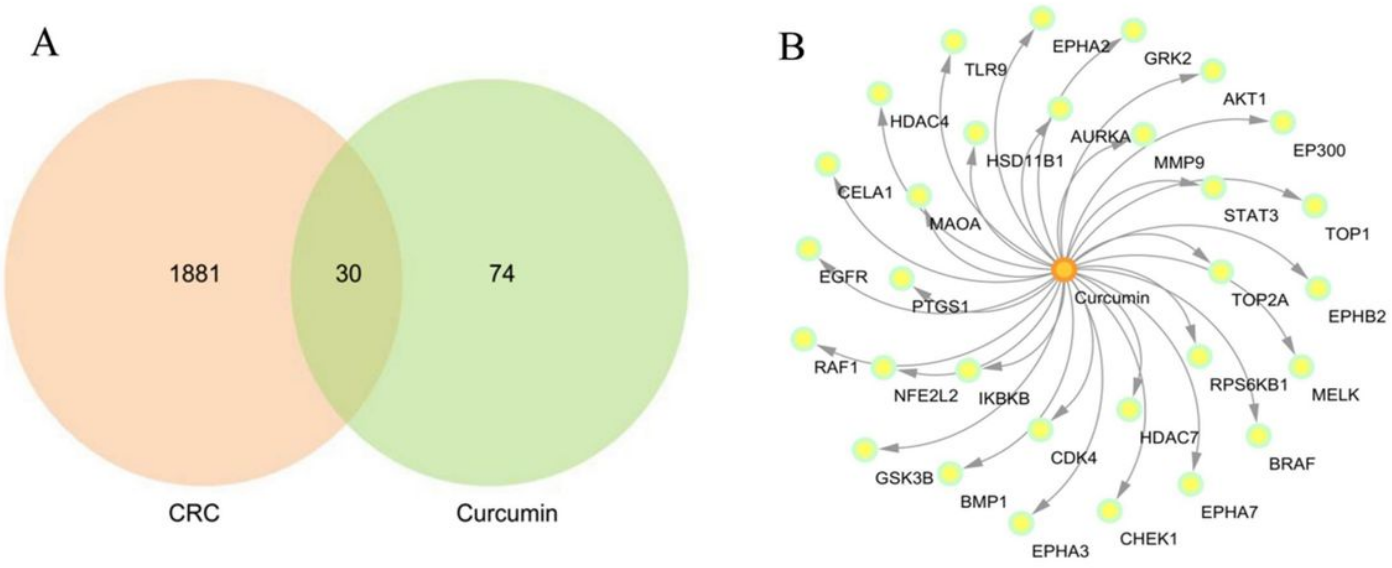

Figure 3

Common targets network. (A) Intersection of Curcumin-related targets and CRC-related targets. (B) Composition-targets network.

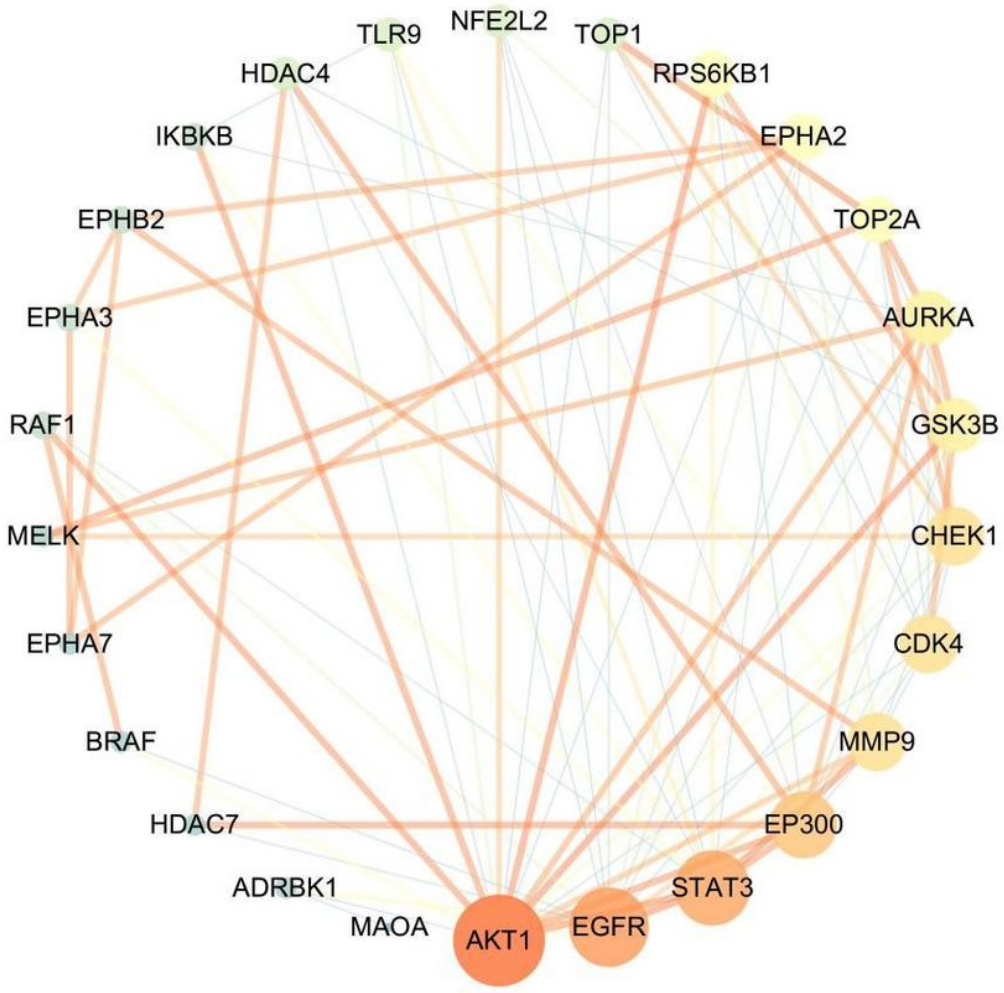

Figure 4

Protein-protein interaction network. The size of the circle represents the node degree of the target protein.

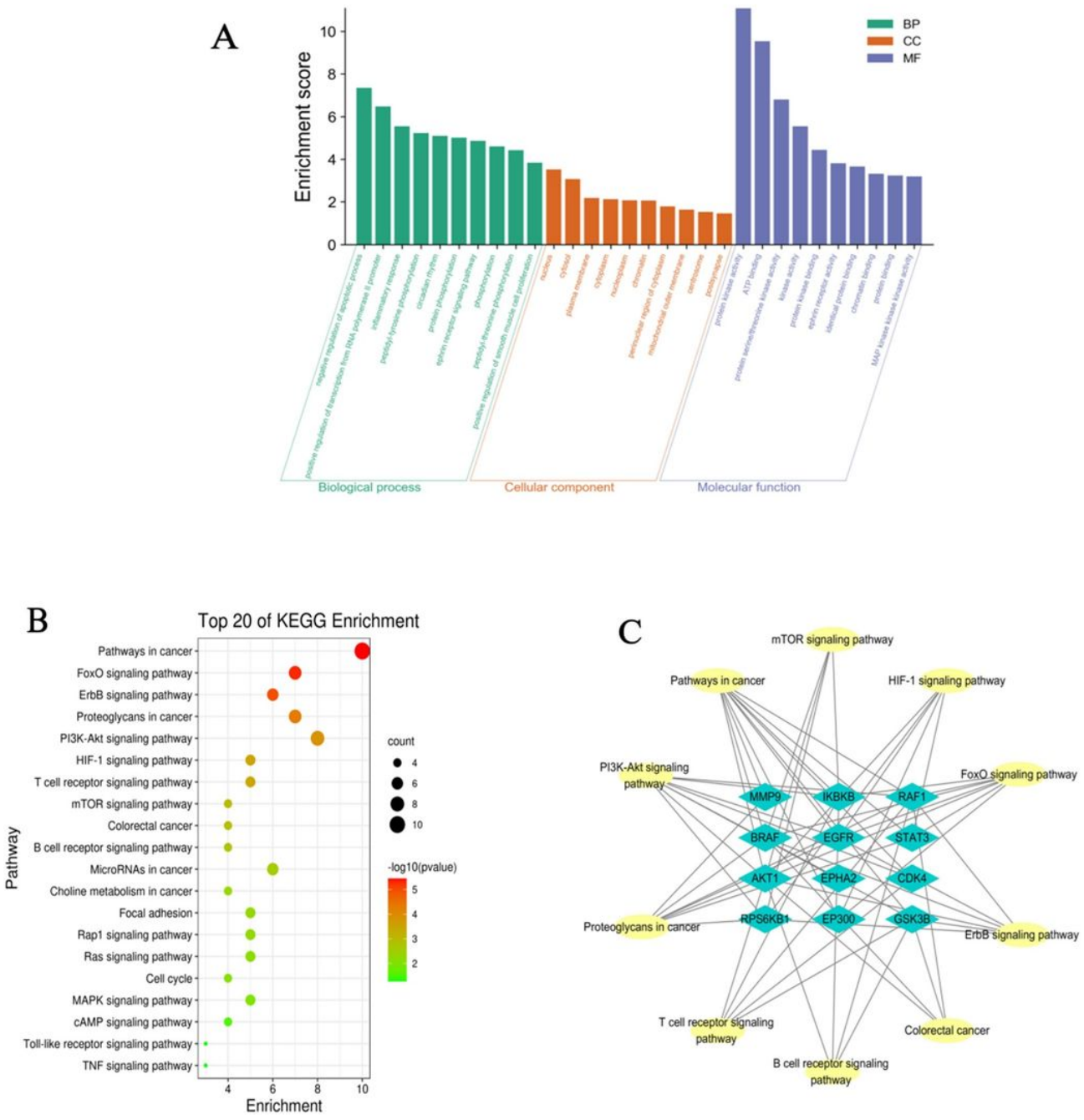

Figure 5

(A) Go enrichment analysis. The top 10 biological processes, the top 10 cellular components, and the top 10 molecular functions. (B)KEGG enrichment analysis. The top 20 KEGG pathways. The color scales indicate the different thresholds for the p-values, and the sizes of the dots represent the number of genes corresponding to each term. (C) Pathways-targets network.

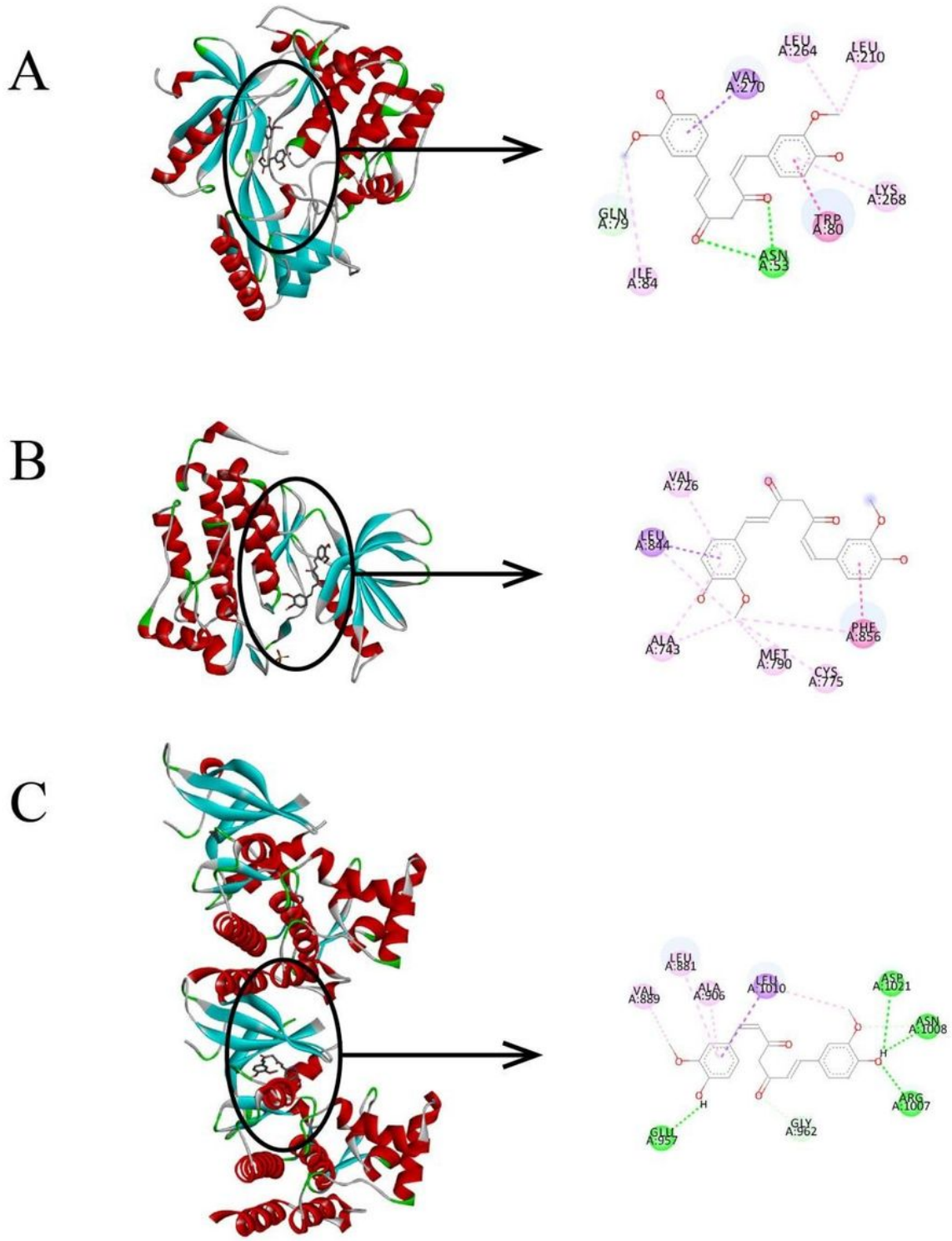

Figure 6

Three-dimensional and two-dimensional representation of the interaction between curcumin and key targets. (A) Or AKT1; (b) or EGFR; (c) and STAT3.

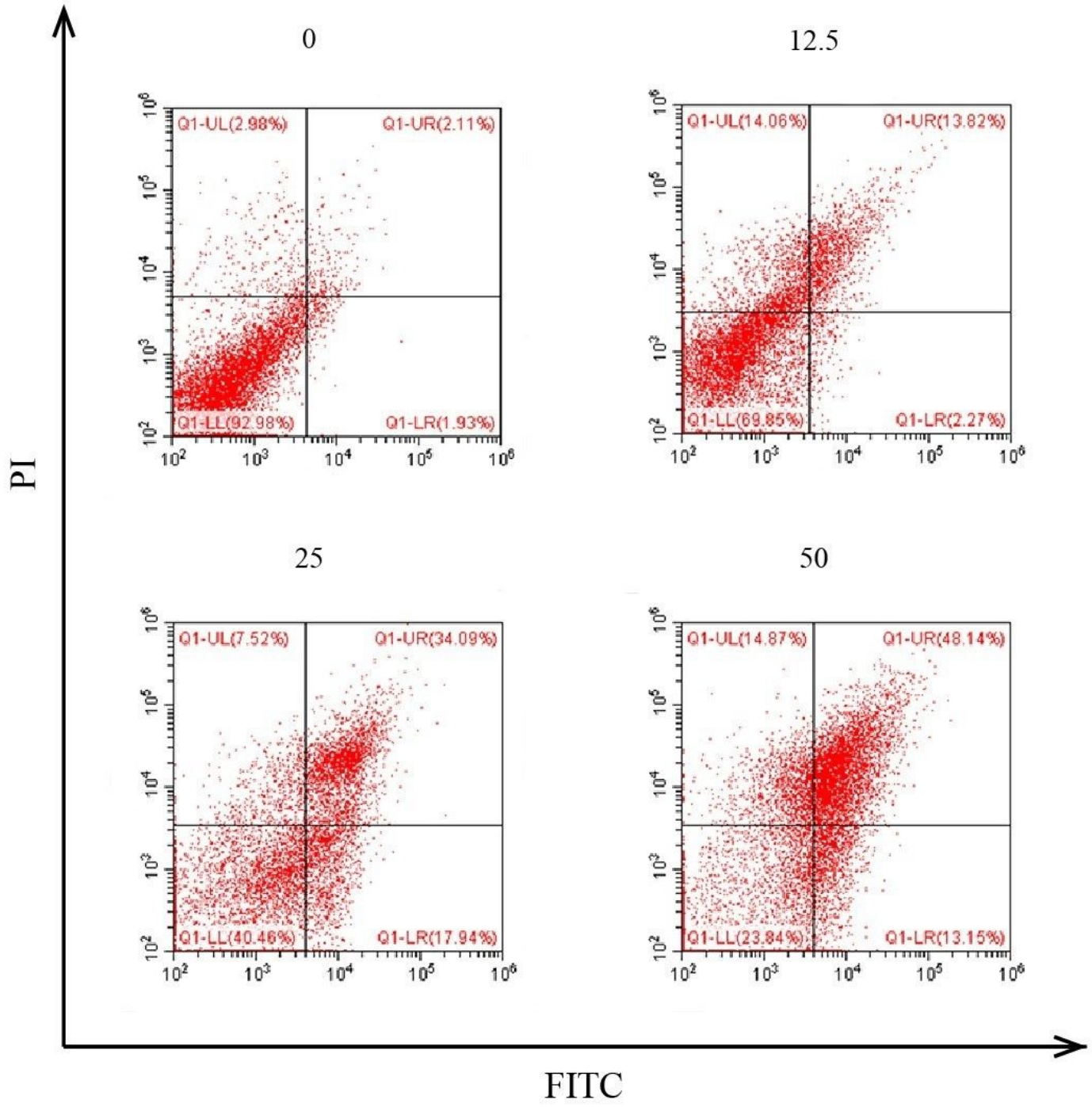

Figure 7

Curcumin promotes apoptosis of HCT116 cells.

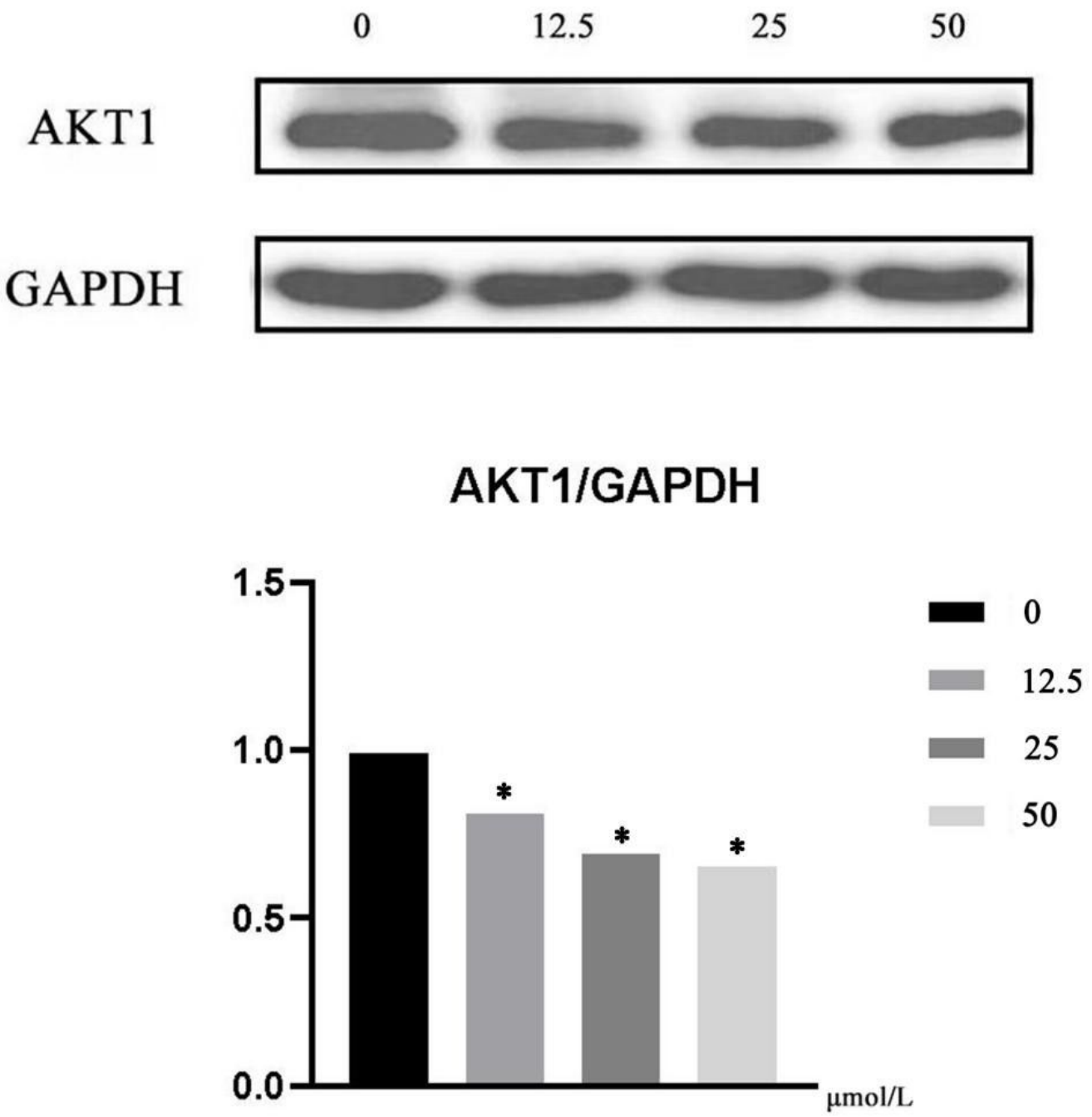

Figure 8

Western Blotting Assay. GAPDH was used as an internal control.