Quercitrin suppresses hepatocellular carcinoma metastasis and angiogenesis by targeting the Nrf2 signaling pathway

Hong Guang Li1, Heng Jun Gao1, Fang Feng Liu1 and Jun Liu1

1Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, China

Correspondence to: Jun Liu, email: liujunljunl@163.com

Keywords: HCC; quercitrin; metastasis; angiogenesis; Nrf2

Received: August 24, 2017 Accepted: November 14, 2017 Published: December 30, 2017

ABSTRACT

NF-E2-related factor 2 (Nrf2) abnormally accumulates in multi-types of cancer, and its expression is closely associated with a poor prognosis for cancer patients. A large body of research has demonstrated that Nrf2 can protect cancer cells from chemotherapeutic agents and facilitate the malignant progression of tumors. Quercitrin, which is found in flavonoids from Sedum sarmentosum, has been associated with potential anti-cancer roles in the initiation and progression of several types of cancer. In this current study, we mainly investigated whether quercitrin had a therapeutic effect against hepatocellular carcinoma (HCC) using in vitro and in vivo studies. Herein, we demonstrated that quercitrin inhibit human HCC cell growth, migration, and invasion. In vivo experiments showed the growth and metastasis of HCC cells was markedly inhibited by quercitrin treatment. Additionally, quercitrin inhibited HCC cell-induced mobility and invasion, as well as the tube formation of human umbilical vascular endothelial cells (HUVECs) in vitro. In vivo, quercitrin markedly suppressed microvessel density and increases HCC cell apoptosis in tumors from nude mice inoculated with HCC cells. Finally, we demonstrated that Nrf2 was involved in cell growth and metastasis in HCC cancer cells. In summary, we demonstrated for the first time that quercitrin inhibited human HCC growth, angiogenesis and metastasis by targeting the Nrf2 signaling pathway.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-associated deaths worldwide [1]. Although advances in HCC treatment, including hepatectomy and liver transplantation, have been achieved, the rapid development of HCC leads to a poor prognosis and a five-year survival rate below 5% [2]. The main challenges in treating HCC include early postoperative detection, distal metastasis, and intrahepatic recurrence. Cancer metastasis is a complex multi-step process that consists of a series of consecutive steps, including cell migration, invasion, and movement of cancer cells through circulation, as well as the re-implantation of cancer cells in distant organs, which can be followed by colonization and associated tumor-induced angiogenesis [3, 4]. The ability to inhibit initial cancer cell migration and invasion will generally prevent metastasis. Therefore, improving our understanding of the molecular mechanisms of HCC metastasis may provide a new effective therapeutic target for the treatment of HCC and improve patient prognoses [5, 6].

NF-E2-related factor 2 (Nrf2) is a critical regulator of various cytoprotective genes [7]. After translation, Nrf2 is degraded by the proteolytic ubiquitin-proteasome system in the cytoplasm of cells. Kele-like ECH-associated protein 1 (KEAP1) is a component of the Cullin 3 (CUL3)-based E3 ubiquitin ligase complex and it controls both Nrf2 stability and accumulation [8, 9]. Abundant evidence indicates that Nrf2 either functions as a proto-oncogene or supports the transformation other oncogenes [10]. Additionally, Nrf2 promotes tumor cell survival under oxidative stress conditions and a potential model would posit that the up-regulation of Nrf2 activity...
HepG2 and PLC/PRF/5 HCC cell lines were used. As cell proliferation by colony formation analysis. After 24 h treatment. We further confirmed the inhibitory effects on exerted 50% inhibition under 20 μM after 24 or 48 h times were calculated, which showed that quercitrin manner. The IC50 values for both HCC cells and incubation PRF/5 cell proliferation in a dose- and time-dependent shown in Figure 1A, quercetin inhibited HepG2 and PLC/PRF/5 cancer cells mechanistically, we evaluated the anti-cancer roles of quercetin in HCC remain not well be elucidated [20]. Therefore, this present study aimed to determine whether quercetin could inhibit the growth and metastasis of HCC [21]. Additionally, we attempted to uncover the underlying cellular and molecular anti-cancer mechanisms of quercetin. Previous studies have shown that quercetin can inhibit the production of reactive oxygen species (ROS), enhance the activity of nuclear factor E2-related factor 2 (Nrf2), and drive antioxidant response element (ARE) activity in APAP-treated HepG2 cells. Consequently, we predicted that quercetin may inhibit HCC growth and metastasis by down-regulating the Nrf2 signaling pathway. Herein, we demonstrated that quercetin inhibit HCC cell invasion and migration in vitro. Using the nude mouse model, we shown that quercetin inhibit the metastasis of HCC cells in vivo. To investigate how quercetin regulated cancer cells mechanistically, we evaluated the anti-cancer activity of quercetin on HCC cells and found that the inhibition of tumor growth and metastasis were related to the inhibition of the Nrf2 signaling pathway. RESULTS

Quercitrin inhibits HCC cell growth and induces apoptosis

To assess the anti-cancer activity of quercetin, the HepG2 and PLC/PRF/5 HCC cell lines were used. As shown in Figure 1A, quercetin inhibited HepG2 and PLC/PRF/5 cell proliferation in a dose- and time-dependent manner. The IC50 values for both HCC cells and incubation times were calculated, which showed that quercitrin exerted 50% inhibition under 20 μM after 24 or 48 h treatment. We further confirmed the inhibitory effects on cell proliferation by colony formation analysis. After 24 h pre-treatment with quercetin, cells were incubated for three weeks and the colonies were counted. As shown in Figure 1B, quercetin markedly reduced the formation of HCC cell colonies in a concentration-dependent manner. To determine whether quercetin-mediated anti-cancer activity in HCC was associated with the induction of apoptosis, HepG2 and PLC/PRF/5 cells were treated with 2, 5, or 10 μM quercetin for 24 h. Apoptosis was then quantified by flow cytometry using Annexin V-FITC/PI double staining. As shown in Figure 1C, our data suggested that treatment with quercetin resulted in the dose-dependent induction of apoptosis in HepG2 and PLC/PRF/5 cells. In general, these data suggested that quercetin inhibit HCC cell growth and induces apoptosis.

Quercitrin inhibits HCC cell invasion and migration

To investigate the effect of quercetin on HCC metastasis in vitro, we determined the migration capacity of HepG2 and PLC/PRF/5 cells treated or not with quercetin. As shown in Figure 2A, the control group of cells closed the wounded area by 24 h, whereas quercetin treatment significantly inhibited cell wound closure in a dose-dependent manner at 24 h. Moreover, in Transwell invasion assays, quercetin treatment markedly reduced HepG2 and PLC/PRF/5 cell invasion (Figure 2B). Subsequent to this confirmation of the inhibition of quercetin in HCC cell migration and invasion in vitro, we tested whether quercetin inhibited pulmonary metastasis by intravenously injecting HCC cells into nude mice by tail vein injection. In control mice, HCC cells metastasized to the lungs and formed multiple metastatic lesions, whereas in quercetin-treated mice the number of metastatic foci was significantly reduced (Figure 2C). These data suggested that quercetin effectively inhibited HCC cell metastasis in vivo. Epithelial interstitial transformation (EMT) is a key mechanism of cancer metastasis and is closely associated with cell migration and invasion. Thus, we analyzed the expression of the EMT-associated E-cadherin/N-cadherin by western blotting and qRT-PCR using the total cell lysates of HepG2 and PLC/PRF/5 cells that had been exposed to quercetin. Quercetin-treated HepG2 and PLC/PRF/5 cells showed significant down-regulation of N-cadherin and up-regulation of E-cadherin (Figure 2D). Together, these data suggested that quercetin treatment reduce the metastasis of HCC cells both in vitro and in vivo. Quercitrin suppresses HCC development in murine models

To assess the anticancer effect of quercetin in vivo, we investigated the potential role of quercetin in a mouse model of HCC. Quercetin was orally administered, as shown in Figure 3A, and we found that
quercetin significantly prolonged survival (Figure 3B) and inhibited tumor development (Figure 3C). Additionally, immunohistochemistry results suggested that Ki67 staining in cancer tissues could be reduced by the administration of quercetin (Figure 3D). Furthermore, quercetin treatment increased the percentage of caspase-3 positive cells in tumor tissues (Figure 3E), suggesting that quercetin induced tumor cell apoptosis. The CD31 index represents a marker of microvessel density. We finally tested whether quercetin could regulate tumor

**Figure 1: Quercitrin inhibits HCC cell proliferation.** (A) HepG2 and PLC/PRF/5 cells were exposed to the indicated concentrations of quercitrin for 24 or 48 h, and cell viability was determined. Data are presented as means ± SD. Values are expressed as the percentage of viable cells normalized to the percentage of viable cells in the untreated group. (B) Effects of quercitrin on HCC cell colony formation *in vitro*. HepG2 and PLC/PRF/5 cells were pre-treated with quercitrin for 24 h. Cells were seeded in a 6-well plate in the absence of quercitrin and were cultured for three weeks. (C) HepG2 and PLC/PRF/5 cells were treated with quercitrin and Annexin V-FITC/PI apoptosis assays were performed. Representative plots of apoptotic HepG2 and PLC/PRF/5 cells are shown. Data are presented as means ± SD; *p < 0.05, **p < 0.01 compared to untreated control cells.
angiogenesis. Compared with the control group, we found that quercetin could down-regulate the expression of CD31 in HCC tumors (Figure 3F).

**Quercitrin suppressed tumor cell-induced angiogenesis**

Tumor-induced angiogenesis, which is critical to tumor cell growth, migration, invasion, and distant metastasis, depends upon angiogenic factors, including VEGF-A and bFGF secreted by tumor cells. To determine the impact of quercetin on tumor angiogenesis, we found that human umbilical vein endothelial cells (HUVECs) formed tube-like structures on Matrigel after treatment with conditioned medium (CM) from quercetin treated or untreated HepG2 or PLC/PRF/5 cells. As shown in Figure 4A, CM from both HepG2 and PLC/PRF/5 untreated quercetin cells induced obvious tube formation of HUVECs. However, quercetin-treated CM did not evoke a prominent increase in the tube network, suggesting that

---

**Figure 2: Quercitrin inhibits HCC cell metastasis.** (A) HepG2 or PLC/PRF/5 cells were wounded by scraping with a pipette tip. Cells were incubated with quercitrin for 24 h. The percentage of wound closure at 24 h was calculated based on the width of wound closure at 0 h. (B) Cells pre-treated with quercitrin were allowed to invade the Transwell membrane. The cells that invaded were stained and quantified. Data are representative of three experiments and expressed as means ± SD of five random fields per well. Data are presented as means ± SD; *p < 0.05, **p < 0.01 compared to untreated control. (C) BALB/c nude mice were injected with HepG2 or PLC/PRF/5 cells via the tail vein. After daily oral administration of vehicle (control) or quercitrin for 21 days, mice were sacrificed and lung tissues were subjected to H&E staining. Images show metastatic colonies of the lungs. (D) Both HepG2 and PLC/PRF/5 cells were treated with quercitrin, and the expression levels of E-cadherin/N-cadherin were analyzed by western blotting and qRT-PCR. Data are presented as means ± SD; *p < 0.05, **p < 0.01 compared to untreated control cells.
quercetin reduced the length of capillary-like structures in a dose-dependent manner. During the process of tumor angiogenesis, cancer cells vigorously attract endothelial cells by releasing angiogenic factors into microenvironment. To identify the effects of quercetin on tumor-mediated acceleration of endothelial cell mobility and invasion in vitro, HUVECs were inoculated in the upper chamber of the Transwell, whereas CM from quercetin-treated HepG2 or PLC/PRF/5 cells were plated in the lower chamber of the Transwell, respectively. As shown in Figure 4B, control CM in the lower chamber markedly induced HUVEC invasion. CM from quercetin-treated HCC cells significantly inhibited HUVEC invasion, suggesting that quercetin inhibited tumor cell-derived endothelial cell chemotaxis in a dose-dependent manner. As expected, the control group of cells that were treated with control CM closed the wounded area by 24 h, whereas CM from quercetin-treated cells significantly delayed cell wound closure by 24 h (Figure 4C). Finally, we found that quercetin attenuated VEGF-A and bFGF expression, which regulated tumor angiogenesis. As shown in Figure 4D, quercetin significantly inhibited VEGF-A and bFGF levels in HCC cells in a dose-dependent manner.

**Anti-cancer of quercitrin effects mediated by Nrf2 signaling**

We analyzed levels of Nrf2 in 47 HCC cancer tissues and 29 cases of corresponding adjacent normal tissues by qRT-PCR. Nrf2 was more frequently found in HCC tissues than in adjacent normal tissues (Figure 5A). Western blot and qRT-PCR analysis also revealed overexpression of Nrf2 in HepG2, SNU-423, SNU-387, SNU-449 and PLC/PRF/5 HCC cell lines in comparison to LO2 cell line (Figure 5B). To investigate whether Nrf2 promotes growth and metastasis in HCC cells, we knocked-down or over-expressed the Nrf2 gene in HepG2 or PLC/PRF/5 cells (data not shown). Our data demonstrated that the growth and invasion/migration of HCC cells could be significantly inhibited by Nrf2 knock-down (Figure 5C–5E), and increased by Nrf2 over-expression (Figure 5F–5H). To investigate whether quercitrin suppressed the growth and metastasis of HCC cells via Nrf2, a Nrf2 knock-down assay was conducted. Our findings suggested that the down-expression of Nrf2 markedly abolished quercitrin-induced inhibition of HCC cell colony formation, migration, and invasion (Figure 6A–6C). Additionally, immunoblotting and qRT-PCR analysis demonstrated that

---

**Figure 3: Quercitrin affects HCC cell growth in vivo.** (A) As a murine HCC cancer model, mice were implanted with HCC cells subcutaneously and administered quercitrin, as shown in the schematic diagram. (B) The survival time was evaluated. (C) Tumor weight was evaluated. The percentage of positive cells is presented. Scale bar: 200 μm. (E) Caspase-3 expression in tumor tissues was evaluated by immunostaining. The percentage of positive cells was presented. (F) Immunohistochemical analysis of the tumor angiogenesis marker CD31 in tumor tissues. Percentages indicate positive staining for the indicated biomarker.
Figure 4: Quercitrin suppresses tumor cell-HUVECs tube formation, migration and invasion. (A) Tube formation by HUVECs induced by control CM or quercitin-treated HCC cells CM was examined. The total length of capillary-like structures was counted using ImageJ software. (B) CM from HepG2 or PLC/PRF/5 cells that were treated or not with quercitrin were plated into the lower chamber and HUVECs cells were added to the upper chamber. After 24 h, HUVECs invaded were stained and quantified using ImageJ. (C) HUVECs cell monolayers were wounded by scraping. Cells were incubated for 24 h in the presence of control CM or quercitin-treated CM of HepG2 or PLC/PRF/5 cells. The percentage of wound closure at 24 h was calculated based on the width of the wound at 0 h. Data are presented as means ± SD; *p < 0.05, **p < 0.01 compared to control CM. (D) HepG2 and PLC/PRF/5 cells were treated with quercitin, and the expression levels of VEGF-A/bFGF were examined by western blotting and qRT-PCR. Data are presented as means ± SD; *p < 0.05, **p < 0.01 compared to controls.
the down-expression of Nrf2 abrogated the influence of quercetin on E-cadherin/N-cadherin levels in HCC cells (Figure 6D). To confirm that quercitrin restrained tumor-induced angiogenesis was dependent on Nrf2, a Nrf2 knock-down experiment was conducted. We found that the effect of quercitrin on tumor-induced angiogenesis was abolished by Nrf2 knock-down (Figure 6E-6F). These data strongly implied that quercitrin suppressed the proliferation, invasion, and migration capacities of HCC cells by targeting Nrf2.

Figure 5: Effects of Nrf2 in the regulation of HCC cell growth and metastasis. (A) The mRNA expression levels of Nrf2 in HCC cancer tissues and corresponding adjacent normal tissues were determined by qRT-PCR. (B) Expression levels of Nrf2 in a panel of HCC cell lines compared to LO2 cells were assessed by immunoblotting and qRT-PCR. (C) Colony-formation assay. Colonies of HepG2 or PLC/PRF/5 cells transfected with shNrf2 were higher compared with those transfected with control shRNA. (D) HepG2 and PLC/PRF/5 cells transfected with shNrf2 or control shRNA were wounded by scraping. The percentage of wound closure at 24 h was calculated on account of the width of the wound at 0 h. (E) HepG2 and PLC/PRF/5 cells that were transfected with shNrf2 or control shRNA were subjected to a Transwell invasion assay. Figures are representative of three experiments and are expressed as means ± SD of five random fields per well. (F) Colony-formation analysis. Colonies of HepG2 or PLC/PRF/5 cells transfected with Nrf2 were lower compared with those transfected with the control vector. (G) HepG2 and PLC/PRF/5 cells transfected with Nrf2 or control vector and were wounded by scraping. The percentage of wound closure at 24 h was calculated based on the width of the wound at 0 h. (H) HepG2 and PLC/PRF/5 cells that were transfected with Nrf2 or vector were subjected to an invasion assay.
Overexpression of Nrf2 rescues HCC cell metastasis that could be suppressed by quercitrin

To confirm that quercetin inhibited the migration and invasion of HCC cells by inhibiting Nrf2, Nrf2 rescue experiments were conducted. Our data suggested that the ectopic-expression of Nrf2 significantly attenuated quercetin-mediated inhibition of cell colony formation and metastasis by HepG2 and PLC/PRF/5 cells (Figure 7A–7C). Additionally, western blot analysis and qRT-PCR results revealed that the over-expression of Nrf2 reversed the effects of quercetin on E-cadherin/N-cadherin levels in HCC cells (Figure 7D). To confirm that quercetin inhibited tumor-induced angiogenesis, Nrf2, HepG2, or PLC/PRF/5 cells were transfected with a Nrf2 construct or an empty vector. In tube formation analysis, HUVECs formed tube-like structures on Matrigel, which could be rescued by the over-expression of Nrf2 in the presence of quercetin (Figure 7E). Finally, western blot and qRT-PCR analysis demonstrated that the over-expression of Nrf2 reversed the effects of quercetin on VEGF-A and bFGF levels in HCC cells (Figure 7F). Our findings suggested that the overexpression of Nrf2 significantly reversed quercetin-mediated angiogenesis inhibition.

DISCUSSION

Quercetin is an active ingredient in flavonoids that is abundantly found in Chinese herbs, onions, and fruits [22]. Extensive studies have demonstrated that quercetin exhibits various biological activities, including antioxidant, anti-inflammatory, and anti-cancer properties [23]. Several studies suggest that the anti-tumor effects of quercetin are mediated by inhibiting the metastatic potential of prostate cancer cells and melanoma and by restraining tumor angiogenesis [24, 25]. Additionally, previous studies establish that quercetin could suppress lipopolysaccharide- or 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced matrix metalloproteinase-9 (MMP-9) expression in glioma cells [26]. Although some pharmacological studies of quercetin have been carried out [20], the anti-growth and anti-metastasis capacities of quercetin in HCC have not yet well been analyzed. In this present study, we demonstrated that quercetin could inhibit the migration, invasion, and metastasis of HCC cells in vitro and in vivo, and revealed the underlying molecular mechanisms. Quercetin inhibited the expression of Nrf2 and thereby suppressed Nrf2 target genes (E-cadherin/N-cadherin), leading to inhibition of the metastasis of HCC cells. Moreover, we confirmed...
that quercitrin efficaciously suppressed HCC cell colony growth in vitro and xenograft tumor growth of HCC cells in vivo. Finally, we established an inhibitory effect of quercitrin on the angiogenic properties of HCC cells. Thus, our study suggested that quercitrin represents a novel candidate as an anti-cancer agent against HCC that was at least partially mediated by the inhibition of Nrf2.

Cancer cell metastasis is a multi-step process, which includes local invasion, intravasation to blood systems, survival in the bloodstream, extravasation from the micro-vessels, and colonization at distant organs [6, 27]. Many studies have yielded insights into the molecular mechanisms of each step of cancer cell metastasis [28]. The multistep process of metastasis and the known associated mechanisms provide numerous options for chemical interventions. Herein, we demonstrated that (1) quercitrin significantly attenuated HCC cell migration and invasion; (2) quercitrin suppressed HCC cell metastasis to the lung in vivo; and (3) quercitrin inhibited EMT-associated markers in HCC cells. Additionally, quercitrin markedly affected the prognosis and delayed tumor growth in a mouse xenograft model. Interestingly, quercitrin exhibited anti-angiogenic activities, whereas it exhibited lower CD31 expression in quercitrin-treated mouse. These data indicated that quercitrin may represent an angiogenesis inhibitor.

Solid tumors require persistent angiogenesis, which involves the formation of new vessels from pre-existing ones to allow for tumor cell growth and metastasis [29]. Without angiogenesis, cancer cell growth is restricted to a few millimeters and the cancer cells cannot escape into blood circulation to cause distant metastases [30]. In vivo, tumor-induced angiogenesis begins when tumor cells open an ‘angiogenic switch’ by releasing pro-angiogenic factors, including VEGF-A and bFGF, which, in turn, provoke pre-existing vessels to initiate angiogenesis [31]. In this present study, culture media (CM) from HCC cells were applied as an inducer of endothelial cells for an in vitro angiogenesis assay. In contrast to CM from HCC cells that were not treated with quercitrin, CM of quercitrin-treated HCC cells decreased the production of VEGF-A and bFGF by tumor cells; consequently, it inhibited the migration/invasion of endothelial cells. Quercitrin also suppressed the tumor-induced capillary-like tube formation of endothelial cells. Overall, these findings implied that quercitrin exhibited anti-angiogenic activities.

Furthermore, the expression of Nrf2 was assessed in HCC patient specimens and HCC cell lines. We found that its expression was elevated in HCC. The ectopic expression of Nrf2 promoted HCC cell metastasis, whereas silencing of Nrf2 resulted in inhibited metastasis.

![Figure 7: Upregulation of Nrf2 rescues the suppressive effects of quercetin on the growth and metastasis of HCC cells. (A) HepG2 and PLC/PRF/5 cells expressing Nrf2 or an empty vector were treated with 5 µM quercitrin and a colony formation assay was performed. (B) Nrf2-overexpressing HCC cells were treated with or without 5 µM quercitrin. Cellular migration was measured by wound healing assay. (C) HCC cells that overexpressed Nrf2 were treated with or without 5 µM quercitrin. Cellular invasion was detected by a Transwell invasion assay. (D) Nrf2-overexpressing HepG2 and PLC/PRF/5 cells were treated with or without 5 µM quercitrin. Expression levels of E-cadherin/N-cadherin were assessed by western blotting and qRT-PCR. (E) Tube formation by HUVECs induced by control CM or quercetin-treated CM from Nrf2-overexpressing HepG2 or PLC/PRF/5 cells were examined. The total length of capillary-like structures were measured using ImageJ. (F) HepG2 and PLC/PRF/5 cells that overexpressed Nrf2 or an empty vector were treated with or without 50 µM quercitrin. Expression levels of VEGF-A/bFGF were assessed by western blotting and qRT-PCR. Data are presented as means ± SD; “p” < 0.01 compared to control.](www.impactjournals.com/oncotarget)
Furthermore, the up-regulation of Nrf2 expression markedly stimulated altered expression of EMT-associated markers. Conversely, down-regulation of Nrf2 expression significantly decreased the expression of N-cadherin and Nrf2 target genes. To determine whether quercitrin inhibited HCC metastasis and angiogenesis in a manner dependent on Nrf2, both Nrf2 knock-down and over-expression analyses were performed. We found that in the presence of Nrf2 shRNA, quercitrin did not influence HCC migration or invasion. We also found that quercitrin did not inhibit angiogenesis after HCC cells were transfected with Nrf2 shRNA. As expected, Nrf2 over-expression rescued the inhibitory effect of quercitrin on HCC metastasis and angiogenesis. Overall, we showed that quercitrin effectivity inhibited human HCC cell growth and metastasis. We also confirmed that quercitrin exerted inhibitory effects on the process of HCC cell-induced angiogenesis. As such, quercitrin exhibited the potential to be used as a potent anti-cancer agent in therapeutic strategies to inhibit the growth and metastasis of HCC.

MATERIALS AND METHODS

Cell culture and HCC tissues

HepG2, SNU-423, SNU-387, SNU-449 and PLC/PRF/5 HCC cell lines and normal liver cells (LO2) were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RMPI-1640 or DMEM supplemented with 10% fetal bovine serum (FBS). In total, 47 HCC tissues and 29 normal tissues were collected from Superchip Biotechnology Co. (Shanghai, China). The shRNA against Nrf2 (shNrf2, sc-37030-SH) and control shRNA (shCTL) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). HepG2 and PLC/PRF/5 cells were seeded in 6-well plates the day prior to transfection and then were infected with shNrf2 or shCTL using Lipofectamine 2000 (Invitrogen). An Nrf2 expression plasmid (PCMV-Nrf2) was constructed with synthetic oligonucleotides and a PCMV vector (control vector) were purchased from Guangzhou FulenGen Co., Ltd (Guangzhou, Guangdong, China). HepG2 and PLC/PRF/5 cells were seeded in 6-well plates the day prior to transfection and then were infected with shNrf2 or shCTL using Lipofectamine 2000 (Invitrogen). An Nrf2 expression plasmid (PCMV-Nrf2) was constructed with synthetic oligonucleotides and a PCMV vector (control vector) were purchased from Guangzhou FulenGen Co., Ltd (Guangzhou, Guangdong, China). HepG2 and PLC/PRF/5 cells were transfected with Nrf2 plasmid or empty vector as control plasmids (0.1 μg/well for 96 well culture plates and 2 μg/well for 6 well culture plates) using the X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). After 48 h post-transfection, the cells were subjected to future experiments.

Cell proliferation assay

The MTT assay kit (Beyotime, Shanghai, China) was used to assess HCC cell viability after quercitrin treatment. Quercitrin was isolated from Sedum sarmentosum and purified in house; the purity exceeded 90.52%, as determined by a HPLC method. Briefly, HepG2 or PLC/PRF/5 cells were seeded into a 96-well plate overnight. Complete medium containing different concentrations of quercitrin was added into each well. Then, cells were cultured for 24 or 48 h. Cell viability was measured by adding MTT agent and absorbance was determined using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm.

Colony formation assay

HepG2 or PLC/PRF/5 cells were pre-treated with quercitrin or DMSO for 24 h before seeding into a 6-well plate. After 21 days of culture, tumor colonies were visualized by 0.1% crystal violet staining and visible colonies were counted.

Wound healing assay

A confluent monolayer of HepG2, PLC/PRF/5, or HUVECs cells was wounded gently by scratching with a pipette tip. Complete media containing either quercitrin or DMSO was added into each well. For each group, two photographs were taken at 0 and 24 h after wound scratching. The percentage of wound closure in HCC cells at 24 h was calculated and compared to the width of the wound at 0 h.

Transwell assays

Fresh media containing 15% FBS was placed into the lower chamber of a Transwell (this acted as a chemo-attractant). HepG2 or PL/PRF/5 cells were allowed to invade for 24 h with or without quercitrin. Then, invasive cells that had attached to the lower surface of the Transwell membrane were visualized by 0.1% crystal violet staining. Photographs of 5 random fields were selected, and the number of cells that invaded was counted.

Apoptosis analysis

Cell apoptosis was determined using an Annexin V–FITC apoptosis detection kit (KeyGen Biotechnology, Nanjing, China) according to the manufacturer’s instructions. In brief, cells were treated with quercitrin for 24 h. Cells were then harvested by trypsinization and washed twice with PBS. Cell pellets were re-suspended in binding buffer and stained with Annexin V-FITC/propidium iodide. After incubation at 15°C for 15 min in the dark, samples were analyzed using a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ, USA). Data were analyzed using CellQuest software (Becton Dickinson).

qRT-PCR

Total RNA of HCC cells or tissues was extracted using TRIZOL® reagent (Invitrogen), and 1 μg RNA
was used for cDNA synthesis with TaKaRa PrimeScript RT reagent kit (TaKaRa Biotechnology, Dalian, China). A Roche Light Cycler 480 PCR and SYBR® Premix Ex Taq™ Green I (TaKaRa) were used for real-time PCR. The mRNA level of GAPDH was set as an internal control and the final mRNA levels of genes were normalized to those of the control group. Primer sequences of target genes were listed in Supplementary Table 1.

Western blotting

Protein samples were lysis using RIPA buffer. Proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with TBST containing 5% non-fat milk powder, membranes were incubated overnight with anti-VEGF-A, anti-bFGF, anti-Nrf2, anti-E-cadherin, anti-N-cadherin (Cell Signaling Technology, MA, US), or anti-GAPDH (Cell Signaling Technology, MA, USA). Membranes were incubated with goat anti-rabbit secondary antibody (Boster, Wuhan, Hubei, China) for 2 h and enhanced chemiluminescence was (ECL) used to visualize protein bands with a Bio-Rad ChemiDoc XRS System.

Xenograft model of HCC

BALB/c nude mice were purchased from the SLAKE Experimental Animal Company (SLAKE, Shanghai, China). The experimental protocol was approved by the Medical Experimental Animal Care Commission of Nanjing University of Chinese Medicine. A total of 6 × 10⁶ HCC cells were implanted by subcutaneous injection (100 μl per mouse) to yield subcutaneous tumors. Quercitrin (20 mg/kg) or vehicle (0.5% methylcellulose) was administered orally using a feeding tube. The tumor volume was calculated according to the following formula: Tumor volume = (largest diameter × perpendicular height²)/2. Mice were euthanized at the end of the experiment to obtain tumor tissues. The tumor tissues were subjected to immunohistochemical staining with primary antibodies against Ki67, CD31, and caspase-3 (Abcam, Cambridge, MA, USA).

Statistical analysis

Data are presented as means ± SD. Differences between two groups were evaluated using one-way ANOVA followed by Dunnett’s post-hoc test. Differences with P < 0.05 were considered to be statistically significant.

ACKNOWLEDGMENTS AND FUNDING

None.

CONFLICTS OF INTEREST

None.

REFERENCES