Investigation of Quercetin-Induced HepG2 Cell Apoptosis-Associated Cellular Biophysical Alterations by Atomic Force Microscopy

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Summary: Quercetin, a wildly distributed bioflavonoid, has been proved to possess excellent antitumor activity on hepatocellular carcinoma (HCC). In the present study, the biophysical properties of HepG2 cells were qualitatively and quantitatively determined using high resolution atomic force microscopy (AFM) to understand the anticancer effects of quercetin on HCC cells at nanoscale. The results showed that quercetin could induce severe apoptosis in HepG2 cells through arrest of cell cycle and disruption of mitochondria membrane potential. Additionally, the nuclei and F-actin structures of HepG2 cells were destroyed by quercetin treatment as well. AFM morphological data showed some typical apoptotic characterization of HepG2 cells with increased particle size and roughness in the ultrastructure of cell surface upon quercetin treatment. As an important biophysical property of cells, the membrane stiffness of HepG2 cells was further quantified by AFM force measurements, which indicated that HepG2 cells became much stiffer after quercetin treatment. These results collectively suggest that quercetin can be served as a potential therapeutic agent for the investigation of anticancer drugs. SCANNING 9999:1–13, 2015. © 2015 Wiley Periodicals, Inc.

Key words: quercetin, apoptosis, atomic force microscopy, biophysical property

Introduction

Hepatocellular carcinoma (HCC) is the most common cancer in some parts of the world in terms of incidence with 626,000 new cases being diagnosed each year, accounting for 5.7% of all new cancer cases (Parkin et al., 2005). Due to the poor prognosis of the disease, the number of deaths per year is almost the same as new cases, making HCC the third most common cause of cancer-related death. The only curative treatment methods for HCC are surgery, percutaneous ablation, and liver transplantation. Unfortunately, the majority of patients have unresectable disease at diagnosis so that effective treatment options are needed for patients with advanced HCC (Song and Bae, 2014). As other options for HCC treatment, chemotherapy and radiotherapy are found to induce plenty of side effects. Thus, it is imperative to explore and evaluate new agents for treatment of HCC.

With a long history of clinical use, Chinese herbal medicine (CHM) is emerging as a noticeable choice for its multi-level, multi-target, and coordinated intervention effects against HCC. With the development of phytochemistry and molecular biological approaches, some CHM-derived compounds have been found to show great anti-cancer effects in HCC treatment. Quercetin (Fig. 1(A)), a bioflavonoid widely distributed in a variety of plants, can inhibit proliferation and induce apoptosis in different kinds of cancer cells (Kim et al., 2005; Zhang et al., 2005; Lim et al., 2007; Kim et al., 2008; Tanigawa et al., 2008; Chou et al., 2010; Jakubowicz-Gil et al., 2010; Jung et al., 2010; Pratheeshkumar et al., 2012). What is more, quercetin exerts apoptosis induction effect in a selective manner because the same concentrations to induce apoptosis of cancer cells cannot induce apoptosis of normal cells (Chowdhury et al., 2005).
Previous studies have demonstrated that quercetin is a potent inhibition agent against HCC cell lines through the induction of apoptosis via ROS production, caspase activation, Bcl-2 regulation, p53 stabilization, PI-3-Kinase/Akt, and ERK inhibition (Granado-Serrano et al., 2006; Tanigawa et al., 2008; Chang et al., 2009; Zhou et al., 2009). Quercetin can also significantly inhibit H22 hepatoma growth in mice model in a dose-dependent manner, which results in improving survival time of tumor-bearing mice (Yuan et al., 2006a,b), demonstrating the potential use of quercetin for HCC treatment.

The biophysical information of cancer cells is an important indicator of the basic functions and metabolism states of cancer cells, as the unique physical properties of cells are closely related to the cell functions of adherence, motility, transformation, and invasion (Suresh, 2007; Kim et al., 2012). As a nondestructive cell surface imaging tool, atomic force microscopy (AFM) can provide us subtle information about cell morphology, cell surface ultrastructure, and cellular biophysical properties with nanometer accuracy (Jin et al., 2011a; Zhang et al., 2012). Given these advantages, AFM has been widely used in the imaging of cellular morphology, membrane ultrastructure, and the determination of cell surface biophysical properties to investigate the anticancer effects of drugs (Ye et al., 2006; Jin et al., 2011b; Kim et al., 2012) or to diagnose cancers (Cross et al., 2007; Plodinec et al., 2012). Quercetin is found to induce apoptosis in HCC cell lines, which is associated with some biochemical alterations, such as ROS production, caspase activation, and p53 stabilization (Granado-Serrano et al., 2006; Tanigawa et al., 2008; Chang et al., 2009; Zhou et al., 2009). However, the effects of quercetin on the biophysical property of cancer cells are still poorly understood.

In the present study, to investigate the anticancer effects of quercetin against HCC cells, we conducted a battery of evaluations on the basis of HepG2 cells, which included cell viability, apoptosis, cell cycle, mitochondrial membrane potential, F-actin structure, and nuclei morphology. And notably, we investigated the morphology, ultrastructure, and stiffness of HepG2 cells upon quercetin treatment using AFM, which extended our understanding of the biophysical information about quercetin-induced cancer cell apoptosis.

Materials and Methods

Materials

Quercetin (≥98%, HPLC) was obtained from Feibo Biotechnology (Guangzhou, China). Fetal calf serum (FCS), penicillin/streptomycin, Dulbecco’s modified eagle medium (DMEM), and trypsin kit were obtained from Gibco (Grand Island, USA). Paraformaldehyde was purchased from Sigma (St. Louis, USA). 3-(4,5)-dimethylthiazol-(2)-2,5-diphenyltetrazolium bromide (MTT), annexin V-FITC/PI apoptosis detection kit, rhodamin 123, actin-tracker green (phalloidin-FITC), 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), and cell cycle analysis kits were purchased from Beyotime Institute of Biotechnology, China.
Cell Culture

HepG2 cell line was a generous gift from Dr. Jianru Guo (State Key Laboratory of Quality Research in Chinese Medicines, Macau University of Science and Technology, Macau, China), which were cultured with DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Cell Viability

MTT assays were used to test the cell viability changes of HepG2 cells exposed to quercetin. The cells were seeded into 96-well plates with a density of 5 × 10³ for 24 h and incubated with different concentration of quercetin for 24 h and 48 h, respectively. After quercetin treatment, MTT reagents (10 μL, 5 mg/mL) were then added into each well for 4-h incubation, the medium was removed, and the cells were suspended in 100 μL SDS–HCl solution to incubate overnight. A spectrophotometer (Tecan, Männedorf, Switzerland) was used to test absorbance at 570 nm.

Determination of Apoptosis and Necrosis

Annexin V-FITC/PI apoptosis detection kit was used to detect the apoptosis of quercetin-treated HepG2 cells according to the manufacturer's instructions. After being incubated with quercetin for 48 h, HepG2 cells were harvested, washed triple with PBS, suspended in annexin V binding buffer, and incubated with FITC-labeled annexin V and PI for 5 min at room temperature in dark. Then, the samples were immediately analyzed by flow cytometer (BD, Franklin Lakes, USA).

Cell Cycle Analysis

After 48-h treatment with quercetin, HepG2 cells were harvested, washed with PBS, and fixed with 70% ethanol overnight at 4 °C. The fixed cells were washed three times with PBS, treated with RNase A, stained with propidium iodide (PI, 50 μg/mL) for 30 min at 37 °C, and analyzed by a flow cytometer (BD).

Mitochondrial Membrane Potential (MMP) Analysis

Rhodamine 123-based flow cytometry was used to determine the alterations of MMP of HepG2 cells before and after quercetin treatment. After treated with quercetin for 24 h, the harvested and washed HepG2 cells were incubated with rhodamine 123 for 60 min in dark at 37 °C. Flow cytometer was used to detect the fluorescence signal of rhodamine 123 after the cells were collected and washed twice with PBS (BD).

F-Actin and Nuclei Analysis

The structure of F-actin and nucleus in HepG2 cells was investigated by fluorescence microscopy with special staining of F-actin with actin-tracker green and nucleus with DAPI. After being treated with quercetin for 48 h, washed HepG2 cells were fixed by 4% paraformaldehyde for 10 min, washed with PBS (1% Triton X 100), and then incubated with 200 μL actin-tracker green for 30 min in dark at room temperature. After washed triple with PBS, cells were then incubated with 50 μM DAPI for 4 min and washed triple with PBS. A fluorescence microscopy was used to image the nuclear morphology and the organization of F-actin cytoskeleton structure.

AFM Sample Preparation

For AFM measurements, HepG2 cells were harvested with 0.25% trypsin and cultured at a density of 5 × 10⁴ cells/mL on glass coverslips in a 6-well plate (2 mL/well). After overnight incubation, designed concentrations of quercetin were added into the culture medium for 48 h stimulation. Cells were then washed triple with PBS, fixed with 4% paraformaldehyde solution for 10 min, washed triple with distilled water, and dried in air for morphology imaging in air or immediately used for force measurements in PBS solution.

AFM Analysis

AFM (Bruker, Rheinstetten, Germany) was used to investigate the topographical and ultrastructural changes of HepG2 cells induced by quercetin treatment. Organic contaminates of the silicon nitride tips used in all measurements were removed by ultraviolet irradiation. The spring constants of the AFM cantilevers were calibrated using the thermal noise method implemented in the Nanoscope software on AFM (Veeco, New York, USA). Firstly, the calibration of deflection sensitivity was carried out on the glass cover slips at a small vertical deflection (~0 V) in air. Then the thermal tune curve was fitted by the simple harmonic oscillator fitting to calculate the spring constant. The curvature radius of the AFM tips (BudgetSensors, Sophia, Bulgaria) used for morphology imaging is 10 nm, and the spring constant of tip is 0.42 ± 0.09 N/m with a deflection sensitivity of
51.19 ± 2.12 nm/V. The morphological and ultrastructural images of HepG2 cells were measured in air at room temperature in contact mode. The ultrastructure of HepG2 cells were all obtained in the areas surrounding the nuclei and the topographical image processing and data analysis were performed using the instrument equipped Nanoscope analysis software. For the particle size and roughness analysis, more than 18 different 1 × 1 μm ultrastructure images on 10 different HepG2 cells were calculated by the instrument equipped Nanoscope analysis software.

The adhesion force curves between HepG2 cells and AFM tips were acquired by AFM (Bruker) in PBS buffer. The curvature radius of the tips used for force measurements is 15 nm, and the spring constant of the tip is 0.36 ± 0.06 N/m with a deflection sensitivity of 20.12 ± 1.23 nm/V. For adhesion force measurements, more than 4500 force-distance curves were recorded for each group from at least 15 different cells at the nuclei area of HepG2 cells in PBS solution at room temperature in force volume mode (16 × 16). To reduce the error originating from different detecting condition, the measurements of samples in each group were performed under the same conditions. The force curve analysis was also performed using the instrument equipped Nanoscope analysis software.

Youngs Modulus Calculations

In the contact region, the behavior of the cell–tip–cantilever system can be described by the continuum mechanics of elastic contact. The Young’s modulus is calculated from the basic Sneddon model, which describes the behavior of a known geometry indenter calculated from the basic Sneddon model, which demonstrates a dose-dependent inhibition manner of quercetin in HepG2 cells, cell viability had no significant changes both in control and quercetin-inhibited HepG2 cell proliferation. In response to quercetin treatment, cell cycle of HepG2 cells was arrested in G2/M phase, which could be served as an important reason for quercetin-inhibited HepG2 cell proliferation. In response to quercetin treatment, cell cycle of HepG2 cells was arrested in G2/M phase, which was associated with a parallel rise in p21 level and a reciprocal fall in cyclin D1 (Tanigawa et al., 2008).

Results and Discussion

Inhibition Effects of Quercetin on HepG2 Cell Proliferation

As a member of the bioflavonoids family, quercetin is widely distributed in plants and fruits, and the chief dietary sources of quercetin are from apples, onions, and tea. Quercetin has been found to possess many biological activities, including antioxidant effects (Coskun et al., 2005), pro-oxidant effects (Metodiewa et al., ’99), anti-inflammation effects (Nakamura and Omura, 2008), and anticancer effects (Kim et al., 2008). The anti-proliferative effects of quercetin on human HCC cell line HepG2 cells were evaluated using MTT assay. As shown in Figure 1, the viability of HepG2 cells upon 24-h quercetin exposure was 85.1 ± 3.7%, 80.5 ± 4.8%, 57.9 ± 4.0%, 55.9 ± 4.3%, 51.5 ± 5.9%, and 41.5 ± 5.9% for 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, and 50 μM, respectively (Fig. 1(B)). And after 48-h quercetin treatment, the viability of HepG2 cells was 86.2 ± 1.6%, 65.5 ± 2.1%, 45.2 ± 2.9%, 22.2 ± 3.2%, 18.4 ± 3.6%, and 14.7 ± 1.7% for 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, and 50 μM quercetin, respectively (Fig. 1(C)). For DMSO-treated HepG2 cells, cell viability had no significant changes both in 24-h and 48-h treatment groups. These results demonstrated a dose-dependent inhibition manner of quercetin on the viability of HepG2 cells.

Quercetin Induced Cell Cycle Arrest in HepG2 Cells

Flow cytometry was performed to assess the effects of quercetin on cell cycle distribution in HepG2 cells with PI staining after 48-h quercetin treatment. As shown in Figure 2(A), the increased cells in G2/M phase were observed in the representative cell cycle distribution maps. And the average percentage of cells in G2/M phase increased from 11.9 ± 3.1% for control HepG2 cells to 12.7 ± 5.3%, 48.8 ± 12.6%, and 42.9 ± 8.2% for 5 μM, 20 μM, and 40 μM quercetin-treated HepG2 cells, respectively (Fig. 2(C)). The obtained results demonstrated that quercetin significantly arrested the cell cycle of HepG2 cells at G2/M phase, which could be served as an important reason for quercetin-inhibited HepG2 cell proliferation. In response to quercetin treatment, cell cycle of HepG2 cells was arrested in G2/M phase, which was associated with a parallel rise in p21 level and a reciprocal fall in cyclin D1 (Tanigawa et al., 2008).

Statistical Analysis

All results are representative of three or more independent experiments and the data presented are expressed as mean ± S.E.M. Statistical analysis was performed using Student’s t-test, and p < 0.05 was regarded as statistically significant.
Quercetin Induced HepG2 Cell Apoptosis

Flow cytometry analysis was further performed to assess the induction of apoptosis in HepG2 cells by quercetin treatment using annexin V-FITC and PI staining. In a representative apoptosis map, Q2 meant the late apoptotic and necrotic cells, Q3 presented the live cells, and Q4 was the percentage of early apoptotic cells. After 48-h treatment, the live cells decreased with the increasing dosage of quercetin, from 93.6% to 89.7%, 43.1%, and 20.3% for 5 μM, 20 μM, and 40 μM quercetin-treated groups, respectively (Fig. 2(B)). The percentage of HepG2 cells suffering from early apoptosis increased from 0.37 ± 0.31% to 0.76 ± 0.2%, 33.1 ± 4.4%, and 54 ± 8.7% after 5 μM, 20 μM, and 40 μM quercetin treatment, respectively (Fig. 2(D)). And the late apoptotic and necrotic cells increased from 1.6 ± 0.6% to 3.1 ± 0.8%, 25.9 ± 1.8%, and 21.9 ± 5.8% after 5 μM, 20 μM, and 40 μM quercetin treatment, respectively (Fig. 2(D)).

The strategy for treatment of HCC could be multiple, and the induction of apoptosis in cancer cells had become one of the most important approaches. Quercetin was found to induce apoptosis in a variety of cancer cells, including melanoma cells, glioma cells, breast cancer cells, prostate cancer cells, colon cancer cells, and HCC cells (Kim et al., 2005; Zhang et al., 2005; Lim et al., 2007; Kim et al., 2008; Tanigawa et al., 2008; Chou et al., 2010; Jung et al., 2010; Pratheeshkumar et al., 2012). Our study showed that quercetin could induce significant apoptosis in HepG2 cells, which implied that quercetin-induced toxicity in HepG2 cells was mainly...
through the induction of apoptosis. Apoptosis is a kind of programmed cell death that can kill cancer cells without inflammations in the surrounding tissues. The strong ability of quercetin to induce apoptosis in HepG2 cells, thus, makes quercetin a potent apoptosis induction agent for HCC treatment.

**Quercetin Induced Disruption of Mitochondrial Membrane Potential in HepG2 Cells**

To determine whether the dysfunction of mitochondria was also involved in quercetin-induced HepG2 cell apoptosis, we examined the mitochondrial membrane potential (MMP) in response to quercetin exposure by flow cytometry using rhodamine 123 as a fluorescence probe. As shown in Figure 3, the fluorescence signal decreased from 92.3 ± 0.7% to 90.6 ± 1.2%, 80.8 ± 1.4%, and 77.3 ± 0.6% after 24-h treatment of 5 μM, 20 μM, and 40 μM quercetin, respectively. The decrease of fluorescence signal after quercetin treatment indicated that quercetin could induce the disruption of MMP in HepG2 cells. As well known, quercetin could act as an effective donor of electrons for scavenging reactive peroxyl radicals to form quercetin radicals (Quercetin-O), which could enter the redox cycle to potentially increase ROS level and subsequently cause oxidative damage of cancer cells (Jeong et al., 2009). Moreover, the increased level of ROS in cancer cells would then destroy the function of mitochondria, which was the main place of redox reaction happened, and finally induced the disruption of mitochondrial membrane potential. The disruption of MMP would lead to mitochondria-dependent apoptosis, including the release of cytochrome c, the decrease of adenosine triphosphate (ATP) generation, the activation of caspases, and finally the irreversibly initiation of apoptotic cascades (Waterhouse et al., 2001; Hu and Kavanagh, 2003). Previous works had demonstrated the ability of quercetin to induce apoptosis by targeting the function of mitochondria in cancer cells (Liang et al., 2011; Kim et al., 2013). In addition, quercetin was also reported to induce apoptosis through mitochondria-mediated pathway in HepG2 cells (Granado-Serrano et al., 2006). Our data also demonstrated that quercetin, as a strong apoptosis-inducing agent, could induce mitochondria-mediated apoptosis in HepG2 cells by acting on the mitochondrial membrane potential.

**Quercetin Induced F-Actin Structure Damage and Accumulation in HepG2 Cells**

The structural changes of F-actin and nuclei induced by quercetin in HepG2 cells were tested by specific staining of the F-actin cytoskeleton with phalloidin-FITC and the staining of nucleus with DAPI for the localization of nucleus. As shown in Figure 4(A), the staining of control HepG2 cells showed remarkable actin filaments, which were arranged in parallel structures with thick stress fibers. There were no significant changes of F-actin structure between control and 5-μM quercetin-treated HepG2 cells, which both showed some remarkable actin filaments (Fig. 4(B)). But after 20-μM and 40-μM quercetin treatment, the actin filaments disappeared in HepG2 cells and the F-actin tended to aggregate in the apoptotic cells (Figs. 4(C and D)). The results implied that 20-μM and 40-μM quercetin treatment for 48 h significantly disturbed the organization of F-actin in HepG2 cells, making the parallel distributed actin filaments into aggregates in apoptotic cells. The cytoskeleton of cells, an intricate polymer network under cell membrane, was the structural framework that predominantly shapes a cell (Guck et al., 2005), which demonstrated that the morphological damage of HepG2 cells upon quercetin treatment was attributed to the cytoskeleton disruption induced by quercetin. Additionally, F-actin was viewed as the critical component of cytoskeleton that was necessary for cell motility and sustained cell signaling.
because it provided the capability of cells to move organelles within the cytoplasm and to deliver pro-apoptotic molecules to mitochondria (Dustin, 2006; Tang et al., 2006). Thus, the quercetin-induced changes of F-actin in HepG2 cells were also closely related to the apoptotic processes of HepG2 cells induced by quercetin treatment. And it was also worth noting that the fluorescence signal of F-actin increased after quercetin treatment, which could be attributed to the shrinkage of HepG2 cells as the shrinkage of cell body would make the density of F-actin under cell membranes increased dramatically.

Fig 4. Quercetin induced cytoskeleton F-actin disruption and nuclei damage of HepG2 cells determined by fluorescence microscopy. HepG2 cells were treated with (A) 0 μM, (B) 5 μM, (C) 20 μM, and (D) 40 μM quercetin for 48 h and stained F-actin with phalloidin-FITC and nuclei with DAPI. The left panel is the distribution of F-actin of HepG2 cells and the right panel is the morphology of nuclei. The blue arrows show the actin filaments with thick stress fibers, the red arrows indicate the aggregates of F-actin, and the yellow arrows indicate the broken nuclei after quercetin treatment, scale bar: 50 μm.
Moreover, Figure 4 also showed the representative nuclei morphology in HepG2 cells before and after quercetin treatment. Control HepG2 cells showed intact and plump nuclei, which was similar with 5-μM quercetin treated HepG2 cells. However, the nuclei of some apoptotic cells represented typical fragmented or broken morphology of nuclear bodies after 20-μM and 40-μM quercetin treatment. The nuclei damage observed by DAPI staining was another evidence for the apoptosis induced by quercetin, as the broken nuclei and condensed chromatin were important indicators of cell apoptosis.

**Quercetin Induced Morphological Changes in HepG2 Cells**

In recent years, AFM has emerged as a powerful quantitative and qualitative tool for nanoscale morphology imaging and pico-newton sensitivity force measurements of cell surfaces. Applying this high-resolution technique, some important information of cell surface can be resolved, such as the cell membrane ultrastructure, cell membrane adhesion forces, and cell surface stiffness, which are very difficult to be determined by other technologies. AFM has also been recruited to study the morphological and mechanical properties of cancer cells upon drug stimulation in recent years (Jin et al., 2011b; Kim et al., 2012). But until now, the effects of quercetin on the morphological, ultrastructural, and biophysical properties of cancer cells are still not determined by AFM.

To resolve the precise morphological changes of HepG2 cells induced by quercetin treatment, high-resolution AFM was operated to observe a variety of changes in surface morphology and ultrastructure of HepG2 cells treated with different concentrations of quercetin. The typical AFM images of HepG2 cells before and after quercetin treatment are shown in Figure 5. Control HepG2 cells had a regular spindle or oval shape, the cell surface was relatively smooth and intact. After a treatment with 5-μM quercetin for 48 h, there were no remarkable changes in cellular morphology. But after 48-h treatment with 20-μM quercetin, HepG2 cells had shown some typical morphological characteristics of apoptosis, including the shrunk cell bodies and the condensed cytoplasm. And for 40-μM quercetin-treated HepG2 cells, more typical morphological characteristics of apoptosis could be observed besides the shrunk cell bodies and condensed cytoplasm, such as the shrinkage of cells, the smaller cell volume, and the emergence of apoptotic body (indicated by red arrow in Fig. 5(D)). Additionally, the cross line analysis of HepG2 cells also showed the height of cells were about 600 nm, 600 nm, 700 nm, 1200 nm for control, 5 μM, 20 μM, and 40 μM quercetin-treated HepG2 cell, respectively. Through the calculation of more than 15 different cells in each group, the height of HepG2 cells was found to significantly increased after 20-μM and 40-μM quercetin treatment (Supplementary Fig. S1). These results with high resolution demonstrated that quercetin could induce morphological damage of HepG2 cells, which was associated with the apoptosis of HepG2 cells. Additionally, the observed morphological damage of HepG2 cells by AFM could also be attributed to the structure changes of intracellular F-actin, which was very important to shape a cell.

**Quercetin Induced Surface Ultrastructural Changes in HepG2 Cells**

Besides the whole cell morphology, the effects of quercetin on the ultrastructure of HepG2 cells were also determined by AFM analysis to investigate the more detailed morphological changes of quercetin-treated HepG2 cells. As shown in Figure 6, the valley topography of HepG2 cell surface was more remarkable after quercetin treatment as the gully or hole structures became bigger than control cells. And as indicated by the ultrastructure parameters extracted from the ultrastructure images, the particle size and roughness (including Rq and Ra) increased after high dosages of quercetin exposure. The statistical results (Fig. 7(A)) demonstrated that the particle size on the membrane of HepG2 cells remarkably increased from 17.38 ± 1.33 nm to 25.33 ± 2.59 nm, 30.33 ± 4.98 nm, and 32.85 ± 4.62 nm after 5-μM, 20-μM, and 40-μM quercetin treatments, respectively. The increased particle size on the surface of HepG2 cells indicated that quercetin exposure would induce significant aggregation of membrane proteins in HepG2 cells. The changes of cell membrane ultrastructure and membrane particle sizes also implied that some physiological changes had occurred in membrane proteins during quercetin exposure, such as the disruption of cytoskeleton underlying cell membranes, expression or structure regulation of membrane proteins, or opening/closing of ion channels. Zhou et al. had determined differential proteomic profiling of HepG2 cells treated by quercetin using a quantitative proteomic strategy, which found that 11% of the changed proteins in HepG2 cells after quercetin treatment was attributed to membrane proteins and the expression of β-tubulin reduced at a large degree (Chang et al., 2009). Our results also found that the structure of another important part of cytoskeleton, F-actin, was also disturbed by quercetin treatment. Thus, the changes of particle size on the membrane of HepG2 cells after quercetin treatment were mostly due to the alterations of membrane proteins and the cytoskeleton proteins under cell membrane.

The analysis of the surface roughness (including Rq and Ra) could provide some novel quantitative data for the study of the cell ultrastructure as this property of cells indicated the complexity of cell surface structures. Thus, we also quantified the changes of membrane
roughness by AFM analysis and found that the surface root-mean-squared roughness (Rq) of HepG2 cells increased from $6.84 \pm 0.51$ nm to $7.82 \pm 0.62$ nm, $10.05 \pm 1.75$ nm, and $12.94 \pm 1.4$ nm after 5-mM, 20-mM, and 40-mM quercetin treatment, respectively (Fig. 7(B)). The surface average roughness (Ra) of HepG2 cells increased from $5.45 \pm 0.41$ nm to $6.19 \pm 0.50$ nm, $7.74 \pm 1.32$ nm, and $9.97 \pm 1.14$ nm for 5-mM, 20-mM, and 40-mM quercetin-treated groups, respectively (Fig. 7(C)). The increased membrane roughness of quercetin-treated HepG2 cells indicated a rougher surface of quercetin treated HepG2 cells.

As reported, the hole structures were one of the sources for the rougher surface of cells (Kim et al., 2012), which implied that the rougher cell membrane of HepG2 cells induced by quercetin treatment was closely related to the holes emerged on the surface of HepG2 cells. As indicated in the ultrastructure of HepG2 cells, quercetin treatment induced the surface gullies or holes became bigger as a result of membrane damage in HepG2 cells upon quercetin exposure, which was responsible for the increased roughness of HepG2 cell surface. After quercetin treatment, the cell cytoplasm of HepG2 cells lost its osmotic pressure and the membrane collapsed onto the rough cytoskeleton or organelle structures, which thus led to the gully or hole structures. And additionally, the increased particle size on cell membrane, which meant the aggregation of membrane proteins, might also be important reasons for the increased roughness in the membrane of quercetin induced apoptotic HepG2 cells. These remarkable changes in the ultrastructure parameters of HepG2 cells demonstrated that quercetin induced HepG2 cell apoptosis was not only reflected by the whole cell morphological damages, but also could be probed by high resolution AFM to resolve the precise ultrastructure changes at nanoscale.

**Quercetin Induced Stiffness Changes in HepG2 Cells**

Cell stiffness or elasticity is an important factor relating to cell function, adherence, motility, transformation, and invasion. During the past decade, the
changes of stiffness in cancer cells on the progression of tumor have become increasingly clear (Cross et al., 2007; Plodinec et al., 2012), indicating that the stiffness of cancer cells can be served as an important indicator of the invasion or metabolism state of tumors. Thus, the measurements of stiffness in cancer cells are of vital importance to understand the biophysical properties of cancer cells upon drug treatment. In the present study, the stiffness of HepG2 cells was determined by the AFM force indentation analysis. During AFM force indentation analysis of HepG2 cell samples, AFM tip was located on the surface of HepG2 cells, contacted with

Fig 6. Quercetin induced membrane ultrastructure changes of HepG2 cells determined by AFM. AFM images of HepG2 cells treated with (A) 0 μM, (B) 5 μM, (C) 20 μM, and (D) 40 μM quercetin for 48 h. (A1, B1, C1, D1) Topography, scale bar: 20 μm. (A2, B2, C2, D2) Cell surface ultrastructure and (A3, B3, C3, D3) their corresponding 3-D images of HepG2 cells indicated in (A1, B1, C1, D1), scale bar: 200 nm. (A4, B4, C4, D4) Particle size distribution and roughness of cell surface ultrastructure analyzed from (A2, B2, C2, D2).

Fig 7. Statistical results of quercetin-induced membrane ultrastructure changes of HepG2 cells determined by AFM. (A) Particle size distribution, (B) root-mean-squared roughness (Rq), and (C) average roughness (Ra) analyzed from 1 × 1 μm frame ultrastructure images of HepG2 cells, ***p < 0.001, **p < 0.01.
the cell membrane, pressed against the cell surface to make an indentation of tip into the cell sample and finally retracted from cell membrane. During the retraction process of AFM tip from the surface of HepG2 cells, the retraction events occurred and shown as force–distance curves (Fig. 8), which could be calculated into Young’s modulus using the basic Hertz–Sneddon model. As shown in Figure 8(A), the typical indentation force curves acquired on HepG2 cells showed the increase of slope with the treatment of quercetin, which meant that HepG2 cells became stiffer after quercetin treatment. As shown by the Young’s modulus maps in Figure 8(B), the Young’s modulus of HepG2 cells increased with the treatment of quercetin. The distribution analysis of Young’s modulus (Fig. 8(C)) were found to increase from 42.8 ± 0.3 kPa for control HepG2 cells to 50.6 ± 0.6 kPa, 67.2 ± 0.4 kPa, and 105.9 ± 1.2 kPa for 5 μM, 20 μM, and 40 μM quercetin-treated cells, respectively. The statistical analysis results (Fig. 9) demonstrated that the Young’s modulus, a parameter corresponding to stiffness, significantly increased after 48-h quercetin treatment, which implied that HepG2 cells became much stiffer after the exposure of quercetin.

In theory, the Young’s modulus calculated on cell surface is dependent on the intracellular structures of cells, including the strength of cytoskeleton and the presence of underlying organelles under cell surface, such as nuclei and mitochondria. Small and gradual increases of Young’s modulus can be attributed to the hardening of cytoskeleton structures, whereas a larger increase, such as fivefold increasing, is always supposed to be caused by the changes of underlying organelle distributions (Leporatti et al., 2006). In this study, the Young’s modulus of HepG2 cells increased nearly twofold after 48 h 40-μM quercetin treatment, which meant that the influence of underlying organelles was not yet pronounced and the cytoskeleton structure changes might play the most important roles in the stiffness changes of HepG2 cells induced by quercetin.

As the most important part of cytoskeleton, the integrity of F-actin network was regarded as an important indicator of cell stiffness measured by AFM (Rotsch and Radmacher, 2000; Solon et al., 2007). As a result of apoptosis induced by quercetin, HepG2 cells were found to show shrunk cell bodies and condensed cytoplasm, which made the cell volume decrease and the cell height increase (Fig. 5). The staining of F-actin in HepG2 cells demonstrated that the F-actin structure was destroyed after quercetin treatment, which led the parallel distributed actin filaments into aggregates, and the fluorescence signals of F-actin in HepG2 cells became

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Fig 8. Effects of quercetin on Young’s modulus of HepG2 cells. (A) Typical force–distance curves obtained on HepG2 cells. (B) Typical Young’s modulus maps obtained on HepG2 cells. (C) Histogram presenting the distribution of Young’s modulus obtained from HepG2 cells. HepG2 cells were treated with 0 μM, 5 μM, 20 μM, and 40 μM quercetin for 48 h.
much stronger after quercetin treatment (Fig. 4). On the one hand, the increased F-actin fluorescence signals reflected the increased F-actin density in HepG2 cells after quercetin treatment, which might lead to the increased stiffness of cells. On the other hand, the apoptotic cells after quercetin treatment had lost their osmotic regulation and had significantly fragmented actin filaments, became turgid balls of cytoplasmic materials, and had lack of the ability to show their native elastic behavior of cells. F-actin network was thought to generate the tension inside cells and played very important roles in maintaining the biomechanical properties of cells, because an intact actin cytoskeleton would respond elastically to an imposed force (which was evolved into the concept of tensegrity) while a fragmented cytoskeleton would simply respond as an inanimate blot and appeared to be stiffer (Guck et al., 2005). Thus, the changes of F-actin structures in HepG2 cells after quercetin treatment could be set as the main reason for the enhancement of cell stiffness measured by AFM. Additionally, cancer cells or tumor tissues were found to be much softer than normal cells or normal tissues, and a decreased stiffness was able to promote the metastatic spread of soft cancer cells to normal tissues (Cross et al., 2007; Plodinec et al., 2012). These results indicated the possibility that regulating stiffness of cancer cells or tumor tissues might also be a strategy for cancer treatment. Very interestingly, HepG2 cells became much stiffer even after very low-dose quercetin treatment, which made it possible for quercetin to weaken the malignancy level of HCC by regulating the stiffness of cancer cells.

It is also worth noting that other microscopy, like fluorescence microscopy used in this work, could demonstrate the anticancer effects of quercetin against HepG2 cells at high dosages (20 μM and 40 μM), but the changes at lower concentration of quercetin (5 μM) could not be detected. However, AFM demonstrated the marginal influence on the membrane ultrastructure and Young’s modulus (stiffness) of HepG2 cells at 5-μM quercetin treatment, which showed the power of AFM over other microscopy techniques. Based on this advantage, AFM could be served as a powerful tool for the detection of pharmacological action on cancer cells upon low dosage drug treatment, which was difficult to be demonstrated by other microscopies.

**Conclusion**

In summary, our findings highlighted the use of AFM for biophysical property analysis in apoptotic HepG2 cells induced by quercetin for the first time, which would extend our understanding of the anticancer effects of quercetin on HCC cells into nanoscale. The treatment with quercetin could induce cell cycle arrest of HepG2 cells in G2/M phase and apoptosis of HepG2 cells through disrupting the mitochondria membrane potential. The apoptotic HepG2 cells were also proved by the fluorescence staining to show the broken nuclei and disrupted F-actin cytoskeleton, which were further detected by high-resolution AFM to show the shrunken cell bodies and condensed cytoplasm. The changes of HepG2 cells in cell surface ultrastructure were also determined by AFM analysis, which implied that quercetin treatment increased the particle size and roughness of HepG2 cell surface. Additionally, the membrane stiffness of HepG2 cells quantified by AFM force measurements demonstrated the much stiffer surface of HepG2 cells after quercetin treatment, which was attributed to the changes of F-actin induced by quercetin. Although the detailed mechanisms involved in anticancer activity needs further investigations, these findings provide new insights into the anticancer effects of quercetin at nanoscale and suggest that quercetin could be a potential therapeutic agent in HCC, which also highlight the use of AFM for the anticancer investigations of drugs.

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