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Original Article

Quercetin induces apoptosis in triple-negative breast cancer cells via inhibiting fatty acid synthase and β-catenin

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Abstract: Triple-negative breast cancer (TNBC) is an aggressive malignant neoplasia. The increased research interests in TNBC nowadays are due to the lack of clinically validated and molecular-targeted therapy. Fatty Acid Synthase (FASN) is the major biosynthetic enzyme for the synthesis of fatty acids from small carbon substrates. Elevated expression of FASN is frequent phenotypic alterations in many human cancers. The flavonoid Quercetin is one of the most abundant bioflavonoids in the human diet and a known inducer of apoptosis in several cancer cell lines. We aimed to evaluate the effects of Quercetin on TNBC *in vitro* and *in vivo*. Quercetin induced a significant growth-inhibitory effect against TNBC cells and the IC_{50} were 230 ± 3 and 415 ± 4 μM for the TNBC cells, MDA-MB-231 and MDA-MB-157 respectively. Quercetin treatment induced anticancer/apoptotic effects against TNBC cells as shown by morphological alterations, DNA fragmentation, and caspase-3 activation. When compared to controls, decreased protein expression of FASN, β-catenin, Bcl-2, and reduced-nuclear accumulation of β-catenin were detected after Quercetin treatment. Quercetin *in vivo*-treatment induced significant tumor growth inhibition by 41.7% after 25 days of treatment. Immunohistochemical data showed a clear inhibition of FASN expression in tumor xenografts after Quercetin treatment. Quercetin possibly targets the lipogenic enzyme FASN and β-catenin in TNBCs. Quercetin-induced apoptosis via targeting the *de novo* fatty acid synthesis is likely through a caspase-3 dependent mechanism coupled with modulation of FASN and β-catenin expressions.

Keywords: Breast cancer, Quercetin, FASN, β-catenin, phytochemicals, xenografts

Introduction

Triple-negative breast cancer (TNBC) patients represent 15-20% of patients with breast cancer. The reasons for the increased research interest in TNBCs nowadays are the lack of clinically validated and molecular-targeted therapy [1, 2]. When compared with other breast cancer types, TNBC is an aggressive malignant neoplasia associated with recurrence within three years of diagnosis, visceral metastases, and death [3]. In addition, advanced TNBC patients have a shorter survival rate, 12 months, when compared to survival of patients with other advanced non-TNBCs [4]. TNBC cells are distinct from other breast cancers in the absence of estrogen receptor (ER), progesterone receptor (PR) expression, and human epidermal growth factor receptor 2 (HER-2) [5, 6]. The lack of cell surface receptors explains the insensitivity to targeted-therapy [7]. The identification of novel therapeutic targets for TNBCs is important if the outcome of patients with these tumors is to be improved.

In the past decades, a great deal of research has been oriented to discover/investigate plant-derived compounds with potential chemoprevention activity [8]. Flavonoids are phenolic compounds that are ubiquity found in fruits and vegetables [9, 10]. Many studies reported the significant protection activity of flavonoids against all stages of cancer either by preventing, reversing, or impeding carcinogenesis [8, 11]. Mechanisms include scavenging free radicals, altering the mitotic cycle and gene expres-

sion, and inducing apoptosis [12, 13]. Flavonoids are currently considered as a promising chemopreventive agents against various human cancers [9, 10]. The flavonol Quercetin (3,3',4',5,7-pentahydroxyflavone), a well-known flavonoid with no reported carcinogenic activity or significant toxicity *in vivo*, is one of the most abundant bioflavonoids in the human diet including apples, berries, grapes, onions, tea, tomatoes, many seeds, nuts, and leaves, and with fairly high oral bioavailability [14]. It was reported that Quercetin is a known inducer of apoptosis in several cancer cell lines and the incidence of stomach, colon, lung, and breast cancers are inversely associated with high dietary consumption of Quercetin [8, 10, 15, 16]. Besides, Quercetin can inhibit melanoma growth and metastasis as reported previously [13, 17]. Interestingly, Quercetin can induce apoptosis in cancer cells, but not in their normal counterparts [18]. Although over the past 30 years Quercetin has been extensively studied, the exact mechanisms through which Quercetin provides its chemopreventive effects are still not fully known and needs further investigations.

Increased lipogenesis and *de novo* synthesis of the vast majority of fatty acids are a metabolic hallmark of a tumor cell [19]. The poor prognosis and aggressiveness of tumor growth is directly associated with increased lipogenesis that can secure lipids for newly-generated membranes for rapidly proliferating cancer cells [20]. The important role of the *de novo* fatty acid synthesis in cancer has been revealed by several studies. The depletion of fatty acid synthesis enzymes induced cytotoxicity in colon cancer cells that was reversible by supplementing cells with saturated fatty acids, palmitate or stearate [21]. The treatment of several cancer cells with anti-cancer agents, including C75, cerulenin, orlistat, and triclosan, induced anti-fatty acid synthesis dependent apoptosis [22]. The down-regulation of *de novo* fatty acid synthesis machinery have been shown to induce apoptosis in cancer cells and represents an innovative therapeutic approach for cancer according to previous reports [23, 24].

In mammalian cells, fatty acid synthase (FASN), the nutritionally-regulated lipogenic enzyme, is a key enzyme for the synthesis of saturated long chain fatty acids (LCFAs) from malonyl-CoA, acetyl-CoA, and NADPH. With the exception of liver and adipose tissues, which exhibit high levels of FASN, the expression of FASN is unnoticeable in most normal tissues [25]. FASN overexpression is primarily relies on transcriptional activation following oncogenic activation or tumor suppressor machinery loss [19]. The hypothesis that FASN is essential for generating *de novo* cell membranes during the proliferation of tumor cells is supported by the fact that human breast, liver, prostate, colorectal, endometrial, thyroid and ovary cancers exhibit high expression levels of FASN [26]. Many studies demonstrated a role for the increased FASN expression in cell cycle activation and inhibition of apoptosis in many human cancers. In addition, FASN expression is up-regulated in metastatic tumors, and the decreased survival, prediction of poor outcome and disease recurrence in several cancers correlate with high expression levels of FASN [27-30]. It was reported that FASN is up-regulated during the early stages of carcinogenesis [19]. Interestingly, normal cells exhibiting low levels of FASN expression did not show any proliferation arrest after treatment with FASN inhibitors implying a selective anticancer activity for these inhibitors and suggesting FASN as a potential diagnostic tumor marker [31-33]. Chemical inhibitors of FASN, such as C75 and orlistat, have been shown to induce apoptosis in cultured cancer cells and decrease tumor size in animal models of different cancers implying a promising anticancer activity [19, 34]. The toxic effects accompanied by the increase in the malonyl-CoA synthesis pathway and the lack of the end product LCFA by itself may induce apoptosis [22]. Interestingly, Research studies have reported that dietary phenolic compounds can induce apoptosis in different cancer models via suppression of FASN expression; Quercetin, Resveratrol, Piperine, and epigallocatechin-3-gallate (EGCG) have been reported to induce apoptosis in HepG2 cells, breast cancer stem-like, breast cancer, and lung cancer cells, respectively [24, 31-33]. However, the mechanism of cancer-associated FASN up-regulation and how inhibition of FASN by dietary phenolic compounds induces apoptosis in tumor cells is not fully understood and needs further investigations.

β-Catenin was first reported to act as a cell-cell adhesion molecule. However, many studies have indicated that β-catenin could also func-

tion as an oncogene when it is translocated to the nucleus [35]. Nuclear β-catenin and the activation of the Wnt pathway have been reported previously to play a role in breast cancer progression [36]. Furthermore, the expression of different Wnt members and nuclear β-catenin localization have been reported to correlate with abnormal cell proliferation in human breast tumor, suggesting the possible involvement of Wnt and the β-catenin pathway in breast cancer formation and/or progression and may serve as a target for breast cancer therapy [37, 38].

In the present study, we investigated the hypothesis that Quercetin can become one of the FASN and β-catenin inhibitors *in vitro* and *in vivo*. In addition, we aimed to investigate the mechanism underlying behind the inhibitory effect of Quercetin on FASN and β-catenin expression to induce apoptosis in TNBC cells.

Materials and methods

Cell culture

Triple-Negative Breast Cancer (TNBC) cells, MDA-MB-157 and MDA-MB-231, were purchased from American Type Culture Collection (Rockville, MD, USA). Low-passage cultures (below 20 passages) were cultured in Dulbecco's modified essential media (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Lonza Co., Belgium) at 37°C in 5% CO_2 atmosphere until the culture was near confluence. When 70% confluence was reached, cells were treated with Quercetin. DMSO was used as a vehicle to solubilize Quercetin and was added to the control cultures at 0.1-1%.

Cell cytotoxicity (MTT assay)

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to assay mitochondrial activity in viable cells. MDA-MB-157 and MDA-MB-231 were cultured as described above, harvested and re-suspended in growth media to make a stock cell suspension containing 20,000 cells/ml. 100 µl of stock cell suspension were seeded per well of 96-well plate. The cells were allowed to attach and grow for 24 hr and then treated with increasing concentrations (0~550 mM) of Quercetin (Sigma-Aldrich Co. LLC, USA). Each experiment was performed in triplicate in parallel for each concentration. Controls were performed in which only culture media and DMSO were added. The cells were then incubated at 37°C in 5% CO_2. After 48 hr of incubation, the culture medium was removed and the cells were washed twice with phosphate buffered saline (PBS). Then 20 µl of 5 mg/ml MTT was added to each well. The cells were further incubated at 37°C for 4 hr. The supernatant was discarded and 100 µl of DMSO was added to each well. The mixture was shaken on a microvibrator for 5 min and the absorbance was measured at 570 nm (*A*) that served as a measure of cell viability. Inhibition ratio (*I%*) was calculated using the following equation: Formula $I\% = (A_{control} - A_{treated})/A_{control} \times 100$.

Morphological analysis

Morphological alterations of MDA-MB-231 and MDA-MB-157 cells treated with or without different concentration of Quercetin, 0~300 µM for MDA-MB-231 and 0~550 µM for MDA-MB-157 cells, were investigated to determine the changes induced by Quercetin treatment. 2×10^5 cells were cultured in 12-well plate and allowed to adhere for 24 hr, and then the medium was replaced with fresh complete medium containing Quercetin and incubated for 48 hr at 37°C in 5% CO_2. Experiments included untreated cells that were not exposed to any exogenous drug but to an equal content of medium with DMSO during the incubation to serve as controls. After 48 hr, cells treated with or without Quercetin were fixed and photographed at × 200 magnification with inverted phase contrast microscope (Optika, Italy).

DNA fragmentation assay

Cells were incubated with or without Quercetin as the following dosages (0, 150 and 230 (IC_{50}) µM) for MDA-MB-231 and (0, 250 and 415 (IC_{50}) µM) for MDA-MB-157 cells. The apoptotic DNA ladder assay was used for DNA fragmentation assay. Total DNA was isolated from each sample, which was then resolved on a 1.5% agarose gel containing 0.3 mg/mL ethidium bromide in 1X TAE buffer (pH 8.5, 20 mM Tris-acetic acid, 2 mM EDTA). Partial fragmentation of DNA was visualized using gel documentation system.

Caspase-3 activity

Caspase-3 activity was assayed according to manufacturer's protocol of colorimetric assay kit (Sigma Aldrich, Cat No CASP3C-1KT) which provides a quick and efficient detection of caspase 3 activity in cell lysates. 5×10^6 cells were treated with or without different concentrations of Quercetin and lysed in 100 µL lysis buffer containing 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 2 mM EDTA, 0.1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, 5 mM, 350 µg/ml PMSF (phenylmethylsulfonyl fluoride) and 5 mM DTT (Dithiothreitol). Cell were homogenized by three cycles of freezing and thawing and then centrifuged to remove the cellular debris. Each sample was then incubated in buffer containing 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM EDTA supplemented with its substrate (Ac-Asp-Glu-Val-Asp-AFC) Ac-DEVD-AFC for 1 hr at room temperature and then the reaction was stopped with 1N HCl. OD (405) was measured using a spectrophotometer (Jenway Spectrophotometer, UK). Each assay was done in triplicate and standard deviation was calculated.

Western blotting analysis

Cells were plated in 6-well plate for western blotting analysis. Cells were lysed with a 1% NP-40-containing buffer supplemented with a 1 × cocktail of protease inhibitors (Complete Mini, Roche, Germany) and phosphatase inhibitors (phosphatase inhibitor cocktail I and II, Sigma) at 4°C for 30 min. Lysates were centrifuged at 10,000 g at 4°C for 15 min and supernatants were collected. The protein concentration of the supernatant was determined using the BCA assay (Pierce, Rockford, IL). Samples were mixed in a ratio of 1:2 in Laemmli buffer and denatured by heating at 98°C for 5 min. Forty-µg of protein were separated on 10% Tris-SDS-PAGE gels (Bio-Rad Laboratories, USA) at 100 V for 1 hr. For western blotting, the separated proteins were electrophoretically transferred onto polyvinylidenedifluoride membranes (BioRad Laboratories, USA) at 380 mA for 1 hr. Western blot analysis was carried out using specific primary antibodies for FASN, β-catenin, and Bcl-2 (Santa Cruz Biotechnology, USA) antibodies. The expression of β-actin (Sigma-Aldrich, USA) was used as a control for protein loading. The membranes were blocked with TBS plus 5% nonfat milk (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) followed by incubation overnight with primary antibodies diluted in blocking buffer for antibodies (1:1000). This was followed by incubation again for 1 hr in the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA). For detection, an ECL kit was used according to the manufacturer's instructions (Amersham, UK).

Animal gamma irradiation and quercetin treatment

Adult Healthy albino mice (6-8 weeks old, female, 20-25 g) were obtained from the Veterinary Science Institute, Cairo, Egypt. Animal experimental procedures conformed to the relevant regulatory standards and were carried out according to the guidelines of Animal Ethics Committee of Alexandria University, Egypt. To induce immunosuppression in mice, doses of 700 to 1300 cGy are myeloablative [39]. Briefly, albino mice were divided into five groups of 5 animals per group. Mice were irradiated in sanitized pie cage and whole body irradiation (WBI) was administered using a single radiation dose, 700-800 cGy (ELEKTA device, Nuclear radiation department, faculty of medicine, Alexandria University, Egypt), at a rate of approximately 70 cGy/minute for a period of 10-12 minutes. Following irradiation, mice were observed and surviving animals, after 24 hrs, were monitored daily in a HEPA-filtered laminar flow hood provided with standard diet and bottles with long sipper tubes which enable the animals to get to the water source with minimal effort. Severely affected animals displaying obvious weight loss and poor body were removed from the study. For Quercetin treatment, animals were intraperitoneally administered daily with 50 mg/kg [40, 41].

Breast tumor xenografts

6-8 weeks old female albino mice were housed in individually ventilated cages, each cage contains 5 animals. Animals were maintained on standard light cycle and fed standard pellet diet and water *ad libitum*. The mice were allowed one week for acclimatization period prior to commencement of the experiment. Before xenograft transplantation, animals were subjected to a single radiation dose (700-800 cGy) as described above. Xenograft transplantation

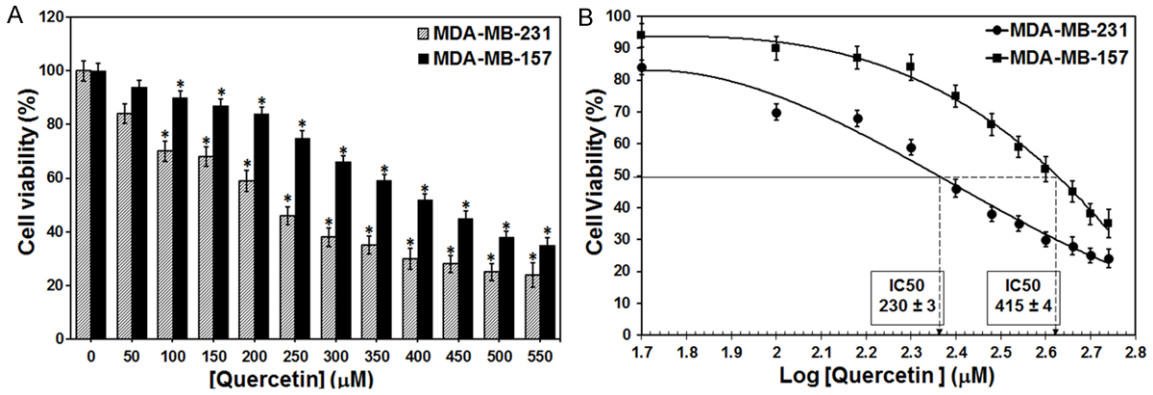

Figure 1. Effects of Quercetin on the viability of MDA-MB-231 and MDA-MB-157 cells. Cells were treated with Quercetin (0~550 μM) for 48 hr. Controls were defined as cells treated with 0.1% DMSO without Quercetin. A: Effects of Quercetin on the proliferation of MDA-MB-231 and MDA-MB-157 cells were expressed as percentage of cell viability. B: Effects of 48 hr exposure to varying concentrations of Quercetin on cell proliferation are expressed as concentrations of Quercetin at which the viability of cells can be reduced to 50% (IC_{50}). Data from at least three independent experiments performed in at least triplicates are presented as means ± SD, $*P < 0.01$.

using MDA-MB-231 breast cancer cell line was performed as described previously [42]. Briefly, each mouse was anesthetized with isoflurane inhalant just prior to inoculation into both sides of the mammary fat pad just inferior to the nipple of a female mouse with human breast carcinoma MDA-MB-231 cells at a dose of 5×10^6 cells (in 300 μl of Matrigel; Biological Industries, CT, USA). Three weeks later, when tumor xenografts grew to an average of 50 mm^3, mice were stratified and randomized into two groups of 5 mice each. The treated mice were administrated intraperitoneally with Quercetin (50 mg/kg of body weight) in 150 μL of DMSO/0.9% physiologic saline (1:0.5) daily for 25 consecutive days. Body weight and tumor volume were measured every 5 days. Tumor volumes were measured by a caliper and calculated according to the following formula: $(width^2 \times length)/2$. A curve was established to the mean tumor volumes against time. The error bars indicated the value of the standard error of the mean. Established tumors were free of evident necrosis at the time of treatment. No death was recorded within the experimented period.

Immunocytochemistry

For MDA-MB-231 cells, monolayer cells were seeded at density of 2×10^5 cells per well on sterile cover slip in a 6-well plate. After 48 hr, untreated and Quercetin-treated cells were fixed in 4% paraformaldehyde for 15 min and permeated with 0.1% Triton X-100 for 10 min at room temperature. Following blocking with 4% FBS for 1 hr, cells were probed with the appropriate primary antibody. Triple washing was followed and incubation with secondary antibody was achieved for 1 hr. Cells were visualized by adding hematoxylin to stain nuclei. Cover slips were mouton on microscope slides. The slides were examined at x400 magnification under Optika microscope (Optika, Ponteranic, BG, Italy). Pictures were taken using a Nikon Digital Camera. For MDA-MB-231 xenograft breast tumors, Formalin-fixed, paraffin-embedded tissue sections of mammary gland or MDA-MB-231 xenograft breast tumors treated with or without Quercetin were cut into 4 μm sections according to standard protocol, and processed for histopathologic examination, deparaffinized in xylene, rinsed in ethanol and rehydrated. Staining was performed using the Ventana XT platform and internal antigen retrieval CC1 standard. FASN primary antibody (Santa Cruz Biotechnology, USA) was diluted 1:1000 and incubated overnight at 4°C with mammary gland or MDA-MB-231 xenograft breast tumor sections. The universal secondary protocol and the DAB MAP kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) were used to detect and amplify the signal.

Statistical analysis

Each experiment was performed at least three times and triplicates were performed in MTT assay. Data were expressed as mean ± stan-

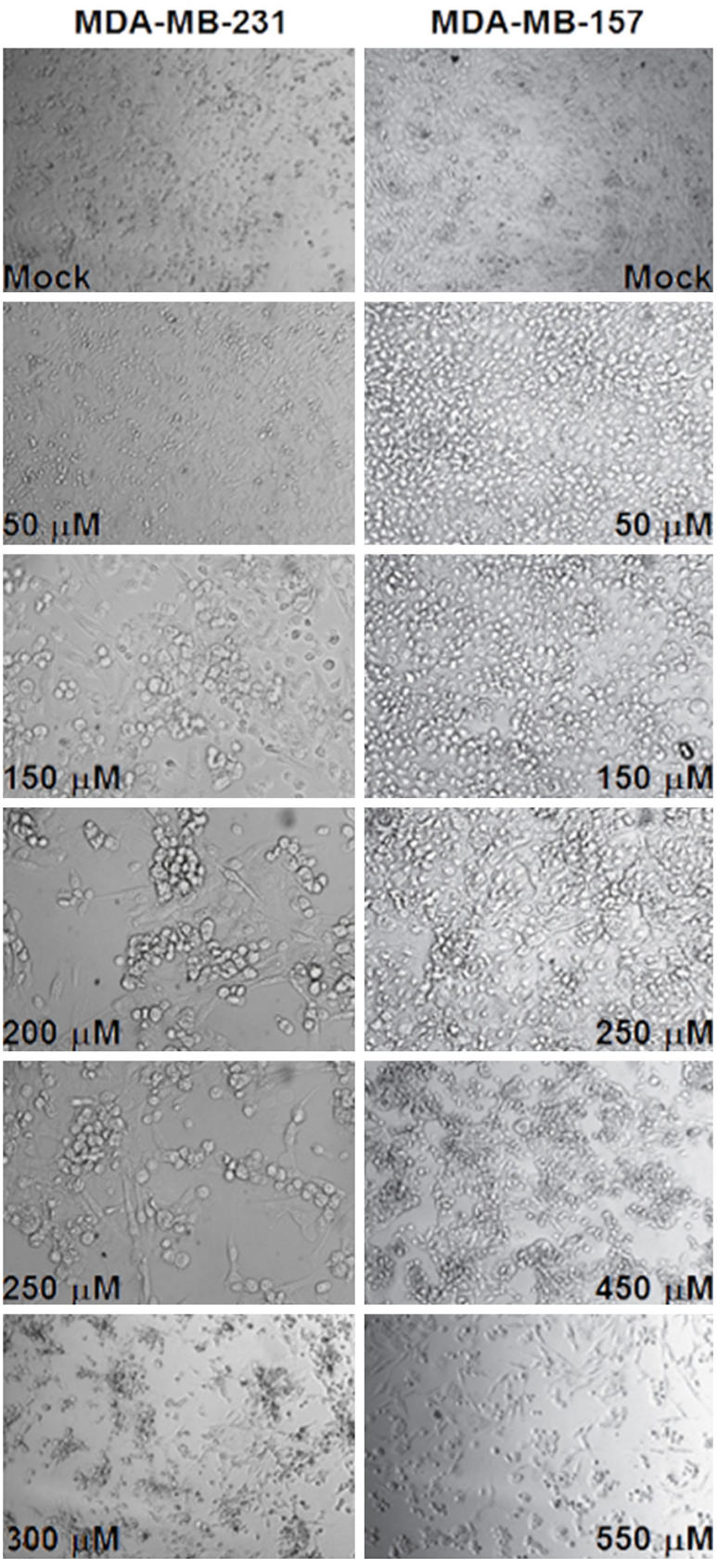

Figure 2. Quercetin induced morphological changes in MDA-MB-231 and MDA-MB-157 cells. Cells were plated onto 12-well plates and treated with the indicated concentrations of Quercetin for 48 hr. The photographs were taken directly from culture plates using a phase contrast microscope at × 200 magnification. Control cells showed a typical polygonal and intact appearance, whereas Quercetin-treated cultures showed pre-apoptotic characteristics including cell shrinkage, cellular irregularity in shape, and detachment. Representative pictures are shown from three independent experiments.

dard deviation (SD), and ANOVA test was used to analyze the difference among various treatments. Statistical significance set at $*P < 0.01$ was compared with vehicle treatment using SPSS ver. 20.

Results

Quercetin inhibits TNBCs proliferation

Exponentially growing TNBCs, MDA-MB-231 and MDA-MB-157 cells, were cultured in increasing concentrations of Quercetin. The effects of Quercetin on cell growth were assessed by MTT assay after 48 hr of incubation with Quercetin as described under materials and methods. The 48 hr treatment by Quercetin in a concentrations range of 0~550 μM showed significant dose-dependent cytotoxic effects on cells when compared to control cells (**Figure 1A**). The concentrations of 50% inhibition of the viability of MDA-MB-231 and MDA-MB-157 cells, IC_{50}, were calculated using a semi-logarithmic plotting of the percentage of cell viability versus concentrations of Quercetin. MDA-MB-231 cells showed a higher sensitive response towards Quercetin than MDA-MB-157 cells and the calculated IC_{50}

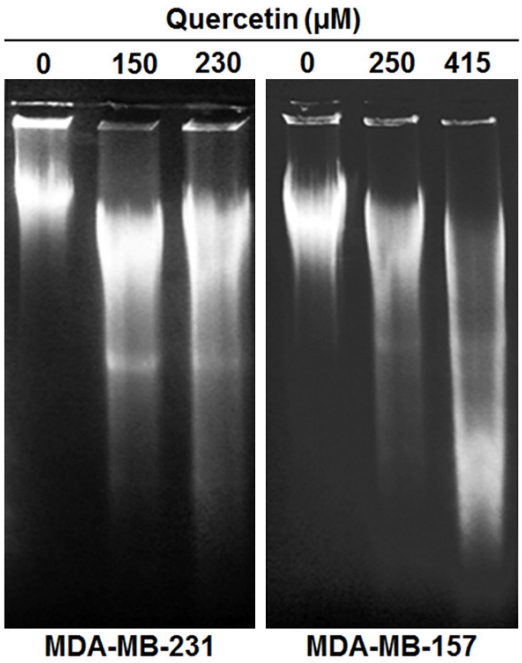

Figure 3. Quercetin-induced partial DNA fragmentation in TNBCs was examined by DNA gel electrophoresis. MDA-MB-231 and MDA-MB-157 cells were incubated with different concentrations of Quercetin for 48 hr, the cells were harvested and DNA was extracted from cells before DNA fragmentation was examined by DNA gel electrophoresis as described in the Materials and Methods.

for MDA-MB-231 and MDA-MB-157 cells were 230 ± 3 μM and 415 ± 4 μM respectively (**Figure 1B**). Our data showed that Quercetin significantly decreased TNBCs viability in concentration-dependent manner starting from the treatment with 100 μM Quercetin compared to control cells ($P < 0.01$, unpaired *t*-test).

Quercetin induces morphological changes in TNBCs

Morphological alterations of MDA-MB-231 and MDA-MB-157 cells after Quercetin treatment for 48 hr were observed under inverted phase contrast microscope with × 200 magnification (**Figure 2**). Changes in morphology were observed in a concentration-dependent manner. Treated cells showed an alteration in normal morphology and cell adhesion capacity in comparison to control cells. The typical morphology of TNBC cells were altered starting from the treatment with 150 μM Quercetin and cells appeared shrunken, smaller in size, and rounded. The response of MDA-MD-231 cells to Quercetin treatment was more dramatic than that of MDA-MB-157 cells when compared to their respective controls.

Quercetin induces DNA fragmentation in TNBCs

To investigate if the inhibition of cell viability after Quercetin treatment was related to apoptosis, MDA-MB-231 cells were treated with 150 and 230 (IC_{50}) μM and MDA-MB-157 cells were treated with 250 and 415 (IC_{50}) μM Quercetin for 48 hr. A comparison of the gel electrophoretic DNA band patterns between control cells and treated TNBCs are shown in **Figure 3**. The Quercetin-treatment resulted in the induction of partial fragmentation of DNA in MDA-MB-231 and MDA-MB-157 cells when compared to controls.

Quercetin induces Caspase-3 activity in TNBCs

Activation of caspases is one of the key events in apoptosis [43]. Caspase-3 activation was evaluated in treated MDA-MB-231 and MDA-MB-157 to investigate if the induced partial DNA fragmentation by Quercetin treatment is in a caspase-dependent manner. Our data indicated that Quercetin treatment showed a significant increase in caspase-3 activity in both treated cell lines when compared with control cells after 48 hr. As shown in **Figure 4A**, Quercetin significantly stimulated caspase-3 activity in MDA-MB-231 and MDA-MB-157 cells. Our data give clear evidence that the proliferation activity and apoptosis induction are induced by Quercetin treatment in TNBCs. In addition, our results confirmed that Quercetin-induced apoptosis of both treated cell lines was in a caspase-dependent manner.

Quercetin induces apoptosis through down-regulation of FASN, β-catenin and Bcl-2 protein expression

The protein expression levels of FASN, β-catenin and Bcl-2, a well-known regulator of apoptosis, were evaluated to further understand the observed increase in apoptosis in Quercetin-treated TNBCs. As demonstrated in **Figure 4B**, the 48 hr treatment of MDA-MB-231 and MDA-MB-157 cells with Quercetin down-regulated

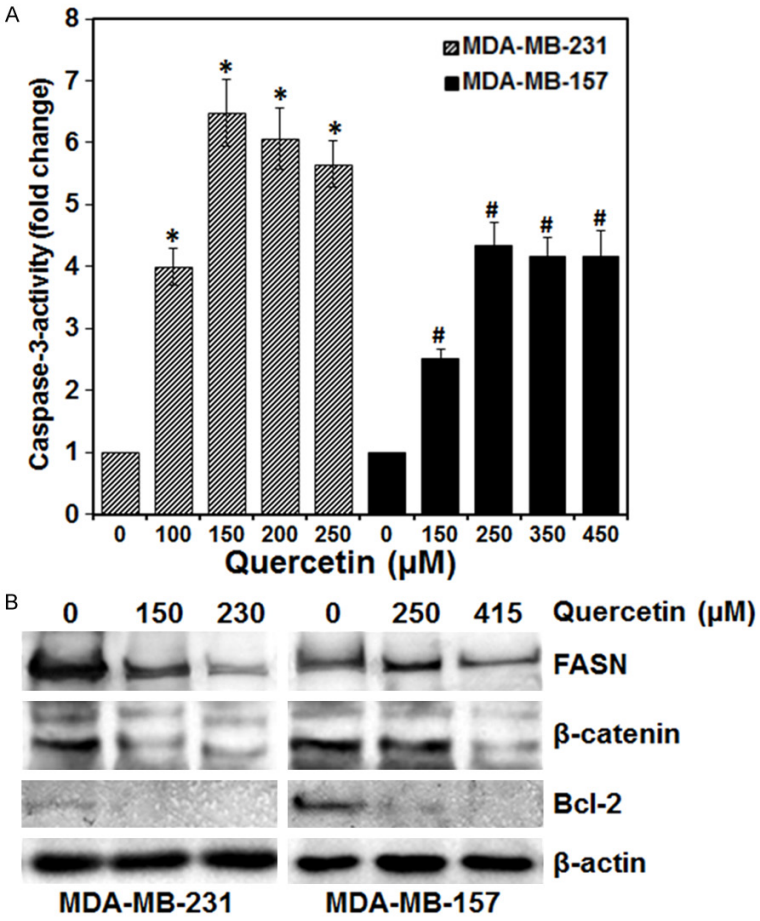

Figure 4. Effect of Quercetin on caspase-3 activity, and FASN, Bcl-2, and β-catenin protein expression levels in MDA-MB-231 and MDA-MB-157 cells. A: Quercetin-induced caspase-3 activity in MDA-MB-231 and MDA-MB-157 breast cancer cell lines. Cells were incubated with different concentrations of Quercetin for 48 hr. Cells were harvested and lysed cells were subjected to caspase-3 activity assay as described under the Materials and Methods. Each assay was done in triplicate and standard deviation was calculated. Data are presented as mean ± SD. * and # are significant differences ($P < 0.05$) with respective controls. B: Quercetin down-regulated FASN, Bcl-2 and β-catenin protein expression levels in MDA-MB-231 and MDA-MB-157 cells. Cells were treated with or without Quercetin for 48 hr. Representative data from three independent experiments are shown. β-actin was used as a control for equal protein loading.

FASN, β-catenin and Bcl-2 protein levels when compared to controls. The down-regulation of FASN, β-catenin and Bcl-2 protein expression levels may trigger the intrinsic mitochondrial apoptotic pathway in treated cells by modulating levels of the pro-survival members.

Quercetin alters the nuclear localization of β-catenin in MDA-MB-231 cells

Wnt/β-catenin pathway regulates cell proliferation and is abnormally regulated in many cancers. A possible effect of Quercetin was investigated here by examining β-catenin cellular distribution, an important indicator of Wnt/β-catenin activity. Since MDA-MB-231 cells showed a higher sensitive response than MDA-MB-157 cells towards Quercetin treatment, therefore, we performed β-catenin immunostaining in MDA-MB-231 cells (**Figure 5**). We observed that control MDA-MB-231 cells showed high nuclear localization of β-catenin suggesting an activation of Wnt/β-catenin signaling in a subpopulation of MDA-MB-231 cells. When 230 μM (IC_{50}) of Quercetin was added to MDA-MB-231 cells for 48 hr, nuclear β-catenin staining was significantly reduced. These results suggest that Quercetin treatment alters the distribution of β-catenin in MDA-MB-231 cells. The decrease in nuclear β-catenin is consistent with a decrease in Wnt/β-catenin signaling activity.

Quercetin suppresses MDA-MB-231-tumor xenografts

To investigate the physiological relevance of the observed reduction in MDA-MB-231 breast cancer cell number and the down-regulation of FASN, β-catenin and Bcl-2 protein expression levels in response to Quercetin treatment, we tested the effect of Quercetin treatment on tumor growth of MDA-MB-231 cells *in vivo*. Xenografts of MDA-MB-231 cells were inoculated into both sides of the mammary fat pad of female-radiated mice three weeks prior to daily treatment with Quercetin (50 mg/kg of body weight) for 25 days. As shown in **Figure 6A**, Quercetin significantly decreased breast tumor xenograft growth compared to the control group after 25 consecutive days of treatment. The decrease in tumor growth of MDA-MB-231 tumor-bearing mice started from day 10 com-

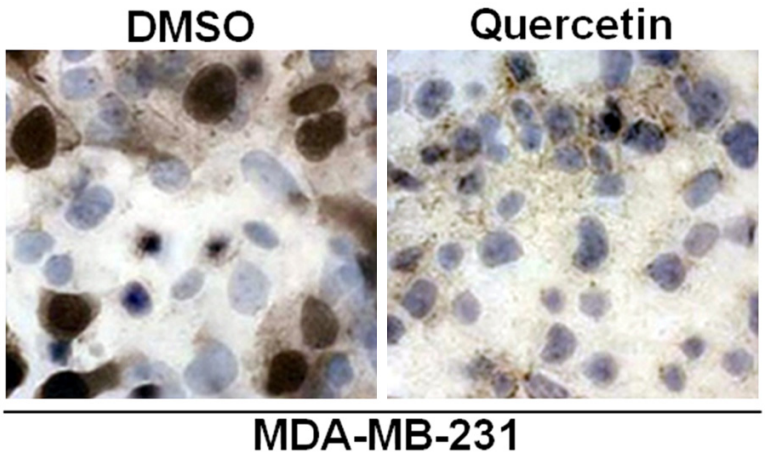

Figure 5. Quercetin affects β-catenin localization in MDA-MB-231 cells. β-catenin immunostaining of MDA-MB-231 cells treated with 230 μM Quercetin (IC_{50}) or DMSO for 48 hr. In control conditions, MDA-MB-231 cells display nuclear β-catenin localization. In cells treated with Quercetin, nuclear β-catenin staining has decreased. Immunostaining of β-catenin was visualized using phase contrast microscope with × 400 magnifications.

pared with the control group. Quercetin showed tumor targeting efficiency evident by a significant tumor growth inhibition by 41.7% at day 25 in comparison to control group (**Figure 6A**). No significant change in body weights was observed during the course of the study indicating that Quercetin and/or tumor burden did not significantly affect weight. Damage to normal skin was not observed in any of the mice. Significant tumor-growth was not evident over the two weeks that followed Quercetin treatment.

Quercetin down-regulates FASN in MDA-MB-231-tumor xenografts

To investigate if FASN is involved in the process of MDA-MB-231 tumor xenografts growth inhibition in radiated albino mice, immunohistochemical staining of FASN expression in MDA-MB-231 xenografted tumors and mammary glands were assessed. As shown in **Figure 6B**, Quercetin-treated mice revealed that the nuclear localization of FASN in MDA-MB-231 xenografted tumors and epithelial ductal components of the mammary gland were significantly decreased after Quercetin treatment for 25 days compared to DMSO-treated counterpart, suggesting that the decreased nuclear localization of FASN may play a role in the process of apoptosis induction.

Discussion

In the present study, we assessed the effect of the flavonoid, Quercetin, on the role of FASN and β-catenin in two Triple-negative breast cancer (TNBC) cell lines. *In vitro* and preliminary *in vivo* studies reported that Quercetin exerts its chemopreventive and antitumor effect through cell cycle arrest, induction of apoptosis, and inhibition of angiogenesis [8]. To date, while chemotherapy remains the regular treatment, no targeted therapy for the treatment of TNBC has been approved. This is not surprising since TNBC is a heterogeneous disease and its subtypes differ on the molecular, pathologic, and clinical levels [5]. Thus, identification of novel, molecular-targeted therapies for TNBC would be of great benefit for patients suffering from this biologically aggressive cancer.

In the present study, MDA-MB-231 and MDA-MB-157 cell lines were selected to investigate the cytotoxicity effect of Quercetin on TNBC cells. Herein, we demonstrated the cytotoxic effect of increasing concentrations of Quercetin and calculated the percentage of cell mortality using the MTT assay. Our data showed that 48 hr treatment with Quercetin significantly reduced the viability of TNBC cells in a concentration-dependent manner with half-maximal cytotoxic doses in micromolar range. The sensitivity of MDA-MB-231 towards Quercetin treatment was higher than that of MDA-MB-157. Our data agreed with previous findings reporting that several flavonols, including Quercetin, are cytotoxic against different human cancer cell types [8]. The morphological changes for the investigated TNBC cells were more prominent in Quercetin-treated cells which exhibited vacuolation and extensive blebbing, suggesting apoptotic mechanism of cell death [44]. After 48 hr of Quercetin treatment, TNBC cells began to display apoptotic morphology, including shrinkage, DNA condensations, and membrane convulsions.

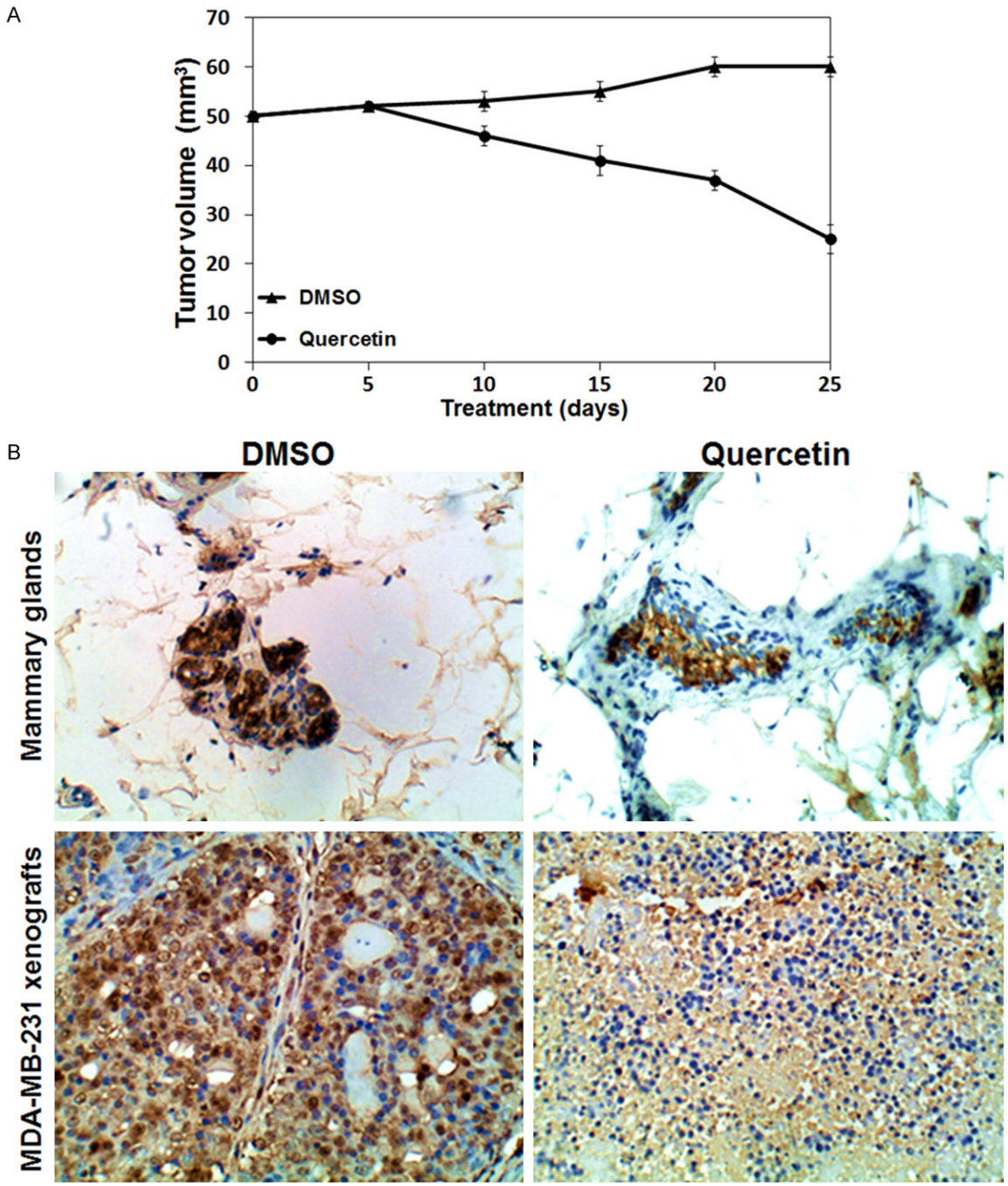

Figure 6. Effect of Quercetin on tumor growth and FASN protein expression in MDA-MB-231-tumor xenografts. A: Female mice were inoculated with MDA-MB-231 cells and after 3 weeks were either treated with DMSO or Quercetin (50 mg/kg body weight). Quercetin was daily administered intraperitoneally for 25 days as described in Materials and Methods. Data are represented as mean of the tumor volume ± SEM, n = 5. B: Immunohistochemical analysis of FASN expression in mammary glands and MDA-MB-231 tumor xenografts treated with DMSO or Quercetin. Brown staining indicates the expression of FASN. Representative data from three independent experiments are shown.

Previous studies have linked Quercetin to the regulation of lipogenesis and adipogenesis via the down-regulation of Fatty acid synthase (FASN) expression, one of the key enzymes that regulate lipogenesis. Quercetin regulates lipogenesis at the level of transcription of several lipid metabolism-related genes, including FASN [45]. The treatment of adipocytes with the hot

water soluble extract from fermented, Quercetin-rich, rooibos (*Aspalathus linearis*) inhibited adipogenesis accompanied by decreased FASN mRNA levels [46]. Very recently, Seo et al. reported that Quercetin prevented adipogenesis by down-regulating FASN expression in OP9 mouse stromal cells [47]. We demonstrated that Quercetin decreased FASN expression in TNBC cells. This is in accordance with previous studies reported that Quercetin-induced apoptosis in HepG2 cells was via the inhibition of FASN biosynthesis [33]. Quercetin inhibited cell proliferation, decreased expression of FASN and exhibited synergistic anti-cancer effects with cisplatin in cultured nasopharyngeal carcinoma cells [48]. Very recently, melanoma cell migration was suppressed by Quercetin, in a dose-dependent manner, and this was associated with decreased expression of FASN [49]. In addition, other published data showed that several flavonoids, including Quercetin, inhibited the activity of purified FASN [50].

The inhibition of lipogenesis may be implicated in the anti-proliferative effects of Quercetin [50]. Brusselmans *et al.* investigated the anti-lipogenic effects of 18 polyphenolic phytochemicals and showed that Quercetin is one of the most potent inhibitors of lipogenesis (via FASN inhibition) in breast and prostate cancer cells [51]. Moreover, the anti-lipogenic effect of Quercetin was strongly associated with its ability to induce apoptosis of breast and prostate cancer cells, indicating that FASN inhibition may be one of the mechanisms by which Quercetin exert its anti-carcinogenic effects [50]. A significant accumulation of malonyl-CoA was observed as a result of FASN inhibition by synthetic inhibitors in tumor cells, as would be expected from the fact that FASN uses malonyl-CoA as a substrate [51, 52]. Indeed, the accumulation of malonyl-CoA as a result of FASN inhibition by the phytochemical capsaicin represented a major cause of mitochondrial-dependent apoptotic induction in HepG2 cells [24]. In the current study, it was found that Quercetin not only exerted an inhibitory effect on FASN expression, but has dose-dependent inhibitory effects on the viability of MDA-MB-231 and MDA-MB-157 cells. The mechanism of apoptosis may be attributed to the accumulation of malonyl-CoA as a result of FASN inhibition, which was likely to trigger apoptosis as suggested by previous studies [52, 53]. Very recently, FASN inhibition, using C75 and EGCG as FASN inhibitors, resensitizes doxorubicin-resistant TNBC cell models to chemotherapy in a preclinical evaluation of FASN Inhibition in TNBC cells [54]. We evaluated the effect of Quercetin on the apoptotic signatures; inter-nucleosomal DNA fragmentation and caspase-3 activation. Our data on the apoptotic effects of Quercetin on TNBC cells support the studied properties of Quercetin as an effective anti-cancer agent [55, 56]. Apoptosis can be activated through two pathways, extrinsic and intrinsic pathways. These pathways trigger caspases to cleave various cellular substrates including PARP [57]. During apoptosis, the cleavage of PARP, mediated through caspases 3 and 7, is a useful hallmark for cell death [58]. Here, the treatment of TNBC cells with Quercetin promoted DNA fragmentation. These results corroborate findings of other reports on breast and other cancer models [59-61]. DNA fragmentation is an irreversible step that often occurs in the early stages of apoptosis [62]. In addition, Quercetin treatment induced apoptosis in TNBC cells by activating caspase 3. In agreement to our results, Quercetin-3-methyl ether induced apoptosis, accompanied by caspases-3 activation, in human breast cancer cells [63]. Quercetin induced apoptosis in MDA-MB-231 and MCF-7 through caspase-3 activation and mitochondrial-dependent pathways [64]. Furthermore, gold-quercetin nanoparticles mediated apoptosis in HepG2 cells by activating caspase 3 via the induction of mitochondrial membrane depolarization and cytochrome c release [65]. The antiapoptotic Bcl-2 protein is an important mediator of mitochondrial apoptosis pathway. Activated Bcl-2 prevents the activation of the proapoptotic Bax protein enabling cancer cells to escape from apoptosis. Activated Bax helps cytochrome-c release thereby activating caspase-3 cleavage leading to cell apoptosis [66]. Quercetin down-regulated Bcl-2 in prostate cancer cell xenograft tumor [67]. Kumar and coworkers reported that Quercetin treatment significantly decreased Bcl-2 and increased caspase-3 activity in PC-3 cells [66]. In agreement with previous studies, herein Quercetin treatment decreased the expression of the anti-apoptotic protein Bcl-2 [68, 69].

β-catenin is a major player in Wnt pathway which has been reported to be modulated by

Quercetin and other polyphenols [12, 70]. Although the Wnt/β-catenin pathway is up-regulated in some types of breast cancers, the mechanisms that lead to the nuclear accumulation of β-catenin in breast cancer cells need further investigations. Many epithelial malignancies are characterized by the loss of E-cadherin-β-catenin complex [71]. In the epithelial-mesenchymal transition (EMT), β-catenin when bound to cadherin complexes enhances cell-cell adhesion in homotypic junctions [72]. β-catenin when localized in the nucleus it interacts with transcription factors to activate transcription of target genes which are associated with cell survival, proliferation, and metastasis [73]. TNBC cells either lack or have very low E-cadherin expression [74]. A regulatory link between FASN and β-catenin has been proposed in which FASN can regulate β-catenin expression [75, 76]. li et al. found that the inhibition of FASN by cerulenin can down-regulate β-catenin expression in MCF-7 cells. Here, we investigated the hypothesis that inhibition of FASN expression by Quercetin may affect β-catenin localization. Interestingly, Quercetin not only down-regulated FASN expression levels, but also down-regulated β-catenin expression levels and altered its nuclear localization in TNBCs. In agreement to our results, very recently, it was reported that Quercetin treatment led to the degradation of the nuclear β-catenin and the inactivation of its downstream signaling in MDA-MB-231 [7]. The nuclear β-catenin staining was reduced in Gbm cells following treatment with isoquercitrin [12]. In addition, it was reported that Quercetin have a strong inhibitory effect on Wnt/β-catenin pathway, particularly through GSK3b [70]. Quercetin may acts similar to non-steroidal anti-inflammatory drugs in induction of β-catenin reduced-nuclear accumulation in TNBC cells. Different studies have connected active Wnt/β-catenin signaling with tumor progression. The results from our study show that Quercetin inhibits TNBC cells proliferation via decreasing FASN expression and the nuclear accumulation of β-catenin. A possible other mechanism have been described recently in which Quercetin down-regulated β-catenin expression and reduced the migration of MDA-MB-231 cells via the induction of E-cadherin expression, down-regulation of vimentin levels, and modulation of β-catenin target genes such as cyclin D1 and c-Myc [7].

Several mechanisms have been proposed very recently on Quercetin-induced apoptosis in MDA-MB-231 and other breast cancer cells. Quercetin dose-dependently treatment decreased cell viability and arrested MDA-MB-231 cells in G2/M phase via the inhibition of the endogenous 26S proteasome activity [77]. Quercetin inhibited MDA-MB-231 and MCF-7 cell proliferation via up-regulating miR-146a expression and inhibited invasion through down-regulating the expression of EGFR [64]. Others reported that, although MDA-MB-231 cells showed much higher cellular uptake of folic acid-tagged Quercetin-loaded mesoporous silica nanoparticles than MCF-7, Quercetin induced cell cycle arrest and apoptosis in the two tested cell lines through the regulation of Akt and Bax signaling pathways [78]. The reported mechanisms for the Quercetin-induced apoptosis in MCF-7 cells are through suppression of the Twist protein via p38MAPK pathway [79], via up-regulation of ERα combined with down-regulation of Her-2 [80], by attenuating the expression of the receptor for advanced glycation end-products (RAGE) and the high-mobility group box proteins-1 (HMGB1) proteins [81], via PTEN/Akt Pathway [82], or by inducing G0/G1 phase arrest through a mechanism that involves the down-regulation of the expression levels of survivin [83]. In addition, Quercetin blocked five glycolysis pathway molecules (GLUT1, HKII, PFKFB3, PDHK1 and LDH) in a panel of breast cancer cell line models (MDA-MB-231, MCF-7, HBL100 and BT549) [84]. Also, Quercetin inhibited the clonogenic survival and the proliferation of HER2-overexpressing BT-474 breast cancer cells in a dose- and time-dependent manner. These growth inhibitions were accompanied with induced caspase-dependent extrinsic apoptosis through inhibition of STAT3 signaling and an increase in sub-G0/G1 apoptotic populations [85].

The apoptosis-inducing activity of Quercetin was further assessed against MDA-MB-123-xenograft in albino mice. Together with the *in vitro* results, this study clearly demonstrated that Quercetin exhibited apoptotic activities in both cell culture system and xenografts as well. Our data showed that Quercetin mediated an apoptotic action independent of mechanisms involving the ER, PR, or HER-2. These results are in accordance with published data by So *et al.* which reported that quercetin exerts its anti-

proliferative activity via other mechanism than binding to the estrogen receptor [86, 87]. Herein, Quercetin induced significant tumor growth inhibition by 41.7% when compared to the control group, which in turn is consistent with the report of the drug-evaluation branch of the National Cancer Institute that considers treatments with tumor growth inhibition values of ≤ 42% as having significant anti-tumor activity [88]. Very recently, it was reported that the administration of Quercetin at 15 mg/kg body weight resulted in a ~70% reduction in tumor growth of MDA-MB-123-xenograft in SCID mice and the mechanism may involve Akt/mTOR inhibition [89]. Additionally, although Quercetin had showed limited or no effect on normal breast cells, the dietary flavonoid interacted with DNA, arrested cell cycle at S phase, and caused tumor regression of mouse breast cancer cell lines (EAC)-xenograft in mice by activating the intrinsic mitochondrial pathway of apoptosis [90]. The underlying mechanisms of the role of Quercetin may be concomitant with the anti-inflammatory and antioxidative activities of Quercetin as recently reported [91]. Recently, it was illustrated that the reduced expression of β-catenin in MDA-MB-231 and HCC38 using lentiviral delivery of β-catenin-specific small hairpin RNAs (shRNAs) correlates with higher sensitivity to doxorubicin or cisplatin-induced TNBC cell death. And upon implantation of the β-catenin shRNA HCC38 cells in the mammary fat pad of immunocompromised mice, the formed tumors were markedly smaller and grew much more slowly [92]. Furthermore, it was reported that the intraperitoneal administration of Quercetin decreased tumor size in a xenograft mouse model of lung cancer and enhanced the antitumor effect of trichostatin A [93] and enhanced cisplatin antitumor activity in a mouse model of lung cancer [41]. The intravenous administration of the co-encapsulated vincristine and quercetin in liposomes showed a promising anti-tumor efficacy in the ER(-ve), PR(-ve), JIMT-1 human breast tumor xenograft [94]. Furthermore, the combined treatment with dietary polyphenols (quercetin, resveratrol, and catechin) reduced tumor growth of MDA-MB-231 xenografts in a nude mouse model [95].

Conclusion

The present study demonstrated that Quercetin has anti-proliferative and apoptotic effects on TNBC cells, MDA-MB-231 and MDA-MB-157, which are mediated via down-regulation of FASN, β-catenin and Bcl-2, caspase-3 activation, and reduced-nuclear accumulation of β-catenin. Interestingly, in TNBC cell lines studied, a concentration-response correlation was observed between Quercetin treatment and inhibition of cell growth, induction of apoptosis, and morphological alterations. In addition, Quercetin induced DNA fragmentation in TNBC cells. Moreover MDA-MB-231 cells exhibited higher sensitivity towards Quercetin than that of MDA-MB-157 with half-maximal cytotoxic doses in micromolar range. This study demonstrated that the apoptosis-inducing effects of Quercetin on MDA-MB-231 cells *in vitro* were effectively extrapolated to the *in vivo* situation. Our results suggest that Quercetin may be a potential candidate for treatment of TNBCs. Our ongoing studies will further validates the effective chemopreventive capabilities of Quercetin in other cancer cell lines as well as in a xenograft assays. If Quercetin is indeed proven to exert an effective chemopreventive potential *in vivo*, it may be an ideal therapeutic agent for breast cancer. Our findings showed that the ability of Quercetin to induce apoptosis in TNBC cells, MDA-MB-231 and MDA-MB-157, is directly associated with its FASN inhibitory activity, thereby providing a new mechanism by which the dietary plant compound, Quercetin, may exert its anti-cancer effects.

Disclosure of conflict of interest

None.

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