Quercetin reduces cyclin D1 activity and induces G1 phase arrest in HepG2 cells

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Abstract. Quercetin is able to inhibit proliferation of malignant tumor cells; however, the exact mechanism involved in this biological process remains unclear. The current study utilized a quantitative proteomic analysis to explore the antitumor mechanisms of quercetin. The leucine of HepG2 cells treated with quercetin was labeled as d3 by stable isotope labeling by amino acids in cell culture (SILAC). The isotope peaks of control HepG2 cells were compared with the d3-labeled HepG2 cells by mass spectrometry (MS) to identify significantly altered proteins. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses were subsequently employed to verify the results of the MS analysis. A flow cytometry assay was designed to observe the influence of various quercetin treatment concentrations on the cell cycle distribution of HepG2 cells. The results indicated that quercetin is able to substantially inhibit proliferation of HepG2 cells and induce an obvious morphological alteration of cells. According to the MS results, the 70 credibly-changed proteins that were identified may play important roles in multiple cellular processes, including protein synthesis, signaling, cytoskeletal processes and metabolism. Among these functional proteins, the expression of cyclin D1 (CCND1) was found to be significantly decreased. RT-PCR and western blot analyses verified the SILAC-MS results of decreased CCND1 expression. In summary, flow cytometry revealed that quercetin is able to induce G1 phase arrest in HepG2 cells. Based on the aforementioned observations, it is suggested that quercetin exerts antitumor activity in HepG2 cells through multiple pathways, including interfering with CCND1 gene expression to disrupt the cell cycle and proliferation of HepG2 cells. In the future, we aim to explore this effect *in vivo*.

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Abbreviations: SILAC, stable isotope labeling by amino acids in cell culture; CCND1, cyclin D1; CCND2, cyclin D2; CCND3, cyclin D3; Leu-d3, deuterated leucine; Leu-d0, normal leucine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ACN, acetonitrile; MS, mass spectrometry; ESI, electrospray ionization; LC, liquid chromatography; MS/MS, tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ion-time of flight

Key words: quercetin, cell cycle, cyclin D1, SILAC-MS, quantitative proteomics

Introduction

Flavonoids have recently drawn extensive attention due to their interesting biological activities (1). This family of compounds comprises a large class of low-molecular weight, natural products of plant origin that are ubiquitously distributed in edible fruits and vegetables. Quercetin is one of the most important flavonoids and exhibits a wide range of biological activities. The significant antitumor, anti-allergy and anti-inflammatory effects of quercetin have been extensively reviewed (2,3). Evidence indicates that quercetin is able to target various types of malignant tumor cells, including leukemia, breast, esophagus, colon, prostate, nasopharyngeal, endometrial and lung cancers (1,4). The proliferation of these malignant cells may be inhibited by quercetin; however, the exact molecular mechanisms underlying the effects of quercetin are unknown.

Recently, a new quantitative method has demonstrated great promise for the simultaneous, accurate identification and quantitation of complex protein mixtures. This method is termed stable isotope labeling by amino acids in cell culture (SILAC)-mass spectrometry (MS), and identifies proteins by MS based on stable isotopes (5). Using this method, we have previously studied total proteomic expression changes of HepG2 cells and identified certain significant differences in HepG2 cells following their cultivation with quercetin. These proteins may serve important roles in multiple cellular processes, including protein synthesis, signal transduction, cytoskeleton formation and metabolism (6).

One of the most important goals of anticancer research is to inhibit proliferation of malignant cells. Normal cell

proliferation requires successful transition through cell cycle checkpoints (7). Access to the mitotic stages of the cell cycle is strictly controlled by growth-promoting and growth-inhibitory signals; these signalling pathways ensure that cells do not unnecessarily commit to DNA replication and cell division (8). In eukaryotes, cyclins and cyclin-dependent kinases (CDKs) are principally responsible for controlling cell cycle regulation (7). Cyclins are the regulatory subunits, while CDKs are the catalytic subunits, and each cyclin contains a special region that allows binding to form a cyclin-CDK complex (8). When this complex is in an active state, it permits cells to move past the check points of particular cell cycle phases through the phosphorylation of unique protein substrates. The G1/S transition is the most important check point of the cell cycle that is regulated by cyclin-CDK complexes (8).

In mammalian cells, G1 cyclins serve a major a role in cell cycle regulation. The G1 cyclins are composed of two families of phase regulatory proteins: D-type cyclins and cyclin E (9). Cyclin D consists of three subunits that are central to the transition of cells from one phase to the next: Cyclin D1 (CCND1), D2 (CCND2), and D3 (CCND3). These proteins share a cyclin box and PEST sequence, but bind to different chromosomes (10). Recently, the majority of research on the structure and function of cyclin proteins has focused on CCND1. Overexpression and rearrangement of CCND1 has been found to be associated with tumor progression in numerous different tumor types, including carcinomas of the esophagus, prostate and breast (11,12). In the present study, total protein identification by SILAC-MS revealed that the peak of CCND1 in cells treated with quercetin was significantly different compared with the CCND1 expression in control HepG2 cells. Due to the essential function of CCND1, the influence of quercetin on cell cycle distribution of HepG2 cells was investigated. Combined with the SILAC-MS results, the current study reports the possible mechanism by which quercetin may inhibit the proliferation of HepG2 cells.

Materials and methods

Materials. Quercetin (PHR1488), all normal L-amino acids, trypan blue, dimethyl sulfoxide, Tris-HCl, Triton X-100 and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO, USA). The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA, USA). Deuterated leucine (Leu-d3,5,5,5-D3, 98%, lot: l1.8473) was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Potassium phosphate and Tris base were purchased from Merck Millipore (Darmstadt, Germany). Rabbit polyclonal anti-CCND1 IgG, (sc-753) and mouse monoclonal anti-β-actin IgG (sc-130300) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture and SILAC. HepG2 cells were cultured and labeled as described previously (6). Briefly, Eagle's minimum essential medium (Yubo Biotech Ltd., Shanghai, China), including appropriate amounts of amino acids with the exception of leucine, was mixed with 50 mg/l each of the antibiotics gentamicin, penicillin and streptomycin, reconstituted according to the manufacturer's specifications. The leucine-free medium was divided into two equal portions. L-leucine (Leu-d0) and L-leucine-d3 (Leu-d3) were added to each media to reach a final leucine concentration of ~52 mg/l. The mixture with a full complement of amino acids was filtered through a sterile 0.22 μm filter (EMD Millipore, Bedford, MA, USA). Control HepG2 cells were cultured in normal Dulbecco's modified Eagle's medium (DMEM; Yubo Biotech Ltd.) with Leu-d0, and cells treated with quercetin were put in d3-labeling media with Leu-d3. The morphological changes of the HepG2 cells were observed following the addition of quercetin. Briefly, the growth rate and cell morphology of HepG2 cells was evaluated using an inverted microscope (IX50; Olympus Corporation, Tokyo, Japan) after 24, 48 and 72 h. The results of our previous research (6) indicated that the proliferation and apoptotic properties of HepG2 cells were significantly different when the cells were treated with 50 μmol/l quercetin for 48 h compared with untreated cells. Thus, this condition was selected to perform the Leu-d3 labeling treatment. Cells were grown in DMEM containing 10% fetal bovine serum in humidified air with 5% CO_2 at 37˚C. The d3-labeling medium was replaced after four days, and cells were passaged for >10 generations in order to produce adequate proportions of Leu-d3-labeled cells.

Protein preparation and gel digestion. As described in our previous study (6), HepG2 cells were treated with 50 μM quercetin for 48 h and collected to analyze protein changes. HepG2 cells were cultured with and without quercetin and lysed using radioimmunoprecipitation assay buffer [10 mM Tris-Cl (pH8.0); 1 mM ethylenediaminetetraacetic acid; 0.5 mM ethylene glycol tetraacetic acid; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 140 mM NaCl] to extract the protein. Equal amounts of the protein extraction were then separated with 12% Tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE), stained with Coomassie Blue. The gel lanes were cut to 1 mm^3 pieces and destained with 1:1 acetonitrile (ACN) and 50 mM ammonium bicarbonate solution, followed by dehydration in 100% ACN for ~15 min. The gel pieces were completely dried under vacuum centrifugation. Subsequently, samples were incubated overnight in a 10 ng/μl MS-grade trypsin solution (Promega Corporation, Madison, WI, USA). Finally, peptides were extracted with a formic acid solution (5% formic acid, 50% ACN) and resuspended in 50% ACN and 0.1% trifluroacetic acid for the MS analysis.

Liquid chromatography (LC)-tandem MS (MS/MS) analysis and protein quantification. LC-MS/MS analysis was designed as described in our previous paper (6). Briefly, LC-MS/MS analysis was performed with nano-ultra-performance liquid chromatography (nanoUPLC) and a 4x tandem mass spectrometer (UPLC-Q-TOF; Waters Corporation, Milford, MA, USA) coupled with an electrospray ionization (ESI) source. NanoUPLC separations were performed on a nanoAcquity™ C18 column 3.5 μm, 0.75 μm x 100 mm (Waters Corporation). The chromatographic separation was performed at a flow rate of 0.3 μl/min with the mobile phase, consisting of solvents A [water:formic acid, 100:0.1 (v/v)] and B [ACN:formic acid, 100:0.1 (v/v)], using a linear gradient elution from 95% A:5% B to 60% A:40% B in a 45-min initial step; then decreased to 10% A:90% B at 55 min, and finally increased to 95% A:5% B at 60 min. Subsequently, 2 μl of sample was injected for the

nanoUPLC-MS/MS analysis. The ESI source was performed in positive ion mode at a spray voltage and cone voltage of 2.8 kV and 40 kV. The source temperature was set at 90˚C, and 0.45 l/h argon was used as the collision gas. The speed of nano gas flow was controlled at 0.36 l/h. Spectra were accumulated until a satisfactory signal/noise ratio had been obtained from a range of 400-1,600 mz. Three MS/MS ions with charged states of 2+ and 3+ were selected for each replicate; trypsin autolysis products and keratin-derived precursor ions were automatically excluded.

The MS/MS data (pkl files), including mass values, intensity and charge of precursor ions, were acquired by the software ProteinLynx v2.25 (Waters Corporation). Mascot Program 2.0 (Matrix Science Ltd., London, UK) was used to analyze the pkl files. A strict standard was set to ensure a high confidence level for identification. Peptides with Mascot scores below the threshold value were excluded from the data ($P<0.01$). Protein abundance was calculated as the peak intensity of the fragment ions from the labeled vs. unlabeled peptides. A protein was considered significantly different after treatment with quercetin if its average change ratio was 5x higher or lower than the average standard variation of all quantified proteins.

Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis of CCND1 expression following treatment with quercetin. Semi-quantitative RT-PCR and western blot analyses were conducted to further explore the change in CCND1 expression after the results of the LC-MS/MS analysis were obtained. Briefly, Trizol reagent was used to isolate RNA from control HepG2 cells and HepG2 cells treated with 50 μM quercetin for 48 h. Trizol (1 ml) was added to each tube of 10^7 cell pellets and incubated for 5 min at 4˚C. The lysate was then extracted with 0.2 ml phenol/chloroform for 15 sec and centrifuged at 12,000 x g and 4˚C for 15 min. The concentration of RNA was determined by the OD260/280 absorption value of the solution. Total RNA quality was evaluated by 1% agarose gel electrophoresis.

RT-PCR was performed using a One-Step RNA PCR kit (Promega Corporation) with 500 ng total RNA by using the following primers in the following conditions: 45 min at 45˚C; 2 min at 95˚C; followed by 25 cycles of 30 sec at 94˚C, 30 sec at 54˚C and 30 sec at 72˚C; and a final elongation step of 10 min at 72˚C. The CCND1 (forward, 5'-AGGAACAGAAGTGCGAGG AG-3'; reverse, 5'-CACAGAGGGCAACGAAGGT-3') β-actin (forward, 5'-ACCCCCACTGAAAAAGATGA-3'; reverse, 5'-ATCTTCAAACCTCCATGATG-3') primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). Approximately 5 μl of amplified product was separated on a 1% agarose gel, stained with ethidium bromide and observed under ultraviolet light. The DNA ladder was obtained from Nanjing Jinsirui Biotechnology Co., Ltd. (Nanjing, China). β-actin was used as a positive control for RT-PCR. The Quantiscan 3.0 software used for densitometry was obtained from Azure Biosystems, Inc. (Dublin, CA, USA).

Western blot analysis was performed as described previously (6). Protein extractions from control HepG2 cells and HepG2 cells treated with quercetin for 48 h were electrophoresed on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were washed in phosphate-buffered saline (PBS) containing Tween 20 and incubated with anti-CCND1 and anti-β-actin primary antibodies (dilution, 1:300) after blocking with 5% skimmed milk. Membranes were incubated with polyclonal horseradish peroxidase-conjugated rabbit anti-goat IgG antibody (cat. no. ab6741; Abcam, Cambridge, MA, USA) for 1 h. Finally, membranes were developed with a Western Lightning Chemiluminescence Substrate Kit (PerkinElmer, Inc., Shanghai, China) according to the manufacturer's specifications.

HepG2 cell cycle distribution following treatment with quercetin. Flow cytometric analysis was performed to explore changes in the HepG2 cell cycle following treatment with quercetin. HepG2 cells (~10^6/ml) were exposed to 50 μM quercetin for either 12, 24, 48 or 72 h. After washing in PBS, the cells were fixed in ice-cold 70% ethanol and stored at -20˚C. Then they were stained with propidium in a hypotonic buffer and suspended in 1 ml of hypotonic fluorochrome solution containing 50 μg propidium iodide per ml in 0.1% sodium citrate and 0.1% Triton X-100. All samples were analyzed with a flow cytometer (Elite ESP; Beckman-Coulter, Miami, FL, USA). The cell cycle distribution was estimated with Listmode software (version 3.0; Beckman-Coulter).

Results

Influence of quercetin on HepG2 morphology and proliferation. Our previous studies demonstrated that quercetin can inhibit proliferation of HepG2 cells. Using an inverted microscope (IX50; Olympus Corporation), substantial morphological alteration of the HepG2 cells treated with quercetin was observed. The quercetin-treated cells became circular, detached from their substrate and proliferated slowly (Fig. 1). According to the results of a 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay, quercetin inhibited the proliferation of HepG2 cells in a time- and dosage-dependent manner (6).

The Leu-d3 labeling ratio detection was performed as previously described. After 10 passages of HepG2 cells, the Leu-d3-labeling ratio was nearly 100% (6).

To ensure the accuracy of the results, the two HepG2 samples (the control HepG2 cells and those treated with 50 μM quercetin) were mixed at a 1:1 ratio. The sequence SYELPDGQVITIGNER of β-actin was used for quantification purposes. The details of the Leu-d3 labeling and ratio identification are explained in Zhou *et al* (6).

Quantification of CCND1 by SILAC-MS. In a previous paper, we laid out a classification system to group proteins with significantly different expression levels (6). During the development of tumors, D-type cyclins serve a central role in cell cycle transitions. Recent studies have focussed on the correlations between subunit CCND1 and the poor prognosis of malignant diseases, such as gastric cancer and breast cancer (13). In the current study, changes in CCND1 expression were observed in hepatoma cells following treatment with quercetin using a quantitative proteomics method. There were three Leu-containing peptides of CCND1 to be detected and the MS score was ~158. The average protein expression level of CCND1 was reduced to 0.55 following quercetin treatment; a representative pair of isotope labeling peaks of Leu-containing peptides are shown in Fig. 2.

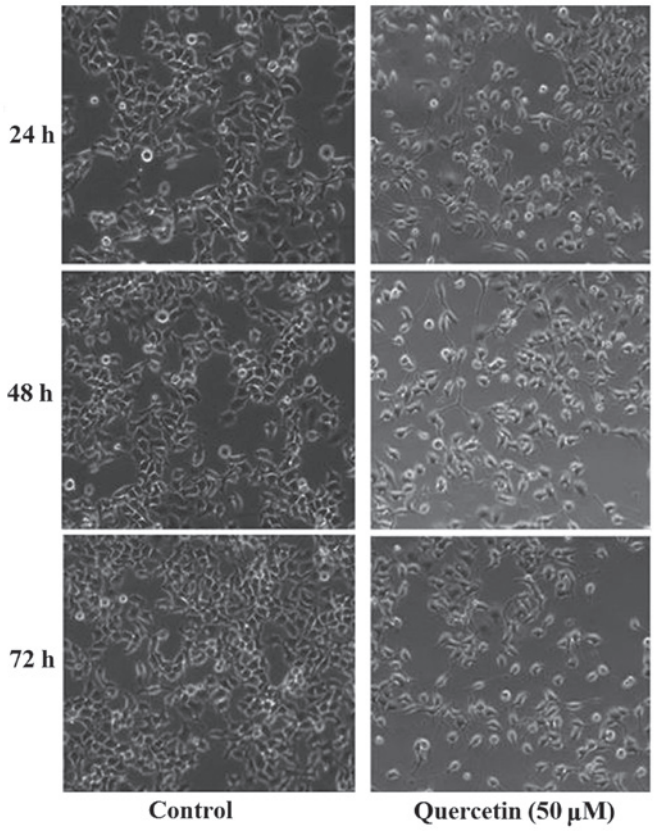

Figure 1. Morphological alteration of HepG2 cells by quercetin. When HepG2 cells were treated with 50 μM quercetin for 48 h, they became rounder, detached from their substrate, and proliferated more slowly. Magnification, x40.

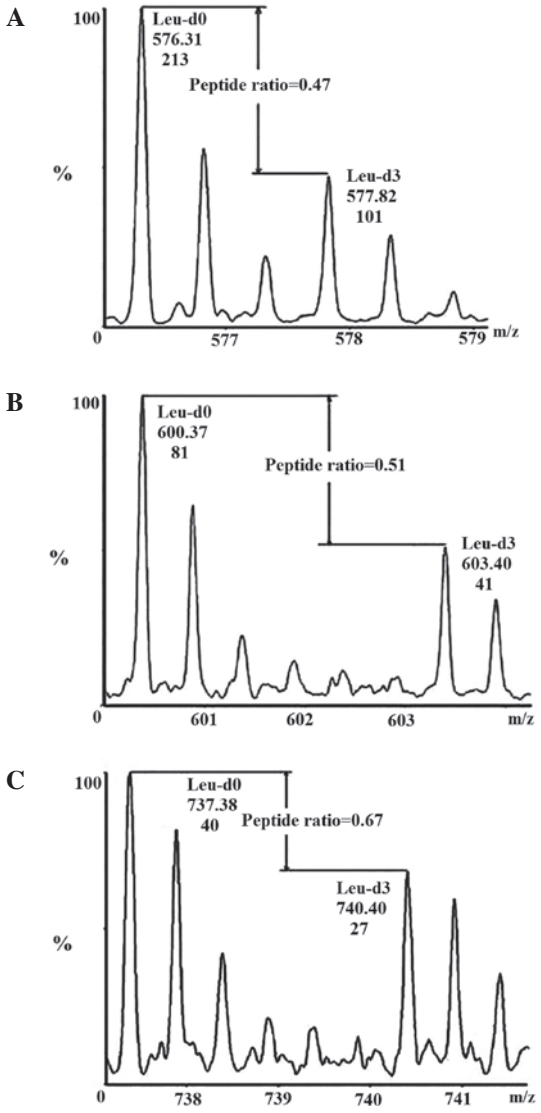

Figure 2. Three representative pairs of isotope labeling peaks for quantification of the downregulated protein CCND1. (A) Representative peptide with AMLKAEETCAPSVSYFK sequence from CCND1. The SILAC ratio is ~0.47. (B) A peptide with LTRFLSRVIKCDPDCLR sequence from CCND1. The SILAC ratio is ~0.51. (C) Another peptide with LKWNLAAMTPHDFIEHFLSK sequence from CCND1. Its SILAC ratio is ~0.67. In this experiment, the SILAC ratio was defined as the ratio of the labeled vs. unlabeled peptide in a protein. CCND1, cyclin D1; SILAC, stable isotope labeling by amino acids in cell culture.

CCND1 expression following treatment with quercetin measured by RT-PCR and western blot analyses. CCND1 gene expression was assessed using RT-PCR and western blotting. The RNA and protein expression levels of CCND1 were consistently decreased in HepG2 cells when exposed to 50 μM quercetin for 48 h, while no change was observed in β-actin expression (Fig. 3). The relative integrated optical density values of RT-PCR with β-actin are indicated in Fig. 3C.

Cell cycle distribution of HepG2 cells when treated with quercetin. Based on the results of the MS quantification, we suspected that quercetin may have effects on the cell cycle distribution of HepG2 cells by influencing CCND1 activity, as the CCND1 is important in cell cycle transitions. HepG2 cells were treated with 50 μM quercetin for 24, 48 or 72 h, respectively, and the cell cycle distribution was detected by flow cytometry. As shown in Fig. 4A, the cell cycle distribution of HepG2 significantly altered following treatment with an effective concentration of quercetin; the percentage of cells in the G0/G1 phase increased, and those in S phase gradually decreased in comparison with the control HepG2 cells. The raw data and statistical comparison can be seen in Fig. 4B.

Discussion

In recent years, the polyphenolic flavonoid compound quercetin has attracted a great deal of attention due to its wide-ranging biological activity and low toxicity (14). Quercetin is naturally found in plants and is consumed in vegetables, beverages, fruits and numerous herbal tonics. It serves important roles in various biological processes, including antioxidant processes, multidrug resistance, liver fibrosis and antitumorigenesis (15,16). Among these activities, the effect of quercetin on carcinogenesis is a highly promising research area. It has been demonstrated that quercetin affects the development of several types of tumors, including leukemia, breast, esophagus, colon, prostate, nasopharyngeal, endometrial and lung cancers, and therefore has potential value in clinical applications (17,18). Quercetin may function in several ways to produce anticancer effects; for example, it may influence apoptosis induction, suppression of heat shock proteins, inhibition of oncogene expression and antiangiogenesis, among others (19,20). Most previous

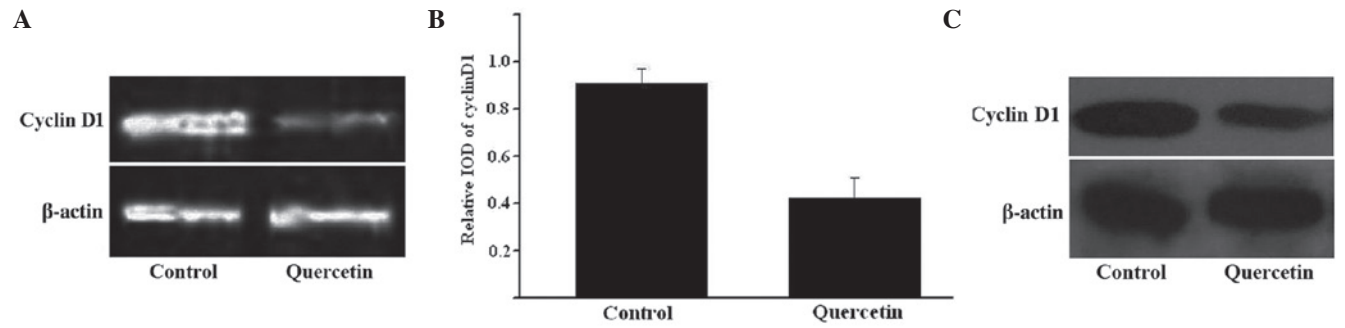

Figure 3. Expression of CCND1 assessed by RT-PCR and western blot analyses. (A) Semi-quantitative RT-PCR of CCND1 from control and quercetin-treated HepG2 cells. (B) Relative IOD value of RT-PCR with β-actin as a reference. (C) Western blot analysis of CCND1 from control cells and quercetin-treated cells. Cells were exposed to 50 μM quercetin for 48 h. Whole lysate was extracted and 20 μg protein was applied to each lane. β-actin was used as the control. CCND1, cyclin D1; RT-PCR, reverse transcription-polymerase chain reaction; IOD, integrated optical density.

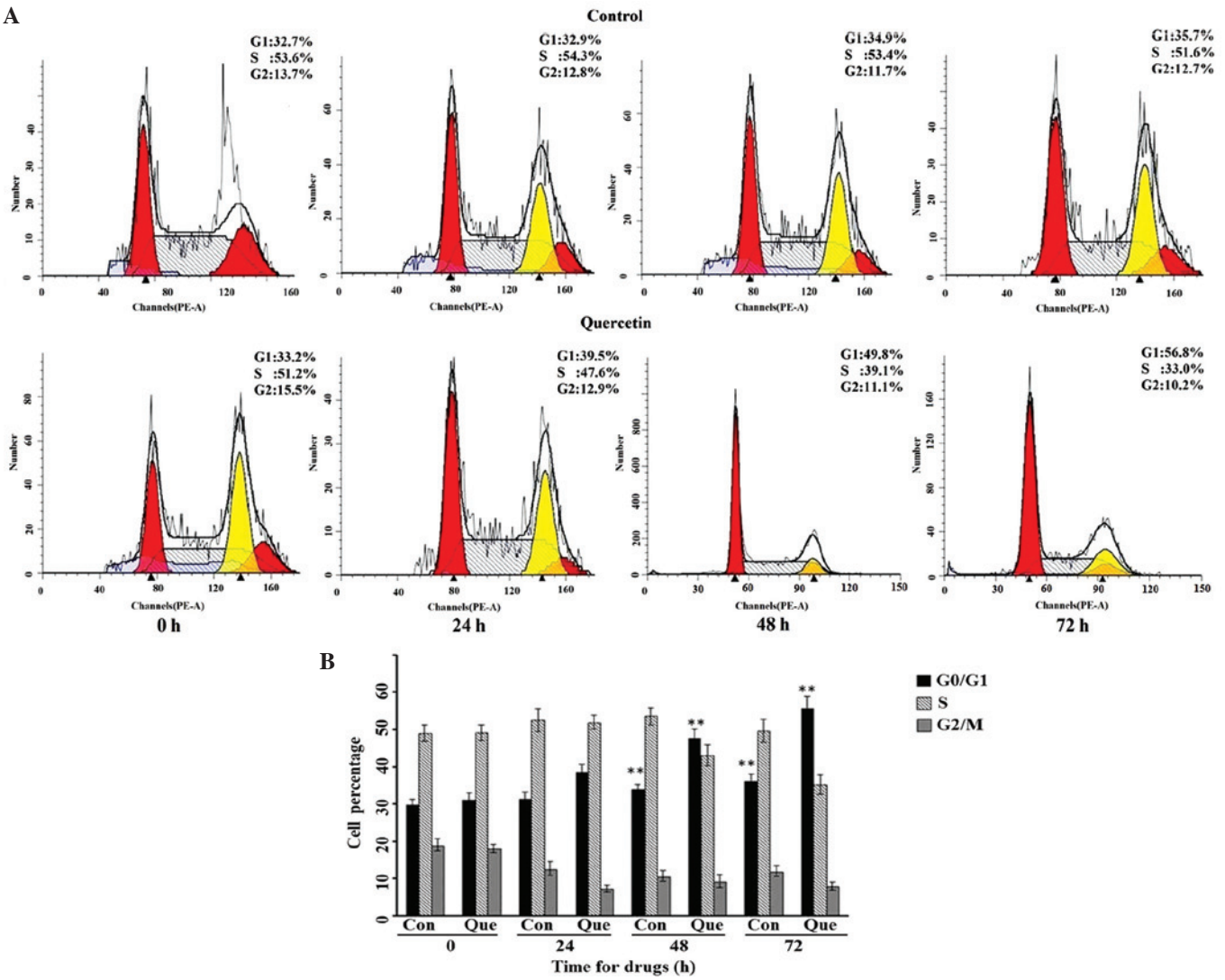

Figure 4. Effect of quercetin on cell cycle progression. (A) Representative analysis of the HepG2 cell cycle performed by flow cytometry after the cells were treated with 50 μM quercetin for 48 h. (B) The percentage of cells in pre-G1, G1, S and G2/M phases of the cell cycle after treatment with quercetin for various times. Each bar is the mean of three separate experiments in triplicate ± standard deviation. **P<0.05 (statistically significant differences) compared with the control. Con, control; Que, quercetin.

reports have focused on a certain gene or pathway, while the exact mechanism of the effect of quercetin on these biological processes remains unknown. In our studies, some of these mechanisms were explored by identifying the differentially expressed proteins in HepG2 cells following exposure to quercetin.

One of the conventional methods used to detect protein expression levels in cells, tissues or organisms is two-dimensional

coupling with matrix-assisted laser desorption ion-time of flight (MALDI-TOF) MS. However, this method provides relatively low sensitivity and low accuracy results on protein expression levels (5). A recently developed protein identification method named SILAC-MS has the benefit of significantly reducing the variation between replicates and results in better quantitative statistics due to the large set of uniformly labeled peptides. SILAC-MS is beginning to be used extensively in the life sciences due to its simple, inexpensive and accurate properties (21). We utilized this method to tag leucine in HepG2 cells with d3, and these cells were treated with quercetin (6). The isotope peaks of the quercetin-treated HepG2 cells were then compared with control HepG2 cells in the same MS image. After three replicate experiments, 70 proteins with significantly different expression were identified, following strictly defined standards. According to annotations in the protein database ExPASy, these proteins can be classified into the following categories: Cytoskeleton, signaling transduction, protein synthesis, chaperone, metabolism and DNA/RNA binding. Some proteins that were identified, such as members of the heat shock protein (HSP) family, were consistent with other studies. Zanini *et al* (22) reported that quercetin is able to inhibit HSP expression in neuroblastoma cells. This may contribute to causing higher sensitization to doxorubicin and invert multidrug resistance. The expression of the Ras GTPase activating-like protein IQGAP1 and β-tubulin, which takes part in the construction of the cell skeleton, were observed to significantly decrease following treatment with 50 μM quercetin for 48 h (6). Combined with a scratch-induced migration assay, we suspected that quercetin may regulate the migration ability of HepG2 cells through its interaction with IQGAP1 and β-tubulin. Other proteins with significant changes to their expression, such as the proto-oncogene tyrosine-protein kinase ABL1 and α-enolase, deserve attention in future research.

The goal of antitumor research is to inhibit the proliferation of malignant cells, and cell proliferation requires the successful transition through multiple cell cycle checkpoints (7). In mammalian cells, D-type cyclins play a central role in integrating extracellular proliferation signals with cell cycle machinery (8). The D-type cyclin family comprises three types of proteins: Cyclin D1, cyclin D2 and cyclin D3. The CCND1 gene is located at 11q13 and codes for the CCND1 protein. CCND1 can be overexpressed in a number of tumor types, particularly in poorly differentiated tumors. It has been reported that CCND1 is likely to be a proto-oncogene (23); deleterious changes to CCND1 may contribute to uncontrolled cell growth characteristic of tumors, and overexpression or mutation of CCND1 are considered to be associated with progression or poor prognosis in numerous different malignant tumors, such as carcinomas of the esophagus, breast, lung and pancreas, as well as mantle cell lymphomas (24,13). In nasopharyngeal carcinoma patients, the abnormal regulation of CCND1 may contribute to carcinogenesis through enabling persistent infection with epstein-barr virus (25). During the development of lymphomas, an overexpression of CCND1 may shorten the time that a cell spends in the resting G phase and accelerate the transition process into the S phase, leading to malignant proliferation (26). A mechanism study revealed that the most common genetic abnormality affecting CCND1 has a large impact on DNA transcription (26). CCND1 can act as a regulatory subunit of CDK4 and CDK6, which are key enzymes in the cell cycle transition process; it is able to phosphorylate CDKs and inhibit the activity of the retinoblastoma protein pRb, leading to the release of E2F-type transcription factors. These E2Fs can regulate the transcription process of CCND1 so as to promote the cell cycle progression. It is notable that CCND1 expression can also be stimulated by underphosphorylated pRb. There may be an autoregulatory loop within the cell cycle transition (26,27).

As is demonstrated by the present results, HepG2 cells treated with quercetin exhibit decreased expression of CCND1, as well as significant changes in the cell cycle distribution in comparison with control HepG2 cells. There have also been studies on the influence of quercetin on the cell cycle distribution in malignant cells, including HeLa human cervical cancer cells and MDA-MB-453 breast cancer cells (28,29). In certain cell types, quercetin may block the cells from transitioning to the G1 or G2 phase. However, Jeong *et al* (30) reported that a low dose of quercetin (10 μM) may induce cell arrest in the G1 phase. In the current study, the concentration of quercetin use was ~50 μM. Although we have observed the same cell cycle arrest phenomenon, the proportion of each cell cycle phase differed among the quercetin concentration treatments. Whether there is a correlation between the concentration of quercetin and malignant cell cycle distribution is the subject of our next study.

In contrast to CCND1, there are relatively few reports on CCND2 and CCND3 and their roles in carcinogenesis. CCND2 was once thought to reside in a chromosomal region that does not readily undergo amplification. However, there has been new evidence revising the location of CCND2. Yamak *et al* (31) reported that CCND2 may enhance the activity of transcription factor GATA4, a key regulator of cardiomyocyte growth and differentiation. Since human mutations in this domain are linked to congenital heart disease, it has been suggested that CCND2 may play an important role in cardiogenesis (31). In chronic myeloid leukemia, Jena *et al* (32) found that BCR/ABL promotes the cell cycle progression by altering expression of CCND2 (32). The overexpression of CCND2 may also be detected in gastric cancer cases and correlates positively with a poor prognosis (33). There have also been limited investigations on CCND3. It is reported that CCND3 is expressed differentially among lymphoma subtypes. In indolent lymphomas, CCND3 overexpression was a marker of poor overall survival and poor relapse-free survival (34). CCND3 is also downregulated in human epidermal growth factor receptor 2 (HER2)-overexpressing breast cancer and is induced by rapamycin to cause G1 arrest (35). Notably, a lack of CCND1 is associated with a compensatory upregulation of CCND3 in mice overexpressing wild-type HER2 (ErbB2) (36). There may be an association between the regulation of these two cyclin D family members. As for the cyclin E gene, its overexpression has been found to be an important predictor of a poor prognosis for patients with tumors of various organs and tissue sources. Over 10% of transgenic mice overexpressing human cyclin E spontaneously developed mammary carcinoma (37).

Given the critical role of D-type cyclins in cell cycle regulation, their abnormal or untimely expression could potentially disrupt the cell cycle and promote the proliferation of malignant cells. In the current study of tumor cell proteomes using the SILAC-MS method, it was found that the expression of CCND1 differed significantly following treatment with

quercetin, but that quercetin did not affect the other cyclin D family members. A western blot assay on HepG2 cells with and without quercetin confirmed the SILAC-MS results. We suspect that the effect of quercetin on HepG2 cell proliferation was partly due to changes in the expression of the CCND1 gene and the subsequent block of the G1 phase. In the future, we plan to study the effect of quercetin on CCND1 and inoculated tumors in an animal model in order to further test the impact of quercetin treatments on tumor proliferation.

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