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Quercetin and sulforaphane in combination suppress the progression of melanoma through the down-regulation of matrix metalloproteinase-9

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Abstract. Malignant melanoma is one of the most common types of cancer in the US and worldwide. The epidemiological data suggest that dietary modification may reduce the incidence of this disease. Quercetin (3,5,7,3',4'-tetrahydroxyflavone), a flavonoid isolated from onion, exhibits anti-oxidant, anti-inflammatory and anti-cancer effects. D,L-sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], a cruciferous vegetable-derived isomer isolated from broccoli, is highly effective in protection against cancer. Matrix metalloproteinases (MMPs), extracellular matrix degrading enzymes, are involved in embryogenesis, inflammation, angiogenesis and cancer. MMP-9 in particular plays a crucial role in the regulation of invasion, tumor growth and metastasis. Previous studies have reported that both quercetin and sulforaphane independently reduce tumor growth and metastasis in breast, prostate, lung and other types of cancers. However, the combined effects of quercetin and sulforaphane on the regulation of tumor growth and the mechanism(s) of actions underlying this process have not yet been investigated. In the present study, we report for the first time that quercetin and sulforaphane in combination inhibit the proliferation and migration of melanoma (B16F10) cells more effectively than either compound used alone. Moreover, these compounds in combination significantly suppressed melanoma growth as compared to their individual use in a mouse model. This combined effect was predominantly due to a decrease in MMP-9 expression in the mouse tumors. Taken together, our findings revealed that the administration of quercetin and sulforaphane in combination rather than alone may be a more effective approach for the treatment of malignant melanoma.

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Abbreviations: QUE, quercetin; SFN, sulforaphane; MMP-9, matrix metalloproteinase-9; DAPI, 4'6-diamidino-2 phenylindole; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Key words: quercetin, sulforaphane, matrix metalloproteinase-9, melanoma growth

Introduction

Malignant melanoma is one of the most common types of cancer in the US as well as worldwide. It is an aggressive disease with high metastatic potential and resistance to many cytotoxic agents (1). Melanoma cells have low levels of spontaneous apoptosis, and chemotherapeutic drugs function by inducing apoptosis (1). Many dietary agents, including kinase inhibitors, have been inversely correlated to the spread of malignant melanoma (2-4). The alkylating agent dacarbazine is currently being used in clinical trials in combination with novel therapeutic agents (4). Quercetin (3,5,7,3',4'-tetrahydroxyflavone) is a flavonoid isolated from onion, whereas sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane] is a member of an isothiocyanate family of chemopreventive agents isolated from broccoli. The anti-cancer properties of these compounds have been demonstrated in a number of malignancies, including prostate, breast, skin and liver cancers (5-9). Previous reports suggest that quercetin is an anti-oxidant and anti-inflammatory compound (10,11). Others have indicated that sulforaphane acts as a potential HDAC inhibitor and an inducer of various pro-apoptotic molecules (12-14). Sulforaphane was also found to inhibit prostate tumor growth and lung metastasis in a melanoma model (15,16).

Matrix metalloproteinases (MMPs) are extracellular matrix proteins known to play a crucial role in normal physiological processes, such as embryogenesis, wound healing, morphogenesis, reproduction, tissue resorption and remodeling, and in pathological processes, such as inflammation, arthritis, cancer, cardiovascular and pulmonary diseases; hence, they are considered therapeutic targets (17,18). Certain MMPs act as tumor suppressors, whereas others act as tumor promoters. MMP-9 is expressed mostly by stromal cells in a tumor environment, although cancer cells do express MMP-9 at low levels (19,20). MMP-9 efficiently degrades native type IV and V collagens, fibronectin, ectactin and elastin. The regulation of MMP-9 activation is more complex than that of other MMPs, as most cells in general do not express the constitutively active form of MMP-9. Rather, its activity is

induced by different stimuli depending on cell type, thereby contributing to specific pathological events (21,22). Previous reports have demonstrated that transgenic mice lacking MMP-9 exhibit reduced keratinocyte hyperproliferation and a decreased incidence of invasive tumors (23). The imbalance between MMP activity and the inhibitory action of tissue inhibitors of metalloproteinase is implied in the regulation of multiple types of diseases. MMP-9 plays a crucial role in the modulation of cytokines and proteases, and in the degradation of the serine protease inhibitor α1-anti-trypsin, which leads to lung destruction. The early stage of a clinical trial has shown promising results using an MMP-9 inhibitor in multiple sclerosis. These observations suggest the hypothesis that MMP-9 is a potential drug target for both chronic obstructive pulmonary diseases and multiple sclerosis. Thus, the further development of highly potent and specific MMP-9 inhibitor is warranted (24,25). In the present study, we report for the first time that quercetin and sulforaphane, when used in combination as opposed to alone, are more effective in suppressing cell migration and tumor growth through the down-regulation of MMP-9 expression, in *in vitro* as well as *in vivo* melanoma models.

Materials and methods

Statement of ethics. Animals were maintained in the experimental animal facilities of the National Center for Cell Science (NCCS), India, in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines (approval no. NCCS/IACUC/2007/B-107).

Cell culture and reagents. Mouse melanoma (B16F10) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine in a humidified atmosphere with 5% CO_2 at 37˚C. Quercetin (purity >98%) was purchased from HIMEDIA (India). D,L-sulforaphane (purity >99%) was a generous gift from Professor S.V. Singh of the University of Pittsburgh School of Medicine, PA, USA. Trypan blue dye was from Cambrex (Walkersville, MD, USA). Gelatin was from ICN (Aurora, OH, USA). Goat polyclonal anti-mouse MMP-9, anti-actin antibodies, horseradish peroxidase-conjugated IgG and Ultra-Cruz mounting media containing 4',6-diamidino-2-phenylindole (DAPI) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Cy2-conjugated anti-goat IgG was obtained from Calbiochem (La Jolla, CA, USA).

Cell viability assay. The effect of quercetin and sulforaphane on cell proliferation either alone or in combination was studied using the trypan blue dye exclusion assay as described previously (26). Briefly, B16F10 cells ($1x10^4$) were seeded in 24-well plates and treated with quercetin (50 μM) or sulforaphane (20 μM) or combinations of two doses of quercetin and sulforaphane (25 and 10 μM or 50 and 20 μM, respectively) and then incubated for 16 h. The number of viable cells were counted, analyzed and represented as the percentage of cell viability vs. treatment. Each of the experiments was performed in triplicate, and data were analyzed by one-way ANOVA and represented in the form of a bar graph.

Wound migration assay. The wound migration assay was performed as described previously (27). Briefly, B16F10 cells were seeded and grown to confluency. Wounds with a constant diameter were made using sterile tips, and the cells were treated with quercetin and sulforaphane either individually or in combination under similar conditions as described above. The wound images were captured at 0 and 16 h using a phase contrast microscope (Nikon). The wound assay data were analyzed, quantified and represented in the form of a bar graph using Image Pro-plus 6.0 software (Nikon) and one-way ANOVA.

In vivo tumorigenicity and immunofluorescence experiments. The tumorigenicity and immunohistochemistry experiments were performed as described previously (28,29). B16F10 cells ($1.5x10^6$) were injected subcutaneously into the right flanks of male C57 BL6 mice (4-6 weeks of age). Animals were randomly separated into 5 groups (6 mice/group). Quercetin (15 mg/kg body weight) or sulforaphane (3.5 mg/kg body weight) was injected thrice a week into the peripheral sites of tumors for 3 weeks. In seperate experiments, a combination of quercetin (7.5 mg/kg body weight) and sulforaphane (1.75 mg/kg body weight) was injected into the peripheral sites of tumors. In another experiment, a combination of quercetin (15 mg/kg body weight) and sulforaphane (3.5 mg/kg body weight) was injected. Animals were maintained in the experimental animal facilities of the NCCS, India, in accordance with IACUC guidelines. At the termination of the experiments, all animals were sacrificed, tumors were photographed, excised and weighed, and the volumes [0.5 (length x $breadth^2$)] were calculated. Paraffin-embedded tumor sections were used for immunofluorescence analysis. Briefly, sections were deparafinized, rehydrated, used for heat-induced antigen retrieval and quenched. The sections were blocked with 2% bovine serum albumin and incubated with an anti-MMP-9 antibody (1:20). The sections were washed and incubated with Cy2-conjugated anti-goat IgG and counterstained with mounting media containing DAPI and analyzed using a confocal microscope (Zeiss).

Western blot analysis and zymography experiment. Western blotting was performed as previously described (30). Tumor samples were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 5 mM iodoacetamide containing 1 mM DTT and 2 mM PMSF), and the protein concentration in the lysates was measured using the Bio-Rad protein assay. The samples containing an equal amount of total proteins (40 μg) were resolved by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto nitrocellulose membranes. The membranes were incubated with goat anti-MMP-9 antibody (1:2,000), further incubated with horseradish peroxidase-conjugated anti-goat IgG (1:4,000) and detected by Western blotting using a luminol reagent (Santa Cruz Biotechnology).

The gelatinolytic activity was measured as described previously (31). To assess the effect of quercetin and

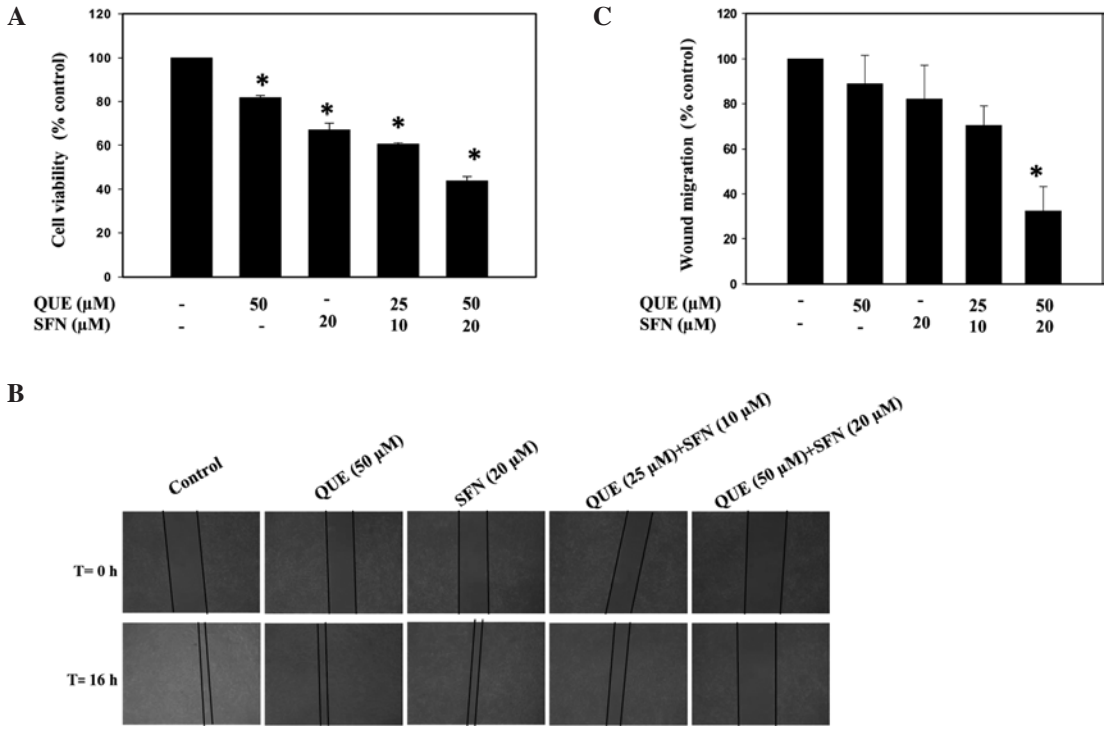

Figure 1. Inhibitory effect of quercetin (QUE) and sulforaphane (SFN) on B16F10 melanoma cell proliferation and cell motility. (A) B16F10 cells ($1x10^4$) were either treated with QUE (50 μM) or SFN (20 μM), or a combination of the two for 16 h. Cell viability was measured by trypan blue dye exclusion assays. The data were analyzed and represented as the percentage of cell viability vs. treatment in the form of bar graphs. The error bars indicate the standard error of the mean in triplicate. The values were analyzed by one way ANOVA ($^*p<0.05$). (B) The wound migration assay was carried out as described in Materials and methods. B16F10 cells were either treated with QUE (50 μM) or SFN (20 μM), or a combination of the two for 16 h. Images of the wounds were captured at 0 and 16 h. (C) Wound migration data were quantified and represented as the percentage of wound migration vs. treatment in the form of a bar graph. The error bars indicate the standard error of the mean. Experiments were performed in triplicate and analyzed by one-way ANOVA ($^*p<0.05$).

sulforaphane on MMP-9 expression, an equal amount of total proteins from tumor lysates was mixed with sample buffer in the absence of a reducing agent, incubated for 30 min and separated by 7.5% zymography-SDS-PAGE containing gelatin (0.5 mg/ml). The gel was washed with 0.25% Triton X-100 and incubated in incubation buffer [50 mM Tris-HCl (pH 7.5) containing 100 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% Triton X-100, 0.02% (w/v) NaN_3]. Negative staining indicated the zones of gelatinolytic activity.

Results

Effect of quercetin or sulforaphane alone or in combination on B16F10 cell proliferation. Previous reports have revealed that quercetin or sulforaphane inhibit cancer cell proliferation in an independent manner (9,32). We therefore examined whether a combination of quercetin and sulforaphane confers a significant effect on the proliferation of B16F10 cells. Accordingly, B16F10 cells were treated with quercetin (50 μM) or sulforaphane (20 μM) either alone or in a combination of two different doses as described in the Materials and methods, and cell proliferation assays were performed using the trypan blue dye exclusion test. The results indicated that quercetin and sulforaphane in combination had a stronger inhibitory effect on the growth of B16F10 cells than either compound alone (Fig. 1A). The data were analyzed by one-way ANOVA and represented in the form of a bar graph ($p<0.05$).

Effect of quercetin or sulforaphane alone or in combination on B16F10 melanoma cell motility. To ascertain whether quercetin and sulforaphane exhibit a combinatorial effect on B16F10 cell motility, a wound migration assay was performed. Cells were grown in monolayer to attain cobblestone morphology and were treated with quercetin (50 μM) or sulforaphane (20 μM), or with a combination of two different doses for 16 h as described above. The data indicated that a combination of quercetin and sulforaphane inhibited melanoma cell migration more significantly compared to treatment with each agent individually (Fig. 1B). The data were analyzed using Image Pro plus 6.0 software (Nikon) and one-way ANOVA. Fig. 1C shows a graphical representation of the percentage of wound migration with respect to the control ($p<0.05$).

Quercetin and sulforaphane in combination suppress melanoma growth in C57 BL6 mice. Our *in vitro* results prompted us to investigate the combined effect of quercetin and sulforaphane on melanoma growth in a mouse model. Accordingly, B16F10 cells were injected into the right flanks of C57 BL6 mice. After 1 week, quercetin or sulforaphane, either alone or in combination, were injected at the peripheral site of the tumors thrice a week for 3 weeks. The data revealed that a combination of quercetin and sulforaphane is more effective in suppressing melanoma growth than each agent used alone (Fig. 2A). Mice were sacrificed by cervical dislocation. Tumors were excised and weighed, and the volume was calculated as described in Materials and

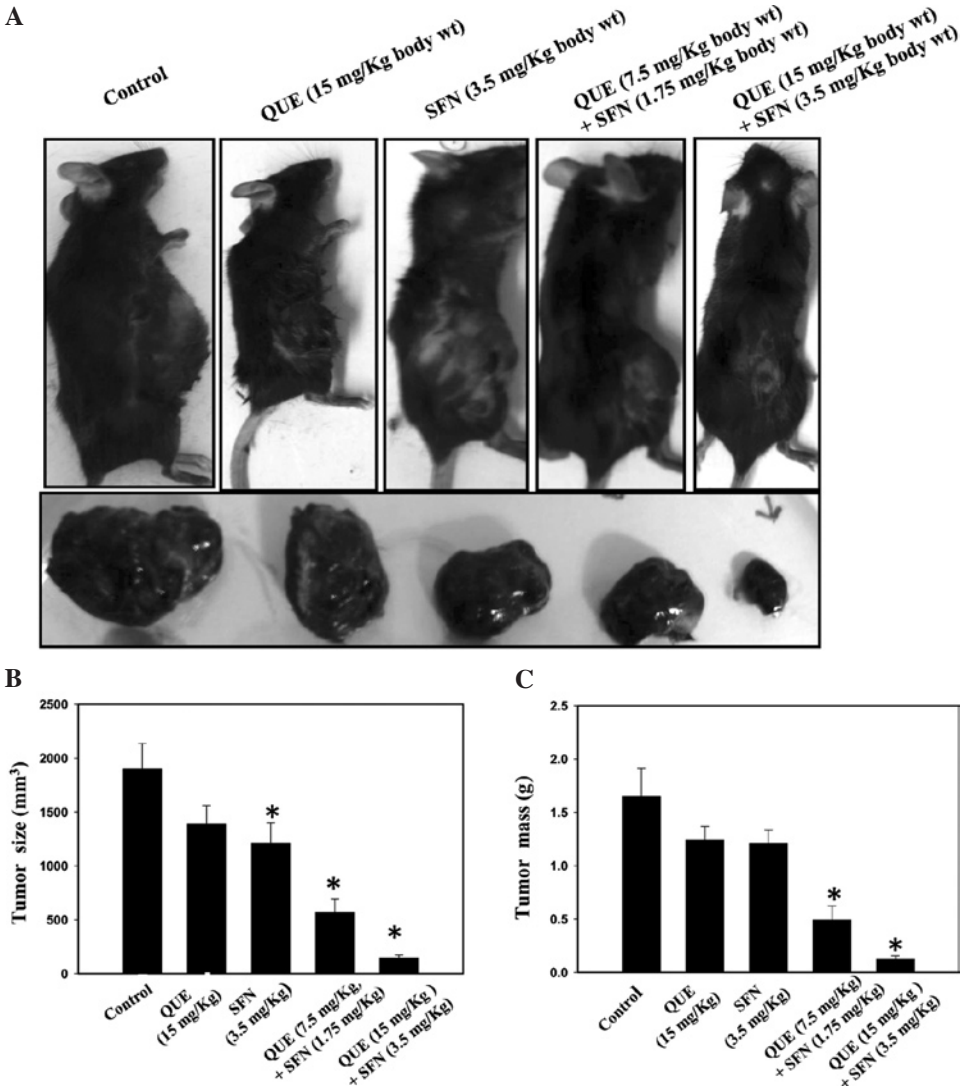

Figure 2. Quercetin (QUE) and sulforaphane (SFN) in combination suppress melanoma growth in a mouse isograft model. B16F10 cells ($1.5x10^6$) were injected subcutaneously into C57 BL6 mice. Various doses of QUE or SFN, either alone or in combination, were injected at the peripheral site of the tumors as described in the Materials and methods. After 3 weeks, the mouse tumors were photographed, excised (A), and tumor volume and weight were measured and represented in the form of bar graphs (B and C). The data were analyzed by one-way ANOVA. The error bars indicate the standard error of the mean (n=6) ($^*p<0.05$).

methods. The tumor volumes and weights were analyzed by one-way ANOVA ($p<0.05$) and represented in the form of bar graphs (Fig. 2B and C).

Quercetin and sulforaphane reduce tumor growth through the down-regulation of MMP-9 expression. To elucidate the molecular mechanism of tumor growth suppression in response to quercetin and sulforaphane, formalin-fixed tumor sections were analyzed by immunofluorescence using an anti-MMP-9 antibody. The data revealed that the expression of MMP-9 in tumor sections from the mice treated with a combination of quercetin and sulforaphane was significantly decreased as compared to tumors from the control mice or mice treated with quercetin or sulforaphane alone (Fig. 3A, panel a). Panels b and c show nuclear staining by DAPI and merged images, respectively.

The expression of MMP-9 in tumor lysates was further confirmed by Western blot analysis and gelatin zymography. Tumor tissues were lysed, and an equal amount of total proteins in the lysates were analyzed by gelatin zymography. The zymography data showed that the expression of pro-MMP-9 in tumors from the mice treated with quercetin and sulforaphane in combination was dramatically reduced as compared to tumors from the control mice or mice treated with either compound alone (Fig. 3B). This was further confirmed by Western blot analysis using an anti-MMP-9 antibody (Fig. 3C). Taken together, these data strongly suggest that quercetin and sulforaphane in combination inhibit pro-MMP-9 expression, which in turn suppresses melanoma progression in a mouse model.

Discussion

Historically, plant-derived compounds have been used as traditional medicines in Asian countries as well as worldwide (33). Many of these plant products have shown great potential to act as anti-carcinogenic, anti-inflammatory and anti-diabetic agents (33). However, higher concentrations of

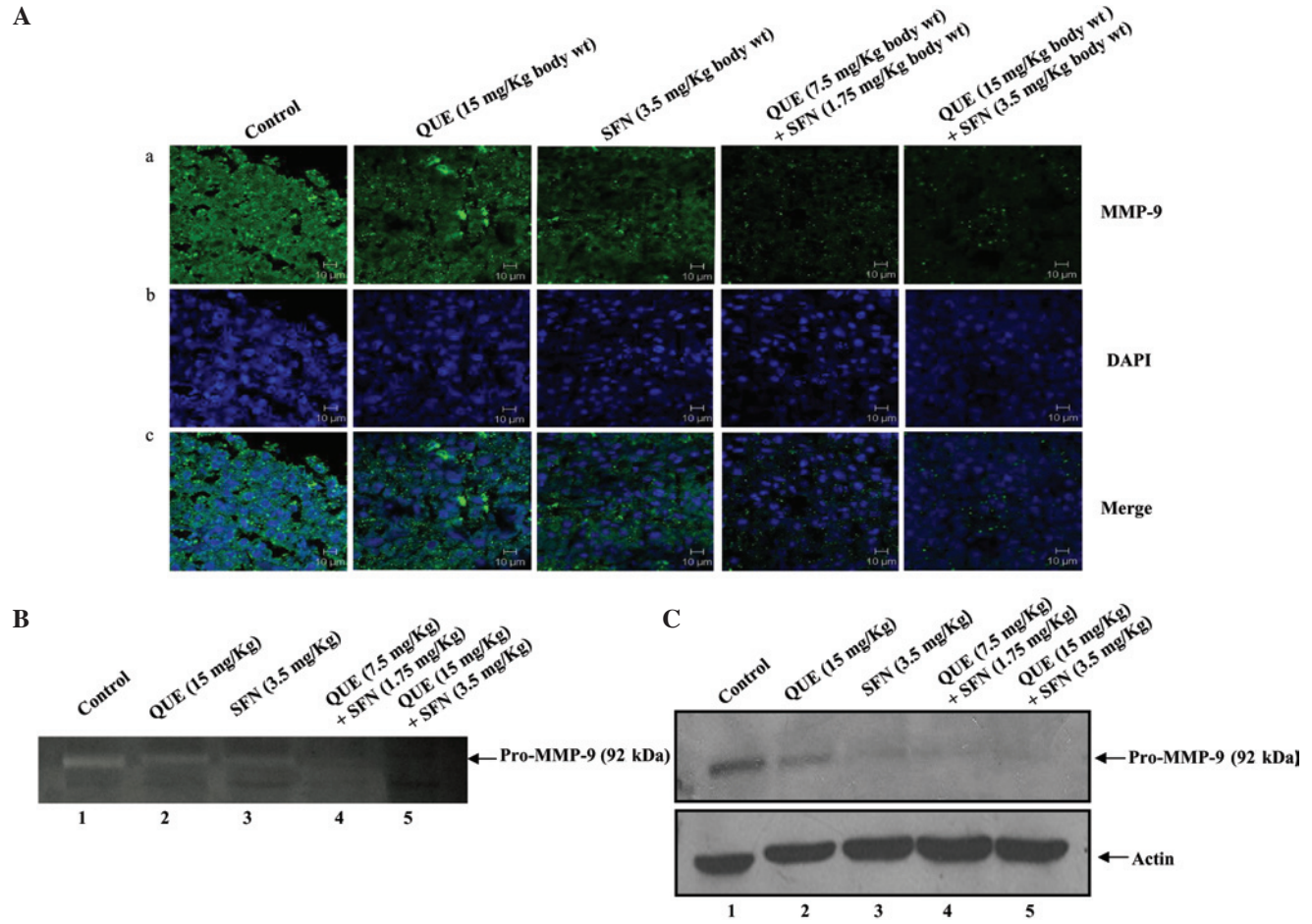

Figure 3. Quercetin (QUE) and sulforaphane (SFN) in combination inhibit the expression of MMP-9 in mouse tumors. (A) The localization of MMP-9 in mouse tumors was visualized by immunofluorescence using an anti-MMP-9 antibody and staining with Cy2-conjugated IgG (green). Nuclei were counterstained with DAPI (blue). Images were captured using a confocal microscope (Zeiss). (B and C) Tumor lysates containing an equal amount of total proteins were analyzed by zymography (B) and Western blot analysis (C). Actin was used as a loading control. Lane 1, control (untreated tumor lysates); lane 2, QUE (15 mg/kg body weight); lane 3, SFN (3.5 mg/kg body weight); lane 4, QUE (7.5 mg/kg body weight) and SFN (1.75 mg/kg body weight); lane 5, QUE (15 mg/kg body weight) and SFN (3.5 mg/kg body weight).

these compounds are required to show significant inhibitory effects. Therefore, combination studies using two or more such pharmacologically active compounds may increase the efficacy of the drug and overcome the hurdle of drug resistance.

The present study demonstrated the anti-carcinogenic/anti-tumor activity of a plant-derived flavonoid, quercetin, and an isothiocyanate, sulforaphane, on melanoma progression when used in combination. Previous reports have revealed that quercetin and sulforaphane act as anti-cancer agents in various cancer models when used individually (5-9). Lin *et al* showed that quercetin inhibits tumor cell motility and invasion through PKCδ/ERK/AP-1-dependent MMP-9 activation in breast cancinoma cells (34). Our *in vitro* data suggest that a combination of quercetin and sulforaphane inhibits melanoma cell proliferation more effectively than each used independently. The combination treatment also significantly inhibits melanoma cell motility. Our results revealed that the use of quercetin and sulforaphane at the ratio 2.5:1 is quite effective against the proliferation and migration of B16F10 cells.

Our *in vitro* results prompted us to examine the effects of quercetin and sulforaphane in an *in vivo* melanoma model. The B16F10 cells were injected subcutaneously into the right flanks of C57 BL6 mice. Tumors were generated, and quercetin and sulforaphane, either independently or in combination, were injected into the peripheral site of the tumors. Notably, quercetin and sulforaphane in combination drastically suppressed melanoma growth as compared to the agents used independently in a mouse isograft model. However, the molecular mechanism by which quercetin and sulforaphane inhibit melanoma growth has not yet been fully understood. Zhang *et al* reported that quercetin inhibits the invasion of murine melanoma B16BL6 cells by decreasing pro-MMP-9 via the PKC pathway (35). Therefore, we examined the status of pro-MMP-9 expression in the tumors treated with quercetin and sulforaphane in combination. As shown using immunofluorescence, zymography and Western blotting, the level of expression of MMP-9 was reduced significantly in tumors treated with lower and higher doses of the two compounds as compared to the control or their individual use. These data demonstrated that combination therapy is more effective than the use of a single compound for the treatment of melanoma growth.

In summary, for the first time we report that quercetin and sulforaphane in combination are much more effective in regulating melanoma progression through the down-regulation of

MMP-9 expression than each compound used alone. Thus, inhibiting MMP-9 expression by quercetin and sulforaphane in combination may be a novel therapeutic strategy for the prevention of melanoma progression.

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