BIOACTIVE COMPOUNDS IN THE PREVENTION OF CANCER

MECHANISMS OF ACTION

A Thesis

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ABSTRACT

Epidemiological studies have consistently shown that increased consumption of fruits and vegetables is associated with reduced risk of various chronic diseases including cardiovascular diseases, hypertension, diabetes and cancers. Phytochemicals in fruits and vegetables have been identified as the primary contributors to these health benefits. However, the molecular mechanisms of the anticarcinogenic effects of fruits and vegetables are not completely understood.

Previous works from our lab clearly showed the antiproliferative activity of quercetin-3-glucoside towards human breast cancer MCF-7 cells. In the project investigating the mechanism of action of the quercetin-3-glucoside, my work demonstrated that quercetin-3-glucoside inhibits MCF-7 cell proliferation by activating p38/MAPK pathway through targeting ASK1. It also induces cell apoptosis via p53-dependent pathway.

In conclusion, we demonstrated that quercetin-3-glucoside exerted anticarcinogenic effects via counteracting antiproliferation and apoptosis induction. These data may be important in further understandings of the protective effects of fruits and vegetables against related cancers.

BIOGRAPHICAL SKETCH

Qiushi Huang was born on October 1987 in Heilongjiang, China. She received her Bachelor of Science in Biological Science at Peking University (PKU) in 2009, working on the single nucleotide polymorphism (SNP) chip for rice genome.

Upon completion of her undergraduate studies in China, Qiushi was admitted into the M.S. program in the Department of Food Science, Cornell University, NY. Qiushi majored in food science and minored in human nutrition and applied economics and management. Her research focuses on the bioactive compounds and the signaling transduction pathways in cancer cells.

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Nowadays, people are focusing more on natural ways in prevention of chronic diseases. Foods, which are necessary for every human being, become the top consideration. Epidemiological studies consistently showed that food pattern is related to disease incidence. A dietary pattern that is high in fruits and vegetables, whole grains, dairy products and fiber and low in fat could lower the risk of various chronic diseases including cancer, hypertension, and cardiovascular diseases.

Cancer accounts for 22.9% of total deaths in United States, exceeded only by heart disease (25.0%) (SEER Cancer Statistics Review 1975-2008). The estimated number of new cancer cases is 1,596,670 in 2011, and the estimated number of deaths due to cancer reached 571,950 in 2011. The overall cost of cancer in 2010 was $263.8 billion, which led to a huge economical and psychological burden for the country (American Cancer Society 2011). The lifetime risk for being diagnosed with all sites of cancers is 41.21% (95% CI 41.11-41.30%). The lifetime risk for dying from cancer is 21.07% (95% CI 21.04-21.10%). (SEER Cancer Statistics Review 1975-2008)

Breast cancer is the first most common malignant tumor among women. In the

United States, nearly every 1 in 3 cancers diagnosed among women is breast cancer. It's also the second leading cause of death resulting from cancer among women. Estimated number for new breast cancer cases in US in 2011 is 57,650 and 230,480 for in situ cases and invasive cases respectively. Total deaths number is estimated to be 39,520 in women. As age goes up, these numbers go up accordingly. The long-term incidence trend shows an increase in breast cancer incidence in early 1980s due to the introduction of mammography screening and changes in reproductive patterns. It dropped sharply between 2002 and 2003 probably due to the decreased use of menopausal hormones. The incidence rates among women older than 50 stayed stable since 2004. The long-term mortality rate dropped approximately 2.2% every year from 1990 to 2007. It could be attributed to improvements in treatments and early detection (DeSantis et al 2011). Risk factors of breast cancer include weight gain after 18, overweight or obese, use of hormones, physical inactivity, alcohol consumption, family history of breast cancer and inherited genetic mutations (American Cancer Society 2011). Commonly used therapies include lumpectomy or mastectomy, radiation, chemotherapy, hormone therapy and target therapy. Patients usually suffered from these therapies both mentally and physically (American Cancer Society 2011). Therefore, a more easily executed method is in serious need. Modified food patterns were proved to be related to breast cancer incidence, though the relationship was not statistically significant (Prentice et al 2006, Pierce et al 2007). It could be used as a supplementary treatment.

1.2 FOOD PATTERN AND REDUCED RISK OF COMMON DISEASES

The American Dietary Guidelines 2010 recommended a food-based eating pattern to promote health and reduce risk of major chronic diseases (Dietary Guidelines for Americans 2010). The increased amount of fruits, vegetables and whole grains was proved to be related to the reduced risk of cardiovascular diseases and various types of cancer (Flock et al 2011). Vegetable oils containing mono unsaturated fatty acids and poly unsaturated fatty acids can improve the blood lipid profile (Dietary Guidelines for Americans 2010). Epidemiology studies have consistently shown that a plant-based dietary pattern could beneficially affect health (Flock et al 2011).

Dietary pattern is associated with risk of cancer. Incidence of lung cancer is clearly proved to be related to smoking status, but it's usually difficult to interpret the observed association of lung cancer and food pattern. Here a case-control study involving 299 never smoker lung cancer patients and 317 controls was conducted to develop the association (Gorlova et al 2011). Dietary data were collected using a modified Health Habits and History Questionnaire. Two major dietary patterns were identified: healthy eating pattern including fruits and vegetables and low-fat food items, and mixed dishes pattern including most foods with positive loadings. Results were controlled for age, gender, supplement use, total caloric intake, second hand smoke expose, family history

of cancer, education, and physical activity score. They found that the lung cancer risk was significantly reduced by the healthy eating pattern (OR = 0.65, 95% CI 0.42-0.98 for the highest compared to the lowest tertile) after adjusting for confounders. Supplement use and physical activity did not modify the effect. For the adenocarcinoma cases in subgroup analysis, a consistent protective effect of healthy eating pattern was also observed [OR = 0.58 (0.34–0.99) in women; OR = 0.71 (0.34–1.49) in men; OR = 0.60 (0.26–1.39) for the highest versus the lowest tertile]. The statistical insignificance might be resulted from the small sample size. (Gorlova et al 2011).

Then a population-based case-control study was conducted to assess the effect of dietary patterns on risk of oesophageal cancers. 365 oesophageal adenocarcinoma (OAC), 426 oesophagogastric junction adenocarcinoma (OGJAC) and 303 oesophageal squamous cell carcinoma (OSCC) cases were selected. 1580 controls were matched to cases. Data on dietary factors, lifestyle and demographic factors were collected using questionnaires. Three dietary patterns were identified: fruit and vegetable, meat and fat, and pasta and pizza. Logistic regression models were used to estimate association of OAC, OGJAC, OSCC and dietary pattern scores. High scores on fruit and vegetable pattern were associated with a decreased risk of OGJAC (OR = 0.66, 95% CI 0.42-1.04, p = 0.07) and significantly reduced risk of OSCC (OR = 0.41, 95% CI 0.24-0.70, p = 0.002). When assessing the effect of high-fat dairy foods, significantly increased risks of OAC and OGJAC were observed (OR 2.46, 95% CI 1.54- 3.94; OR 1.83, 95% CI 1.17-2.86, respectively). These data shown was not

affected by BMI, reflux or smoking (Ibiebele et al 2011). Further research of prospective studies is needed to confirm these findings in case-control studies.

In a cohort study with a follow-up time of 12.9 years, 28082 women with no history of cardiovascular disease, cancer, and hypertension at baseline were recruited. Their fruits and vegetables intake was assessed using semiquantitative food frequency questionnaires (FFQs). Incidence of hypertension was recorded by annual follow-up questionnaires. After adjusted by age, race and total energy intake, the hazard ratio and 95% CI of hypertension were reduced to 0.97 (95% CI 0.89-1.05), 0.93 (95% CI 0.85-1.01), 0.89 (95% CI 0.82-0.97) and 0.86 (95% CI 0.78-0.94) (p trend <0.0001) comparing women who consumed 2-4, 4-6, 6-8 and 8 servings/day of total fruits and vegetables with those consuming < 2 servings/day. Lifestyle factors did not affect the association. When fruits and vegetables were analyzed separately, high intake of all fruits were still significantly associated with reduced risk of hypertension, but the BMI adjustment eliminated all effects (Wang et al 2011). This study suggested that the beneficial effect of a healthy diet containing high intake of fruits and vegetables might be resulting from body weight regulation.

The Dietary Approaches to Stop Hypertension (DASH) diet is recommended by Dietary Guidelines. The relationship between food pattern and coronary heart disease risk was estimated by DASH trial (Chen et al 2010). Individuals with prehypertension or stage-1 hypertension without taking any antihypertensive medication were recruited. They were randomly assigned to 3 diets: control, fruits and vegetables (F/V), and

DASH (rich in fruits and vegetables, low-fat dairy, and reduced fats and cholesterol). The Framingham risk equation was applied to calculate 10-year risk of developing CHD. Results showed that systolic and diastolic blood pressure, total cholesterol and LDL cholesterol were lowered by DASH diet when compared to control. Estimated CHD risk was reduced to 0.93 (95% CI 0.85-1.02) for F/V and to 0.82 (95% CI 0.75-0.90, $p < 0.001$) for DASH. The CHD risk for DASH compared to F/V was 0.89 (95% CI 0.81-0.97, $p = 0.012$). These data indicated a significant reduction in the 10-year estimated risk compared with baseline for those in F/V diet, and the reduction is even more significant for those in DASH diet. The advantage of this study is that it is a feeding study. The effects of the dietary pattern were measured under maximal adherence. The research population is diversified with high rates of internal validity. Thus a credible conclusion can be drawn from this trial that the DASH dietary pattern can substantially decrease estimated 10-year CHD-risk (Chen et al 2010).

Dietary pattern was also linked to insulin sensitivity, which could affect risk of chronic diseases (Anderson et al 2011). This relationship was assessed by a prospective cohort study of 3075 older adults. Food intake was estimated with a modified Block food frequency questionnaire. Blood sample was used to measure fasting glucose and fasting insulin levels. CRP, IL-6 and TNF-α levels were used as inflammation markers. Socio and lifestyle variables include age, gender, race, education, smoking status, alcohol consumption, physical activity. Six clusters were identified in 1751 participants who completed the study. The healthy foods cluster (higher intake of low-fat dairy

products, fruit, whole grains, poultry, fish and vegetables) had significantly lower fasting insulin and insulin resistance values as well as the fasting glucose when compared with other groups ($p \leq 0.05$). Changes in all inflammatory markers showed similar patterns. The means of CRP were lower in the healthy food cluster, indicating a decrease of production of pro-inflammatory cytokines and a reduced metabolic risk. This study suggested that a diet containing low-fat dairy products, fruits and vegetables, whole grains, poultry and vegetables might be beneficial for older adults by inducing insulin sensitivity and lowering systemic inflammation (Anderson et al 2011).

1.3 FOOD PATTERN AND REDUCED RISK OF BREAST CANCER

As early as 1997, breast mammographic changes among women that had a low-fat eating pattern were shown in a randomized trial (Prentice et al 1997). 817 women were assigned either to self-selected diet or dietary intervention with a goal of 15%, 20% and 65% of energy from fat, protein and carbohydrates, respectively. They were then followed for 2 years. Total breast area and total area of dense tissue from each mammographic view were analyzed using mammographic images taken before and at the end of the trial. 2.5% reduction in breast area and 6% reduction in density were observed in intervention group when compared to control group (less than 1% and 2.5% respectively). But the hypothesis that a low-fat well-balanced diet pattern may reduce

breast cancer risk was not adequately tested (Prentice et al 1997).

The feasibility of a high vegetable diet on preventing breast cancer was assessed in another randomized trial (Pierce et al 1997). Women who had been diagnosed with stage I, II and IIIA breast cancer within the previous four years and who had completed initial treatment was recruited. The dietary goals were 5 servings of vegetable, 16 ounces of vegetable juice, 3 serving of fruits, 30 g fiber and 15-20% energy from fat. The adherence to the study diet was assessed using recalls at 6 and 12 months and measurement of circulating carotenoid concentrations in blood samples. The control group was provided with the diet containing 5 servings of fruits and vegetables. At the end of the trial, women in intervention group and control group were not statistically different in baseline characteristics. The difference in vegetable intake occurred by 6 months. Mean intake increased from 2.8 servings/day to 7.4 servings/day compared with 3.0 servings/day in control group. Dietary adherence score did not differ in the two groups at baseline, but was significantly greater for the intervention group at 6 months and 12 months ($p = 0.0001$). The majority of the intervention group was adherent to study goals. Concentration of carotenoids significantly increased in intervention group when compared with control group (66% vs. 52% in β-carotene, 265% vs. 26% in α-carotene, 63% vs. 2% in lutein). Other dietary biomarkers including β-cryptoxanthin, lycopene and total cholesterol remained no change. Results from this study demonstrated that the adoption and maintain of the hi-vegetable, reduced-fat and increased-fiber diet is feasible in breast cancer trials. The methods of

self-modified diet can be achieved with high adherence (Pierce et al 1997).

In the following years, several studies were conducted to test the hypothesis, but controversial results were obtained.

The very first trial to test the association between dietary pattern and invasive breast cancer was the Women's Health Initiative Dietary Modification randomized controlled trial (WHI)(Prentice et al 2006). It was a randomized, controlled, primary prevention trial that conducted at 40 US clinical centers from 1993 to 2005. The objective is to assess the effect of low-fat diet on breast cancer incidence. 48835 postmenopausal women aged from 50 to 79 years without prior breast cancer were enrolled. They were randomly assigned to either the modified diet intervention group or the control group. The intervention goal was to reduce total dietary fat to 20% of total energy intake, and to increase fruit and vegetable intake to at least 5 servings per day and grains intake to at least 6 servings per day. The control group was not asked to make any dietary changes. Invasive breast cancer incidence was measured in the follow-up years. Percent energy from fat was lower in the intervention group by 10.7%, 9.5%, 8.1% at year 1, 3, 6 respectively. Fruits and vegetables intake was increased by 1.2, 1.3, 1.1 servings at 1, 3, 6 years respectively. Grain consumption was higher by 0.9, 0.7, 0.4 servings at these times. The incidence rate of invasive breast cancer was 3.35% and 3.66% in intervention group and control group respectively. The estimated HR was 0.91 (95% CI 0.83-1.01). This number did not change after additional adjustment for calcium and vitamin D. Mortality ratio was 0.77 (95% CI 0.48-1.22), indicating a not

significantly reduced mortality rate in intervention group. Total cancer incidence and mortality and total mortality ratio were slightly less than 1 and were not statistically significant when comparing the two groups (Prentice et al 2006). These data suggests a moderate decrease in breast cancer risk for the intervention group. But subgroup analysis might be useful since breast cancer can be categorized into several subtypes according to tumor hormone receptor. Also, baseline consumption of fat is highly correlated with HR in intervention group.

One analysis on ovarian, breast, endometrial, colorectal and all invasive cancer rates were performed using same data from WHI trial(Prentice et al 2007). Postmenopausal women aged from 50 to 79 years were randomly assigned to a dietary modification intervention or control. The average length of follow-up was 8.1 years. The diet pattern was assessed by the WHI food-frequency questionnaire. Weighted log-rank tests were used to compare incidence of invasive cancers. Percent energy from fat was lower in the intervention group by approximately 10%. Fruits and vegetables intake was increased by about 1 serving per day. Grain consumption was higher by a lesser extent. The hazard ratio for total invasive cancer was 0.95 (95% CI 0.89-1.01), suggesting a moderate protective effect of the modified diet. One thing to be mentioned is that the hazard ratio of ovarian cancer averaged over the entire intervention period was not statistically significantly less than 1. When the follow-up period was divided into the first 4 and the latter 4.1 years, hazard ratio reduced to 0.60 (95% CI 0.38-0.96, $p = 0.03$) in the latter 4.1 years, which was statistically significant (Prentice et al 2007). These

data suggested that the effect of diet on cancer risk might be time dependent, and the protective effect might not be apparent until later in time. Disappointingly, the same analysis was not performed in other types of cancers.

The risk of benign proliferative breast disease was then assessed using the same data set again from the WHI trial(Rohan et al 2008). Women aged 50 to 79 years who reported breast biopsies free of cancer were identified in this study. The dietary intervention was designed to reduce total dietary fat to 20% of total energy intake, increase fruit and vegetable intake to more than 5 servings/day, and increase intake of grain products to at least 6 servings/day. The length of follow-up was 7.7 years in average. One year after randomization, statistical significant differences in intake of these dietary items were evident. The association between dietary modification and benign proliferative breast cancer cases was 1.09 (95% CI, 0.98-1.23). A slight increase in risk of benign proliferative breast disease without atypia (HR = 1.10, 95% CI 0.97-1.25) and a significant increased risk for either atypical hyperplasia or moderately extensive or florid proliferative disease without atypia (HR = 1.16, 95% CI 1.02-1.33) were observed. The overall effect of dietary modification was unchanged by exclusion of the first year of follow-up or exclusion of women with a breast biopsy before the trial, or adjustment for annual measures of height and weight, or use of hormone (Rohan et al 2008). These data suggested that a slightly modified diet with moderately reduced fat, increased fruits and vegetables and fiber, instead of reduce the risk of breast cancer, increased the risk, but the effect is not statistically significant.

In the Women's Healthy Eating and Living (WHEL) Randomized Trial, the effect of a diet very high in fruits and vegetables, fibers and low in fat was studied (Pierce et al 2007). 3088 women aged 18 to 70 years who were treated for stage I, II and IIIA breast cancers were recruited. The intervention group was randomly assigned and had dietary goals of 5 servings of vegetable, 16 ounces of vegetable juice, 3 servings of fruits, 30 g fiber and 15-20% energy from fat. The control group was provided with dietary guidelines containing 5 servings of fruits and vegetables. Invasive breast cancer event and death from all causes were measured. At 4 years, vegetable servings, fruit servings, fiber and energy intake from fat changed +65%, +25%, +30% and -13% respectively in intervention group ($p < 0.01$). Plasma carotenoid concentration increased 43% ($p < 0.001$). Breast cancer incidence was 16.7% in intervention group compared with 16.9% in control group. The hazard ratio adjusted for potential confounders was 0.96 (95% CI 0.80-1.14, $p = 0.63$). Mortality rate was 10.1% in intervention group and 10.3% in control group. More than 80% of all deaths were due to breast cancer. The adjusted hazard ratio was 0.91 (95% CI 0.72-1.15, $p = 0.43$) (Pierce et al 2007). These results showed that the adoption of a diet that was very high in vegetables, fruits, fiber and low in fat did not lead to the significantly reduced risk of breast cancer or mortality risk although the calculated hazard ratio is less than 1.

In the subgroup analysis of WHEL study, breast cancer events among women without hot flashes after treatment of early-stage breast cancer were assessed again (Pierce et al 2009). The risk of secondary breast cancer in women without hot flashes

(HF-) was analyzed. 896 women reported no baseline hot flashes were identified in the HF- group. Dietary intake was assessed by recalls, and plasma carotenoid concentration was performed to validate the dietary self-report. Height, weight and physical activity were also recorded. The crude event rate in the intervention group was highest in quartile 1 (21%, consuming less than 4.9 servings/day) and lowest in quartile 4 (9.6%, consuming more than 8.9 servings/day). When looking at the fruits and vegetables intake, the hazard ratio of additional breast cancer events in quartile 4 is 0.41 (95% CI 0.196-0.86), and the hazard ratio in quartile 1 is 0.8 (95% CI 0.48-1.35). When looking at the fiber intake, the hazard ratio of additional breast cancer events in quartile 4 (consuming more than 25.2 g/day) is 0.48 (95% CI 0.26-0.87), and the hazard ratio in quartile 1 (consuming less than 15.6 g/day) is 0.82 (95% CI 0.45-1.48). When looking at the fiber to fat ratio, the hazard ratio of additional breast cancer events in quartile 4 (> 0.54) is 0.38 (95% CI 0.19-0.77), and the hazard ratio in quartile 1 ($\leq$ 0.25) is 0.82 (95% CI 0.42-1.63). The p values for trend are less than 0.05 in all comparisons. These data suggested that the intervention group experienced fewer cancer events than did the comparison group for all of the baseline quartiles. The difference was significant when comparing upper baseline quartiles. A significant trend for fewer breast cancer events was observed across quartiles of vegetable-fruit and fiber intake (Pierce et al 2009).

Prognosis of breast cancer in women without hot flashes was the second subgroup analysis that derived from the WHEL trial (Gold et al 2009). The identified

intervention and control groups were not statistically different in baseline characters. Using the Cox model in developing hazard ratio, HF-negative intervention women had a 31% reduction in the risk of developing additional breast cancer when compared with the control group (HR = 0.69, 96% CI 0.51-0.93, p = 0.02). Additionally, both the intervention and comparison groups in HF-positive subgroup had a significantly better prognosis than the HF-negative comparison group. The hazard ratio was 0.77 (95% CI 0.59-1.00, p = 0.05) and 0.65 (95% CI 0.49-0.85, p = 0.02) respectively. Significant dietary intervention effect was observed in postmenopausal women with a hazard ratio of 0.53 (p = 0.003). But no effect was observed for premenopausal women in the HF-negative subgroup. In fact, the hazard ratio was 1.16, suggesting an increased risk in additional breast cancer event. When premenopausal women was eliminated from the Cox model, the hazard ratio for HF-negative women in the intervention group reduced to 0.49 (95% CI 0.32-0.75, p = 0.001) (Gold et al 2009). These data suggested that the effect of modified diet on breast cancer depends on the occurrence of hot flashes. Moreover, a significant protective effect was observed in postmenopausal women, suggesting that this sub-population is more sensitive to the dietary pattern.

1.4 CARCINOGENESIS AND REACTIVE OXYGEN SPECIES

A strong association exists between free radicals production and carcinogenesis. *In*

vitro studies showed that some pro-oxidant chemicals promoted tumors in several animal models (Devasenaetal 2006, Liebler et al 2000, Slaga et al 1981, Huang et al 1999), whereas primary endogenous antioxidant enzymes can affect tumor promotion (Zhao et al 2005, Fukuyama et al 2005). The involvement of reactive species (ROS) in tumor progression has also been shown in human cells (Gibellini et al 2010). ROS are a group of highly reactive free radicals produced from molecular oxygen. It can be categorized into four subclasses: singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{-\cdot}$), peroxide anion ($O_2^{2-\cdot}$), and hydroxyl radical ($^{\cdot}OH$). ROS production is constant in cells. For example, hydrogen peroxide can be generated during fatty acids breakdown. Activated leucocytes can produce ROS when responding to immune system(De Duve 1983). A large amount of superoxide is released from macrophages. It can also be generated by the oxidative environment, such as smoking (Babior 1984). The major sources of endogenous ROS are superoxide anion and hydrogen peroxide (Nohl et al 2003). Hydrogen peroxide can be converted to water by enzymes catalase or glutathione peroxidase. Superoxide anion is converted by superoxide dismutase (SOD) to H_2O_2, then participates in 'Fenton reaction' to produce the highly active hydroxyl radical (Waris et al 2006).

ROS normally exists in cells in balance with biochemical antioxidants. Oxidative stress occurs when the balance is disrupted due to excess ROS, antioxidants depletion, or both (Klein et al 2003). Firstly, ROS induces damage of various biomolecules. Cells have developed several antioxidant defenses mechanisms during evolution, including

DNA repair, detoxifying enzymes, and small scavenger molecules. But these systems might not be sufficient to completely remove oxidative stress (Poli et al 2004, Valko et al 2004). Secondly, ROS acts as secondary messengers in several pathways, and results in the resistance to apoptosis, increased proliferation and metastasis (Hussain et al 2003). Thus a significant shift of cellular oxidative balance could result in abnormal physiological conditions (Gibelliniet al 2010).

Free radicals can result in long-term damage and lead to diseases. It has been proved that ROS is associated with various diseases including degenerative diseases, cancers and cardiovascular diseases(Beckman et al 1997). About 20,000 unpaired or misrepaired DNA bases are caused by ROS per day (Beckman et al 1997). Certain types of cancer cells also produce significant amounts of ROS. As a result, oncogenes might be activated, and tumor-suppressor genes might be turned off, resulting in cancer initiation and progression (Ames et al 1993). Thus ROS are considered as potential carcinogens since they facilitate mutagenesis, tumor promotion and progression (Waris et al 2006). Scientific results showed that potentially cancer-inducing oxidative stress resulted from ROS could be prevented or limited by dietary antioxidants found in fruits and vegetables. Phytochemicals in fruits and vegetables exhibit antibacterial and antiviral effects. It can also react with oxidative agents, stimulate immune system and regulate gene expressions and hormone metabolism and serves as chemopreventers (Liu 2002). Among all natural chemopreventers, the most widely studied group of antioxidant compounds are flavonoids (Ramos 2007, Gibellini et al 2010).

1.5 PHYTOCHEMICALS AND FLAVONOIDS

Phytochemicals can be defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases (Liu 2004). They are proved to be the active ingredients in plant food. Based on their chemical structures, phytochemicals could be classified as phenolics, carotenoids, alkaloids, nitrogen-containing compounds and organosulfur compounds (Liu 2004). (Table 1)

Phenolics are characterized as containing one or more aromatic rings, each bearing at least one aromatic hydroxyl and connected with a carbon bridge (Williams et al 2004). Phenolics are plant secondary metabolites. They can function against fungal parasites, herbivores, pathogens and oxidative cell injury to protect plants. Phenolics can be categorized into five subclasses: phenolic acids, flavonoids, stibenes, courmarins and tannins. It is likely to be responsible for decreased risk of cancer, cardiovascular diseases, and other chronic diseases by reducing oxidative stress and affecting cell signaling pathways that involved in cell proliferation and apoptosis. (Williams et al 2004, Liu 2004).

TABLE 1. CATEGORIES OF PHYTOCHEMICALS AND DIETARY SOURCES.

Adopted from Liu 2004

Classes	Subclasses	Phytochemicals	Dietary sources
Terpenes	Carotenoids	α-carotene	Pumpkin
		β-carotene	Carrot
		β-cryptoxanthin	Papaya
		Lycopene	Tomato
		Lutin	Broccoli
	Monoterpenes	D-limonene	Lemon
Phenols	Phenolic acids	Gallic acids	Gall nut
		Caffeic acid	Coffee
	Flavonoids	Quercetin	Apple
		Genisten	Soybean
		Catechin	Tea
		Naringenin	Grapefruit
	Stibenes	Resveratrol	Grape
	Coumerins	Auraptene	Bell pepper
	Tannins	Proanthocyanidins	Blueberry
Alkaloids		Nicotine	Tobacco
		Pyrrolizidine	Comfrey
Organosulfur	Indoles	Indole-3-carbinol	Cabbage
	Isothiocyanates	Isothiocyanate	Brussel sprout
	Allylic sulfur	Diallyl sulfide	Garlic

Among phenolics, flavonoids accounts for two thirds of the total dietary phenolics intake (Liu 2004). It is widely distributed in almost every plant and act as pharmacologically active constituents in many medicines. More than 5,000 flavonoid compounds have been identified (Appleton 2010). All these compounds share the same basic structure. It is formed by two benzene rings (A and B rings) that joined together by a three carbon link, which is formed into a γ-pyrone ring (C ring) (Liu 2004) (Figure 1.1). According to the different connections between B and C rings, the oxidation state and the functional group on C ring, flavonoids can be further divided into six subclasses: flavonols, flavones, flavonols (catechins), flavanones, anthocyanidins, isoflavonoids (Figure 1.2). More convincing evidence these years suggests that flavonoids exhibit various biological, pharmacological and medical properties including platelet aggregation inhibition, free radical scavenging, anti-proliferation and beneficial effects on cancer, cardiovascular disease and neurodegenerative disorders (Amira et al 2008, Liu 2004).

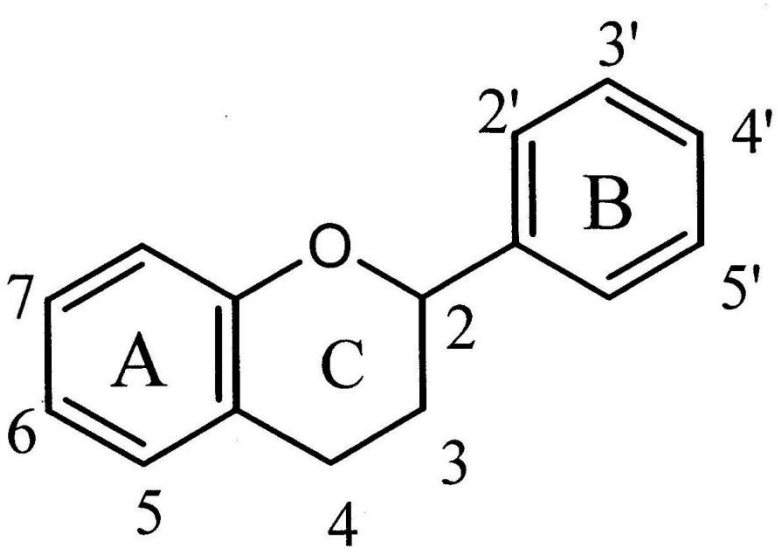

FIGURE 1.1GENERIC STRUCTURE OF FLAVONOID

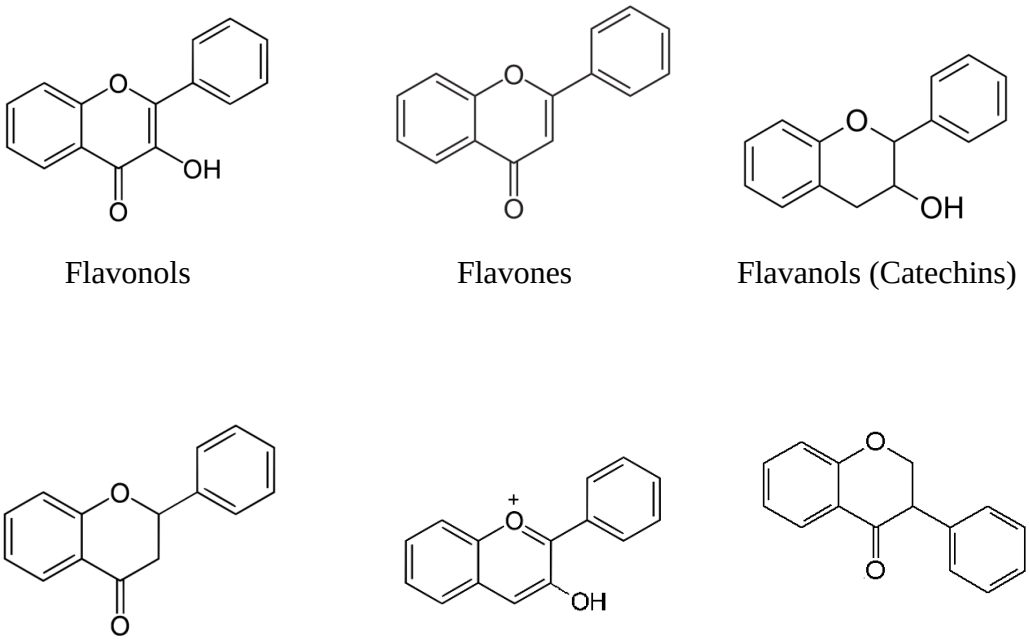

FIGURE 1.2 STRUCTURES OF MAIN CLASSES OF DIETARY FLAVONOIDS

1.6 STRUCTURE, SOURCES AND SYNTHESIS OF Q3G

1.6.1 Structure

Substitution patterns on the basic flavonoid structure occur in natural compounds. Flavonoids usually exist as glycosides, which means one or more hydroxyl groups on the skeleton are joint by a sugar moiety. D-glucose is the most common sugar that appears among the 179 different naturally existing quercetin glycosides. The presence of the sugar in 3-position on quercetin can significantly change its biochemical activities, which leads to the attention of quercetin-3-β-D-glucoside (Q3G) (Cornard et al 1998).

Q3G is also known as isoquercitin, isoquercitrin, and quercetin-3-O-glucoside. It has a molecular formula of $C_{21}H_{20}O_{12}$ with the molar mass of 464.38g/mol (Appleton 2010). Its molecular structure and the conformational possibilities of the sugar moiety were determined in 1998 (Figure 1.3). In Q3G, the sugar substituent on position three results in an out-of-plane bending of the rotatable bonds. All the rotatable bonds adopt a unique preferential position in order to maximize the stability of the molecule (Cornard et al 1998). The structural related activity of Q3G was assessed by its capability of enhancing the endothelial nitric oxide (NO) release of isolated porcine coronary arteries. Taubert et al demonstrated that Q3G only induced moderate NO

release (5 nM to 8.5 nM) when compared to quercetin (more than 8.5 nM), indicating a loss in function due to the three position substitution of sugar (Taubert et al 2002).

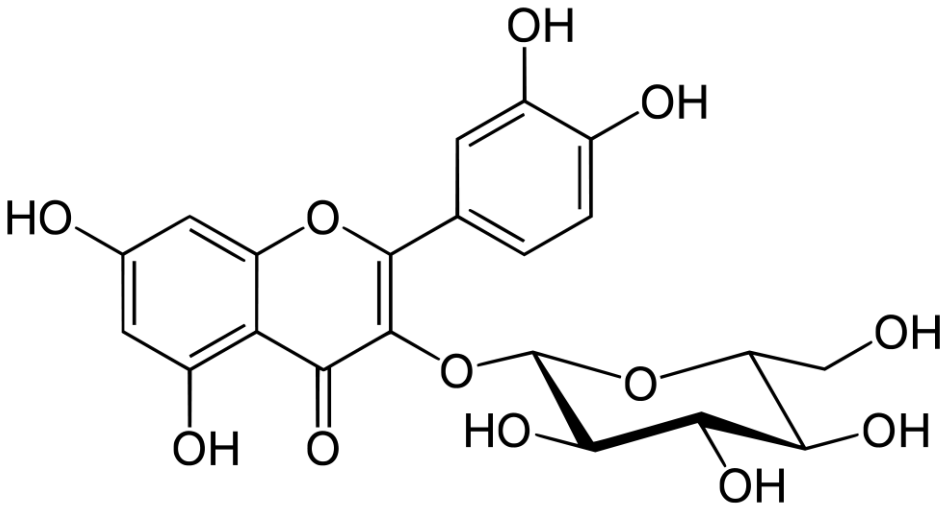

FIGURE 1.3 STRUCTURE OF QUERCETIN-3-GLUCOSIDE

1.6.2 Sources

Q3G exists naturally in apple, onion, broccoli, sophora flower and few other plants as well as herbal medicines, but the concentration is as low as 0.01%. This compound was successfully isolated in some plant species as *Crataegus sp.*, *Argemoneplatyceras*, *Scutiabuxifolia* and *Hyptis fasciculate* (Boligon et al 2009). The amounts of Q3G in different stages of growth as well as different parts of plant are not always the same. Suzuki et al reported the change of Q3G and rutin levels in buckwheat seeds. The seeds were harvested during ripening, and Q3G concentration was measured by HPLC analysis. It clearly increased in the early stages of ripening, reached the maximum at 23 days after pollination (DAP), and decreased rapidly to undetectable at 30 DAP. Rutin level changed following the same trend as Q3G (Suzuki et al 2002).

Then the changes of phenolic compounds of bush butter during ripening were reported by Missing et al. compared to unripe stage, total polyphenol concentration slightly increased to 22.7 mg/g at the preripe stage and decreased to 18.6 mg/g at the soft fruits stage. Q3G content presented in relatively high quantities and increased slightly between the first two stages of ripening and then decreased gradually as ripening progressed (Missing et al 2003).

Several years later, Kalinova et al assessed the Q3G level as well as the quercetin, catechin and myricetin levels within individual plant parts of buckwheat by HPLC analysis (Kalinova et al 2009). Among the compounds tested, Q3G represented the

largest component in buckwheat. Their results indicated that at the flowering stage, Q3G concentration decreased in the following order: flowers > stems > leaves > roots.The level in stems increased gradually during the growing period. And the Q3G level doubled in leaves at the flowering stage compared to the beginning of branching, and continued to increase after flowering. Weather in the given year had an effect on Q3G level. The positive correlations between rutin level and the Q3G level in leaves and roots (0.5053, $p \leq 0.05$ and 0.6674, $p \leq 0.01$, respectively) were also consistent with the previous Suzuki study (Kalinova et al 2009). The above three studies showed consistent results of changes of Q3G during ripening.

Recently Black et al provided evidence of the seasonal variation of fifteen most abundant phenolic contents including Q3G by HPLC in Northern Labrador Tea, *Rhododendron tomentosum* ssp. *Subarcticum*(Black et al 2011). Q3G and quercetin both had relatively low seasonal average concentrations compared to other compounds tested. The seasonal minima were achieved at the end of August as the leaves grew and flowers were produced, and maxima were achieved in September (Black et al 2011).

1.6.3 Synthesis

Q3G can be synthesized by the hydrolysis of rutin.

Biotransformation of rutin to Q3G was conducted in a study by controlling α-L-rhamnosidase and β-D-glucosidase activities from crude enzyme extract of

Aspergillusniger, a food-grade microorganism (You et al 2010). α-L-rhamnosidase activity was most effectively induced by 1% (w/v) rhamnose when compared to other carbon sources. The ratio of α-L-rhamnosidase activity to β-D-glucosidase activity was optimized at pH 6.0 with the buffer containing 15% (v/v) methanol, followed by heat treatment at 70 ℃ for 30 min. After 4 h of biotransformation, 99% of rutin was transformed to Q3G and no quercetin was detected (You et al 2010).

Wang et al then analyzed various acids and enzymes catalyzed rutin hydrolysis (Wang et al 2011). When 2.5% H_3PO_4, 1% HCl and 0.5% H_2SO_4 were used as catalysts under 70 ℃ for 20 hours, transformation yields of Q3G were 9.60, 0.69 and 1.25%, respectively. When hesperidinase, snailase and cellulose-T2440 were used as catalysts, the yields were 43.21, 3.07 and 0.00%, respectively. Quercetin was also produced in all reactions (Wang et al 2011). These results suggested the feasibility of chemical transformation of rutin to Q3G, but the purity and yield is not satisfying when compared to biotransformation.

1.6.4 Bioavailability

Bioavailability is defined as the proportion of the compound that appears in plasma over time when the compound is administered orally. It is expressed as the proportion of an ingested dose that is excreted in urine compared with the proportion excreted in feces over time (Birt et al 2001). The cell uptake and bioavailability of flavonoids

depend on the presence of different substitutions on the carbon atoms of the basic structure (Amira et al 2008). Most flavonoids are present in the diet as glycosides. Conjugation with sugar molecules could stabilize the aglycone and increase its water solubility. Flavonoid glycosides could be converted back to the deglycosylated form and modified during absorption and metabolism (Middleton et al 2000). Q3G is a naturally occurring compound which has a 3-position hydroxyl group on C ring joined by a hemiacetal link to a glucose. Various research results showed contradictory evidences on its absorption inside human body versus quercetin, the aglycone.

In cell model, Caco-2 cells were incubated for 24, 48 and 72 h with Q3G at 70μM. Then the flavonoid content of cells was analyzed by HPLC. A rapid uptake of Q3G by cells after 24 h of incubation was observed. The cellular uptake of Q3G reached a maximum of 0.50 ± 0.20 ng/10^6 cells after 48 h, and disappeared at 72 h (Salucci et al 2002).

The absorption of quercetin aglycone and Q3G were then compared using Caco-2 cells (Boyer et al 2004). The compounds were purified from whole onion and apple peel extracts. The Caco-2 cell monolayers were treated with 100μM Q3G or 50μM quercetin at 37 ℃ for 20, 40, 60 and 90 min in the kinetics design. In the dose-response design, cells were treated with full concentration range from 10 to 100μM of quercetin or Q3G for 40 min. Then the cells were collected, and the cellular extracts were analyzed by HPLC for quercetin and Q3G. Quercetin uptake increased through the 40 nmol doses, while Q3G uptake continued to rise through the maximal

dose of 100 nmol. Peak accumulation of Q3G in cells occurred at 40min after treatment of 100 nmol of Q3G, while unidentified peaks were seen after the treatment of quercetin. Overall, the percentage of pure quercetin absorbed by the cells was higher than Q3G ($p < 0.05$). When the cells are treated with apple peel and shallot extracts that containing quercetin and Q3G, the absorption manner remained the same. The cell took up $45.8 \pm 10.0\%$ of the total quercetin, while only $4.5 \pm 0.4\%$ of Q3G was absorbed. Different from other studies, no quercetin conjugates in cell was detected in this study (Boyer et al 2004).

Interestingly, in human model, Hollman et al observed that 52% of quercetin glucosides from onions was absorbed while only 24% of quercetin was absorbed, which is contradictory to the previous cell model studies. Participants in this study followed a quercetin-free diet for 12 d. On days 4, 8, and 12, three different quercetin-containing breakfasts in random order were provided, including an onion breakfast containing mainly quercetin glucosides, and two other diets containing capsules of quercetin equivalents of 100 mg. Urine and stoma effluent of the participants were collected before breakfast and at the end of the day. Samples were then freeze-dried and extracted before the HPLC analysis. And the authors pointed out that in fact, the 52% absorption might be underestimated because quercetin glucosides need to be released from food and then absorbed, resulting in the loss of amount (Hollman et al 1995). Their findings suggested that quercetin glucosides, rather than the aglycone, is better absorbed in human.

Besides, Cermak et al measured the relative total bioavailability of Q3G in pigs. Pigs were either administered a diet containing 50 mg/kg quercetin or isomolar amounts of Q3G or rutin, or a second diet containing 10 mg/kg quercetin or isomolar amount of Q3G. Jugular and portal blood samples were collected over 24 h and were analyzed by HPLC. The changes in plasma levels of quercetin and its metabolites increase after the administration of quercetin and Q3G, and the proportions of metabolites were similar. But the calculated relative bioavailability of Q3G increased 48% when compared to quercetin aglycone with an oral dose 50 mg/kg, and 67% with a dose of 10 mg/kg (Cermak et al 2003). The absorption mechanism of Q3G remained controversial since the cell model studies and the animal studies showed contradictory results.

1.6.5 Mechanism of Absorption

Two absorption mechanisms were proposed in Q3G absorption. Controversial research results have been obtained.

One possible absorption mechanism of Q3G was investigated by Wolfferam et al (Wolffram et al 2002). They proposed that Q3G was transported by the intestinal sodium-dependent glucose transporter (SGLT1) on the intestine. Pieces of rat jejunum were mounted in Ussing-type chambers and incubated at 37 ℃. Q3G was added to the mucosal or serosal bathing solution, and the samples for HPLC analysis were collected

after 1 and 2 h from both compartments. In the absence of D-glucose from the mucosal medium, Q3G concentration continuously decreased with time. Only 43% and 14 % of the initial concentration were detected after 1 and 2 h, respectively. But the disappearance of Q3G was significantly suppressed by addition of 10 mmol/L D-glucose, leaving 35% of initial concentration in the medium. When the glucose transporter SGLT1 was maximally silenced by omission of Na+ and the specific SGLT1 inhibitor, phloridzin, 54% of the initial Q3G remained in the mucosal compartment after 2 h. Then in order to rule out the possibility of the involvement of sodium-independent fructose transporter GLUT-5, 10 mmol/L D-fructose was added in the sodium free mucosal medium. The disappearance of Q3G was not affected. Therefore, they concluded that Q3G was absorbed via SGLT-1 (Wolffram et al 2002).

Day et al later proposed that the actual quercetin glucoside absorbed through SGLT-1 was quercetin-4'-glucoside (Q4G), rather than Q3G (Day et al 2003). They showed that in their *in vitro* model, Q3G was first hydrolyzed by lactase phlorizin hydrolase (LPH), and then absorbed as quercetin. In their rat everted-jejunal sac model, free quercetin and quercetin conjugates were detected within the tissue at the end of the incubation with Q3G and Q4G. *N-(n*-butyl)-deoxygalactonojirimycin (NB-DGJ), an inhibitor of lactase, inhibited 79% and 83% deglycosylation of Q3G and Q4G respectively. And phlorizin, the SGLT-1 inhibitor, only reduced Q4G hydrolysis. NB-DGJ only inhibited the apical efflux from the enterocyte when Q3G was the substrate, while conversely phlorizin only inhibited the efflux of Q4G metabolites.

Q3G metabolism was significantly reduced (53%, $P < 0.05$), and the concentration of quercetin metabolites in serosal solution was significantly decreased (39%, $P < 0.05$) in the presence of NB-DGJ. Also they found that inhibitor of membrane-bound β-glucosidases significantly inhibited Q3G hydrolysis by 87%. Thus they concluded that Q3G was first hydrolyzed to quercetin by lactase on the membrane of mucosal cells, and then absorbed (Day et al 2003).

1.7 COMPOUNDS RELATED TO Q3G

1.7.1 Quercetin

Quercetin is also a powerful antioxidant that widely distributed in edible plants, including tea, apples, onions and berries. It's not the most predominant flavonoid in diets, but it is the one that has been most widely studies. Its structure is shown in Figure 1.4. Various experimental results showed that quercetin is involved in the regulation of enzymes, carcinogenesis, inflammation, and cardiovascular diseases. Though the absorption and bioavailability of quercetin is usually considered much lower that Q3G, its bioavailability can be influenced by dietary factors (Lesser et al 2004).

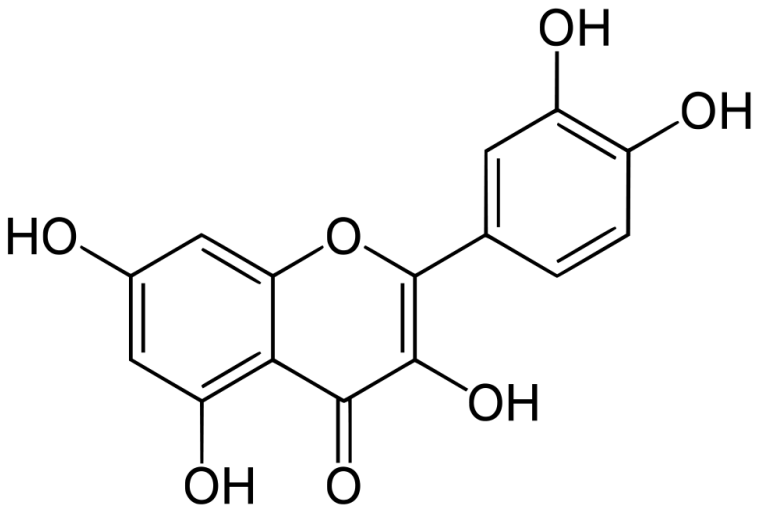

FIGURE 1.4 STRUCTURE OF QUERCETIN

In pig model, the effect of dietary fat content on quercetin absorption was assessed (Lesser et al 2004). Male pigs received either quercetin aglycone or Q3G (30μmol/kg body weight) mixed into a test meal directly before administration. The fat contents of the test meals were 3%, 17% and 32%. Plasma samples were collected after 24 h and then analyzed by HPLC. The peak plasma concentration of quercetin metabolites was detected at 102.9 ±8.0 min after quercetin intake with 3% fat diet. This length was shortened to 70.0 ±8.6 min and 51.4 ±8.0 min by the 17% and 32% fat diet. The elimination of quercetin was clearly delayed by the enriched-fat diets. The significantly higher plasma concentration at 720 min after intake was 0.073 ±0.016μM and 0.070 ± 0.015μM with the 17% and 32% diet respectively compared with 0.015 ±0.015μM with 3% diet ($p < 0.05$). These data suggested that higher fat content can enhance the bioavailability of quercetin. Besides, the early appearance of maximal quercetin plasma concentrations after ingestion might be explained by the formation of quercetin chylomicrons and exported into the peripheral blood via lymph (Lesser et al 2004).

Since quercetin is rapidly metabolized during absorption, the tissue distribution of quercetin is not very clear. One study was conducted to assess the long-term tissue distribution (Boer et al 2005). Rats received 0.1% of 1% quercetin diet for 11 weeks. These diets contained about 50 or 500 mg quercetin/kg body weight. A three-day short-term study was also conducted in pigs receiving 500 mg quercetin/kg body weight. At the end of the study, tissues including kidneys, lungs, spleens, adipose tissues, brains, hearts, and bones as well as blood samples were collected and

homogenized for extraction. All the extraction samples were further analysis by HPLC. The results were corrected for residual blood. In rats, 10-fold increase in the dose of dietary quercetin resulted in a 4-fold increase in plasma and tissue concentrations. The concentration of quercetin was about 30% of the total concentration of quercetin metabolites in most tissues excluding bone, muscle, thymus and adipose tissues. The highest concentrations were found in lungs (1.04 nmol/g tissue), followed by kidneys (0.93 nmol/g tissue) and livers (0.52 nmol/g tissue). In pig, the highest concentration of quercetin was found in liver (3.78 nmol/g tissue) and kidney (1.84 nmol/g tissue) (Boer et al 2005). Based on the biological properties of quercetin that has been well demonstrated, the assumption that quercetin can reduced the risk of lung, kidney, and liver cancers was brought up. The assumption is partly proved by the research results that quercetin regulates proliferation and apoptosis in corresponding cell lines.

1.7.2 Enzymatically modified Isoquercitrin

Apart from pure Q3G, enzymatically modified isoquercitrin (EMIQ), a mixture of quercetin monoglucoside and its α-oligoglucosides, has drawn much attention these years (Nielsen et al 2006, Yamada et al 2006). It mainly consists of Q3G and its α-glucosyl derivatives with 1-7 of additional linear glucose moieties (Figure 1.5). It has been recognized as safe by the Food and Drug Administration (Akiyama et al 2000, Emura et al 2007).

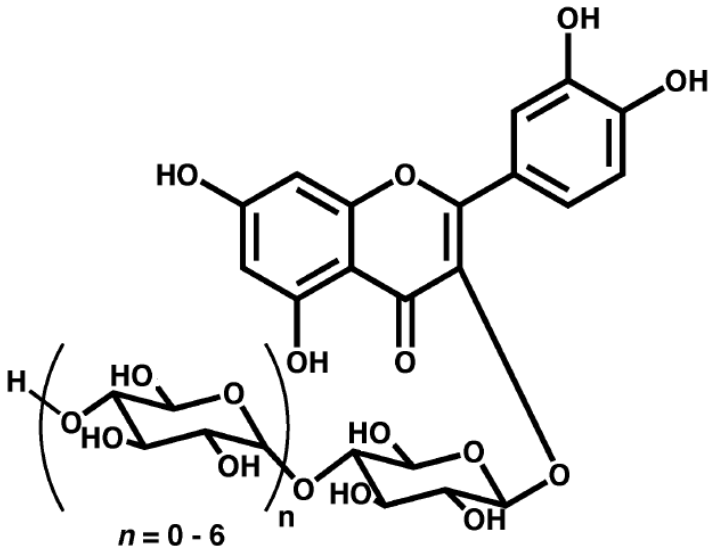

FIGURE 1.5 STRUCTURE OF ENZYMATICALLY MODIFIED ISOQUERCITRIN

Several studies show that EMIQ is absorbed into human body and contributes to human health.

Makino et al first showed that quercetin glucosides were absorbed differently in rat model (Makino et al 2009). Quercetin, rutin, Q3G and EMIQ dissolved in water were orally administered to rats under anesthesia. Bioavailability was calculated from the concentrations of total quercetin in plasma from 0 to 12 h after the administration. A rapid increase in plasma concentrations of quercetin and quercetin metabolites were observed after oral administration of EMIQ, and reached maxima in 15 min. Similar change was found after oral administration of Q3G, but the plasma level was about 30%. Only a trace level of plasma quercetin was found after administration of quercetin and rutin. Enzymatic hydrolysis of rat intestinal epithelium crude extract showed that EMIQ and Q3G were rapidly converted to quercetin during incubation, while no conversion of rutin was observed, suggesting the higher bioavailability of Q3G and EMIQ (Makino et al 2009).

Murota et al tested in human if the introduction of α-oligoglucosides to the sugar moiety could enhance the bioavailability of quercetin glucosides using EMIQ as model reagent (Murota et al 2010). Healthy male and female volunteers aged 21 to 57 years were recruited. All participants were given a flavonoid-free, low-fat meal containing equivalent to 2 mg quercetin aglycone/kg body weight with 200 mL of water. Blood samples were obtained 0.5 h before and 0.5, 1.5, 3, and 6 h after food consumption. Plasma concentrations of quercetin metabolites 1.5 h after the intake of quercetin

aglycone, Q3G, Q3,4'diG and EMIQ were determined by HPLC analysis and the results were compared. Results showed that Q3G exhibited a weak lipophilicity, while EMIQ is more hydrophilic. After Q3G and EMIQ consumption, plasma quercetin conjugates increased simultaneously with a small increase in isorhamnetin conjugates, and the profile of plasma metabolites were nearly identical, indicating the addition of a α-oligoglycosyl chain into the sugar moiety of Q3G did not change the intestinal metabolism of quercetin. The poor absorption of quercetin aglycone was also consistent with the Makino study, which might be attributed to its high hydrophobicity (Murota et al 2010). These data suggested that EMIQ can be used as a substitute of Q3G with even better bioavailability in body.

1.8 GENERAL RELATIONSHIP WITH DISEASES

Epidemiology and animal studies consistently showed that Q3G exhibits various health benefits including anti-inflammation, anti-cancer, reducing oxidative stress and reducing incidence of chronic diseases.

1.8.1 Animal Studies – EMIQ, Q3G and Tumor

There are several animal studies showing Q3G and its effect on inhibiting tumor formation and growth in rats.

Yokohira et al tested the effect of EMIQ and Q3G on liver carcinogenesis. Male rats

were first given a single intraperitoneal injection of N-diethylnitrosamine (DEN, 200 mg/kg body weight), and then fed with diets containing 1.0, 0.1, 0.01 or 0% EMIQ or Q3G starting on week 2. All rats were subjected to two-thirds partial hepatectomy at week 3. At week 8, rats were sacrificed and livers were excised and fixed in 10% neutral buffered formalin. Then immunohistochemical staining for GST-P was performed. Rat serum was analyzed for potential antioxidant power by measuring Cu+-reduction. Results showed that EMIQ or IQ treated groups did not change the number or area of GST-P positive foci compared with the DEN alone group. But higher doses of Q3G or EMIQ *in vivo* (1, 0.1, 0.01%) were correlated with smaller numbers of GST-P positive foci ($p = 0.002$ and $p = 0.049$, respectively). 1% solutions of EMIQ or IQ exhibited high antioxidant power (80953 and 92980μmol/L, respectively). The antioxidant power of rat serum after the treatment was also significantly increased compared to the basal diet (1388 ± 118 and 1387 ± 82μmol/L compared to 883 ± 114μmol/L) (Yokohira et al 2008).

Later Shimada et al investigated again the effect of EMIQ on liver preneoplastic lesions (Shimada et al 2010). Male rats received a single injection of DEN at the concentration of 200 mg/kg body weight. Three diets were provided to rats: basal diet, diet with 0.5% β-naphthoflavone (BNF), diet with 0.5% BNF and water with 0.2% EMIQ. All rats were subjected to two-thirds partial hepatecomy at week 3. After the 6-week treatment, livers were excised and weighed. Then liver samples were fixed and stained with hematoxylin and eosin or elastic-van Gieson (EVG) method for

histopathological analysis. GST-P staining and COX-2 staining were used in immunohistochemical analysis. The number of COX2-positive cells in DEN-BNF group significantly increased compared to DEN group, but EMIQ group suppressed this number by 36% ($p < 0.01$). The area of EVG-positive connective tissue fibers was also reduced by 84% in EMIQ treated group compared with DEN-BNF group. The area and the number of GST-P positive foci increased significantly in the BNF treated group compared with the DEN-alone group. But the co-treatment of EMIQ in water significantly suppressed the area and the number of GST-P positive foci compared to BNF treated group (61 and 47%, $p < 0.01$ and $p < 0.05$, respectively). The inconsistency of the results with Yokohira study might be caused by high solubility of EMIQ in water (Shimada et al 2010).

Nishimura et al used the same model again with a different tumor promoter, oxfendazole (OX) (Nishimura et al 2010). Rats were administered with a single dose of DEN (200 mg/kg body weight). 2 weeks later, rats were fed with different diets: control diet (containing 500 ppm OX), DEN-alone diet, DEN-OX diet, DEN-OX-EMIQ diet (2000 ppm EMIQ in water), and DEN-OX-MLT diet (100 ppm melatonin in water). Rats were subjected to two-thirds of hepatectomy at week 3. Livers were excised and fixed at the end of study. Hematoxylin and eosin staining for histopathological analysis and GST-P staining for immunohistochemical analysis were performed to evaluate hepatocellular preneoplastic lesions. The area of GST-P positive foci in DEN-OX group increased, and the number of GST-P positive foci significantly

increased compared with DEN-alone group ($p < 0.05$). Combined EMIQ administration reduced the area, and significantly reduced the number when compared with DEN-OX group ($p < 0.01$) (Nishimura et al 2010).

Morita et al used a third tumor initiator, phenobarbital (PB), in their study. A single injection of 200 mg DEN/kg body weight was administered to male rats. Diets containing 500 ppm PB with or without EMIQ (2000 ppm) were provided. One week after PB administration, rats were subjected to a two-thirds partial hepatctomy. Hematoxylin and eosin staining for histopathology analysis, GST-P staining, PCNA staining and CAR staining for immunohistochemical analysis were performed. RT-PCR was used to assess the RNA content. PB-EMIQ co-treatment significantly attenuates PCNA positive ratio as well as the number and area of GST-P positive foci compared with PB-alone treatment ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively). Transcriptional expressions of metabolizing enzymes, cell growth-related genes were significantly altered in EMIQ treated group. The ROS production was stimulated in both the DEN-PB and PB-EMIQ groups without significant difference (Morita et al 2011).

In 2011, Kuwata et al confirmed these finding with same results (Kuwata et al 2011). Male rats received an injection of 200mg DEN/kg body weight, and then fed with either diets containing 0.5% BNF with or without 0.2% EMIQ in water, or a basal diet. After 3 weeks of DEN-initiation, rats were subjected to a two-thirds partial hepatectomy. Samples were collected at the end of the 8-week trial. GST-P staining,

additional immunohistochemistry stain for HO-1, PCNA and TRAADD and TUNEL assay were performed. Co-treatment of BNF and EMIQ exhibited significantly decreased number and area of GST-P positive foci compared to BNF alone group (47 and 61% respectively). The numbers of GST-P+ and HO-1+ single cells were also significantly reduced by EMIQ. Both PCNA+ proliferating and TUNEL+ apoptotic liver cells in EMIQ treated group returned to the level of DEN-alone group. Genes for antioxidant enzymes were stimulated by EMIQ. Genes associated with inflammation, apoptosis and cell proliferation were also altered by EMIQ compared with the DEN-alone group (Kuwata et al 2011). These studies showed additional insight into the possible mechanism of action of EMIQ on liver-tumor promotion. And all these animal studies consistently showed that EMIQ can significantly inhibit the tumor generation.

1.8.2 Clinical Trials – Q3G and Common Diseases

A few clinical trials also indicated a relationship between Q3G and diseases.

Herbal medicine red vine leaf extract (RVLE, pharmaceutical extract code AS195) containing large amount Q3G was assessed for its effect on chronic venous insufficiency (Kiesewetter et al 2000). Kiesewetter et al conducted a 12-week, randomized, double-blinded, placebo-controlled, parallel-group, multi-center trial. Male and female patients with stage I and II chronic venous insufficiency (CVI) were

recruited. They were randomly assigned to treatments with placebo, 360 mg AS195 or 720 mg AS195 once daily for 12 weeks with 2-week washout period, respectively. Out of 219 patients that completed the trial, lower leg volume of the placebo group increase by 15.2 ± 90.1 g (water mass, expressed in mean ± SD) and 33.7 ± 96.1 g in week 6 and week 12. Patients receiving 360 mg AS195 and 720 mg AS195 exhibited a clearly reduction in mean lower leg volume of -75.9 g (95% CI: -106.1 to -45.8 g) and -99.9 g (95% CI: -130.3 to -69.6 g) compared with the placebo group, respectively. Calf circumference showed a similar trend. A clear reduction was observed in both treatment groups: -1.40 to -0.56 cm and -1.73 to -0.88 cm for 360 and 720 mg AS 195, respectively, while the placebo group remained no change. All of these differences were statistically significant with a p value less than 0.001. Ankle circumference reduction and improvement of key CVI-related symptoms were also observed in treated groups. No adverse effects different than placebo group were observed. They concluded that AS195 with a daily dose of 360 and 720 mg were confirmed to be safe and effective in the treatment of mild CVI (Kiesewetter et al 2000).

Later in 2003, another 6-week observational trial on AS195 was conducted (Schaefer et al 2003). Schaefer et al reported the tolerability and efficacy of AS195. Participants aged 25-82 years with stage I or II CVI were recruited and were provided with film-coated tablets containing 360 mg AS195 daily for 42 days. All the subjective symptoms of CVI, including tired, heavy legs, sensation of tension in legs, tingling sensations, pain in the leg, were statistically significantly improved in all groups. The

efficacy and tolerability were rated good or satisfactory in both patients and investigators. Therefore, they concluded that AS 195 film-coated tablets can be considered as effective and safe in patients with CVI grade I or II. These two trials provided possibility of Q3G as an effective constituent in herbal medicine treating mild CVI (Schaefer et al 2003).

The most recent clinical trial on AS195 was conducted in 2010 by Rabe et al. It was a multicenter, randomized, double-blinded and placebo-controlled study (Rabe et al 2010). CVI patients with CEAP grades 3-4a and moderate-to-severe clinical symptoms were recruited. They were given 720 mg AS195 per day for 12 weeks. Changes in limb volume were determined by water displacement volumetry, clinical CVI symptoms and global efficacy evaluations. Among the 248 patients that completed the study, the significant difference of leg oedema between AS195 and placebo groups reached -17.0 ±8.6 mL (95% CI -34.1-0.0, p = 0.05) at Day 42. The continuous linear improvement of leg oedema over time was observed in AS195 group. Center effects and treatment by center interactions and calf circumference did not show significant difference between the two groups. Subjective CVI symptoms improved in both treatments, but AS195 was more effective. The symptoms of tired, heavy legs and pain in the legs were increased approximately linearly over time. The largest difference was observed in the symptom of sensation of tension in the legs on Day 42 (p = 0.031) and on Day 84 (p = 0.047). The investigators assessed efficacy as good or satisfactory in 71% of AS195 patients compare to 54% in placebo group. Patient ratings were similar (70% in AS195

and 59% in placebo) (Rabe et al 2010). These results suggested that AS195 was beneficial for a wider range of CVI grades.

Apart from Q3G, Quercetin was also linked to various diseases. It is shown that quercetin can reduce systolic blood pressure and plasma oxidized LDL concentrations in overweight subjects. This was a double-blinded, placebo-controlled cross-over study. Overweight or obese subjects aged 25-65 years with metabolic syndrome traits were selected. They were then randomized to 150 mg quercetin/d in the 6-week treatment periods. Fasting venous blood samples were collected at the first and last day. Clinical safety parameters and haematological parameters were determined. Serum quercetins, lipid parameters, glucose, uric acid, TNF-α, C-reactive protein, LDL, and antioxidant capacity were measured. Fasting plasma quercetin concentration increased from 71 to 269 nmol ($p < 0.001$) during treatment. Systolic blood pressure was reduced by 2.6 mmHg ($p < 0.01$) in entire treated group. HDL-cholesterol and oxidized LDL concentrations were also decreased ($p < 0.001$). No TNF-α or C-reactive protein levels were affected in treated group. Also, blood parameters and haematology parameters indicating liver and kidney function did not reveal any adverse effects (Egert et al 2009). Their findings support the point of view that quercetin might provide protection against CVD.

One prospective randomized, double-blind, placebo-controlled trial showed that quercetin can improve prostatitis status in patients (Shoskes et al 1999). 30 men with category IIIa and IIIb chronic pelvic pain syndrome were recruited and randomized to

placebo or 500 mg quercetin twice daily for 1 month. In a follow-up unblinded, open-label study, 17 additional men were given quercetin supplements as well as bromelain and papain (to enhance quercetin absorption) for a month. The NIH chronic prostatitis symptom score was used to assess symptoms and the quality of life. In the 28 patient who completed the study, an significant improvement of 21.0 to 13.1 in NIH symptom score was observed in quercetin group ($p = 0.003$). 67% of patients in the treated group had at least 25% improvement compared to 20% in placebo group ($p = 0.001$), while in the open-label study, 82% had at least 25% improvement in symptom score. Thus the conclusion is drawn that quercetin can provide significant symptomatic improvements (Shoskes et al 1999).

Another trial assessed the effect of EMIQ on Japanese Cedar Pollinosis(Kawai et al 2009). It is an allergic disease, and about 24-28% of the population is thought to be suffering from this disease. Food additive EMIQ with the highest water solubility among all quercetin derivatives was assessed for effect in the trial. In this randomized, double-blinded, parallel-group, placebo-controlled trial, subjects with the disease were recruited and give two capsules daily of 100 mg EMIQ or placebo for 8 weeks during the pollen season. Symptoms and activities of daily living (ADL) scores and quality of life (QOL) score were recorded. Blood sample collected before and after the study was used to measure serum cytokins, chemokines, IgE, quercetin and oxidized biomarkers. Total symptom score continuously decreased during the whole period, and there was significant therapeutic effect of EMIQ in week 4-5 when a marked increase of the

cypress pollen count was observed ($p < 0.05$). Total ocular score and ocular itching score for the treated group were significantly lower ($p < 0.05$) compared to placebo group. Other scores such as total nasal score ($p = 0.06$), nasal obstruction score ($p = 0.08$), lacrimation score ($p = 0.06$), ocular congestion score ($p = 0.08$) and ADL score ($p = 0.08$) that measured during individual period were also tend to be lower. Oxidized LDL was also significantly decreased in EMIQ group. They concluded that EMIQ was safe and influenced ocular symptoms caused by pollinosis. Since EMIQ was generally recognized as safe and has been approved for use as food additive, this provided the possibility of reducing disease symptoms by food additives (Kawai et al 2009).

1.8.3 Animal Studies -Quercetin and Breast cancer

It is confirmed in one study that dietary quercetin decrease tumor latency in rats(Singh et al 2010). Female ACI rats were obtained at 4 weeks of age. After 1-week acclimatization period, rats were randomly divided into two groups. The E2 + quercetin group was implanted with E2 pellets and fed quercetin enriched AIN76A diet. The control group was implanted with cholesterol pellets and fed with the same diet. Quercetin treatment began 7 days prior to pellet implantation. The length of the study was 240 days and animals were monitored for tumor development weekly. At the end of the study, rats were sacrificed and tissues were collected and analyzed. Tumor incidence and number of tumor nodules were counted and evaluated by pathologists.

Serum samples were obtained for quercetin analysis. Western blotting was used for PCNA analysis. Results showed that ductal carcinoma in situ and microinvasive cancers were present in animals from quercetin + E2 experimental group. The breast tissue was similar in both groups. PCNA levels did not show significantly change, either. However, PCNA expression was significantly induced in both treated breasts compare to control breast. Serum quercetin levels were 6.24 ±0.78μM and 6.68 ± 0.70μM in quercetin + E2 treated group and quercetin group, respectively. Tumor incidence in quercetin + E2 treated group was 100% compared to 82% in the E2 group, but the difference was not statistically significant. These results showed that breast tumors appeared earlier in quercetin + E2 co-treated group compared to quercetin-alone group (Singh et al 2010). This might be explained by the fact that quercetin can induce cell proliferation at low concentrations. Thus the breast cancer cells were promoted, and the latency of tumor was reduced.

In another animal study, rejection of established breast cancer in mice was induced by the co-treatment of quercetin and doxorubicin (Du et al 2010). Female BALB/c and athymic nude mice were obtained. Marine breast cancer cell line 4T1 was cultured and grown in syngeneic BALB/c mice where it metastasized to lungs. After the injection of 4T1 cells into second mammary fat pad and the formation of tumors with 1 cm diameter, mice were randomly assigned to control group, intratumoral doxorubicin injection group, intravenous doxorubicin injection group, quercetin group, co-treatment group. T cells were purified and assessed for intracellular cytokine and

apoptosis. The median survival time in dietary quercetin group was 66 days (95% CI 60-72days), and significantly longer than control group (48 days, 95% CI 45-51 days, $p < 0.01$). The quercetin-alone or doxorubicin-alone treatment did not show changes in the size of tumor, but the co-treatment significantly reduced the initial tumor volumes and led to tumor-free survivals. Serum cytokines including IFN-γ, IL-2, IL-4 and IL-10 were suppressed in the co-treatment group. T cells apoptosis was increased as well. Histopathology examinations showed that more necroric cells existed in combinational treatment group, and no metasatic spread was found. These data indicated a promoted immune system by the co-treatment and quercetin and intratumoral doxorubincin. The two compounds might have synergic effect (Du et al 2010). These two animal studies showed that quercetin as a dietary supplement can function against or promote tumor in rat model. The real effect largely depends on the concentration and the co-treatment of other agents.

1.8.4 Epidemiology Studies – Quercetin and Cancer

In a population-based case-control study in New Jersey, phytoestrogen and its effect on endometrial cancer risk was assessed (Bandera et al 2009). 424 cases and 398 controls were identified. Women with a hysterectomy record were excluded. They completed interviews as well as the Block 98.2 food frequency questionnaire. The individual phytoestrogen intake was calculated by the supplemental questions for

phytoestrogen foods. Risk estimates were derived using an unconditional logistic regression. Results shows that cases tended to consumer lower quantities of phytoestrogens than the controls. A reduction in endometrial cancer risk with quercetin was observed. The adjusted OR was 0.65 (95% CI 0.41-1.01, p = 0.02) in the highest quartile of consumption. The relationship retained even after controlling for total fat and fiber consumption with an OR of 0.63 (95% CI 0.4-0.99) for the highest versus the lowest quartile of consumption. This study showed an inverse association between endometrial cancer and quercetin intake. But the association between total phytoestrogen intake and endometrial cancer risk was not supported by the evidence (Bandera et al 2009).

The effect of quercetin intake on risk of cancer was also part of one prospective cohort study that conducted in middle-aged and older women. The Women's Health Study (WHS) was a randomized, double-blind, placebo-controlled trial that designed for the risks and benefits of aspirin and vitamin E. This subgroup analysis identified the women who completed a 131-item validated semiquantitative food frequency questionnaire (SFFQ). Women with implausible energy intake (less than 600 or more than 3500 kcal/day), or diagnosed with cardiovascular disease or cancers were excluded. The median intake of total flavonoids ranged from 8.88 mg/day in the lowest quintile to 47.44 mg/day in the highest quintile. Quercetin was the major contributor among the 5 individual flavonoids recorded. Median intake ranged from 6.49 to 32.79 mg/day. The multivariate RRs and 95% CIs of total invasive cancer for quercetin from

the lowest quintile to the highest was 1.00 (reference), 0.92 (0.92-1.10), 0.96 (0.85-1.08), 0.94 (0.83-1.06), 0.94 (0.83-1.07). A slightly reduced risk was observed in all quintiles, but not statistically significant (Wang et al 2009). The imperfection of this study includes the lack of use in biomarkers, less accurate flavonoid intake assessment, bioavailability assessment. These were resulted from the original study design. More studies need to be conducted to further investigate the topic.

The specific risk of breast cancer was determined in people with high dietary flavonols and flavonol-rich foods (Adebamovo et al 2005). The study population was identified in the Nurses Health Study II (NHS II), which was designed to investigate the associations between lifestyle factors and diseases incidence. Follow-up with the first dietary assessment of this cohort was conducted. The exclusion criteria included postmenopausal, implausible values of total energy intake, less than half-completed semiquantitative food frequency questionnaire, or previously diagnosed cancer except nonmelanoma skin cancer. Calculated median quercetin intake ranged from 5.3 mg/day to 30.1 mg/day. The multivariable adjusted RR for breast cancer in relation to quercetin intake was then calculated. The RRs and 95% CIs from lowest quintile to the highest quintile were 1.00 (reference), 1.08 (0.85-1.37), 1.02 (0.30-1.30), 1.08 (0.85-1.37), 1.05 (0.83-1.33). In this subgroup analysis, no association between breast cancer risk and quercetin intake was found (Adebamovo et al 2005). In fact, a slightly increase relative risk was observed in groups consuming more quercetin than baseline intake, though it was not statistically significant. But the author admitted the possibility of

chance findings. So more information is in need on this topic.

The true relationship remained unclear when putting these epidemiology and animal studies results together. But the effect of quercetin on cancer is well demonstrated.

1.9 FUNCTION AGAINST OXIDATIVE STRESS

There have been consistent research results showing Q3G exhibits ability to reduce oxidative stress in various cell lines. Free radicals exist in endogenous systems in human body. They are mainly generated from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS) (Darley-Usmar et al 1996). Physicochemical conditions, pathophysiological status and environmental stress could lead to imbalanced oxidants production and cumulated oxidative stress, thus results in DNA damage, lipid peroxidation and protein damage. Q3G can function as an antioxidant to scavenge free radicals, thus relieves the oxidative stress in cells (Ioku et al 1995). Some research results consistently showed this effect.

Q3G can protect fibrosarcoma HT1080 cell against oxidative stress (Kong et al 2008). Key protein involved in the process was matrix metalloproteinase (MMP), enzymes in extracellular matrix degradation and involved in tumor initiation and growth, invasion, angiogenesis, and metastasis. Studies showed that oxidative stress can activate MMPs. Kong et al demonstrated that MMP activity was inhibited by Q3G

in cells treated by H_2O_2 (Kong et al 2008). Hydroxyl radical scavenging activity of Q3G was analyzed by electron spin resonance spectroscopy. Cell viability and cytotoxicity were determined by MTT assay. MMP activity was stimulated by phorbol 12-myristate 13-acetate (PMA), then the inhibitory effect of Q3G on MMP activity was measured by gelatin zymography. Gelatins hydrolyzed by MMPs were visualized and the intensities of the bands were estimated. No toxicity was observed on the cells treated with up to 10μM Q3G. Q3G resulted in a clearly increased scavenging activity ($p < 0.05$), and 94% of hydroxyl radical production was inhibited at the concentration of 10μM compared with control cells. MMP-2 and MMP-9 expressions were dose-dependently inhibited in cells co-treated with Q3G and PMA compared with PMA-alone group. These results suggested that Q3G is a potential chemopreventive agent for cancer (Kong et al 2008).

Q3G can protect neuroblastoma SH-SY5Y, embruonic kidney HEK293 and breast cancer MCF-7 cells against oxidative stress (Soungdararajan et al 2008). Soundararajan et al performed MTT assay, TUNEL assay and ELISA to assess cell viability and apoptotic death. In addition, ROS production was measured by fluorescence to determine oxidative stress in cell. cDNA microarray and quantitative RT-PCR were used to determine gene expressions. Lipid peroxidation and cholesterol assay were used to assess lipid status. Results showed that 10μM Q3G treatment increased cell viability by 55% compared to H_2O_2 treated cells. TUNEL-positive cells were significantly decreased by Q3G treatment. LPH enzyme release reduced 66%,

indicating the improvement of membrane integrity. Lipid peroxidation reduced by 4-fold compared with H_2O_2 treated cells. Genes associated with lipid pathways were modulated by Q3G, and *de novo* cholesterol synthesis was enhanced. The mRNA level of sterol regulatory element-binding protein 2 (SREBP-2) increased 2 times, and the increased transcriptionally inactive SREBP-2 cleavage was also confirmed by western blotting. Therefore they concluded that protection in SH-SY5Y is via inducing SREBP-2 to maintain membrane integrity. But the same activation was not observed in other two cell lines (Soungdararajan et al 2008).

Oxidative stress in transformed cell line can be reduced by Q3G as well. Jung et al demonstrated that Q3G attenuated the oxidative stress in RGC-5 retinal ganglion cells. Cells were exposed to H_2O_2 with or without Q3G. Cell viability and apoptosis were then determined. ROS production was assessed using DHE staining. Total intracellular glutathione and lipid peroxidation were also measured. Results showed that viability of H_2O_2 treated cells increased from 63% to 83% and 90% when co-treated with 10 and 50μM of Q3G, respectively. In addition, western blotting results showed that Q3G significantly reduced the up-regulation of cleaved PARP and p53, and blunted the decrease of Gpx-1, Bcl-2 and catalase proteins, suggesting the suppression of apoptosis. The production of ROS was reduced by Q3G in a dose-dependent manner, and a significant reduction was seen at the concentration of 10μM ($P < 0.001$). GSH depletion was reversed by 10μM Q3G ($p < 0.01$). This study showed the beneficial effect of Q3G on diseases like glaucoma, in which oxidative stress is thought to play a

major role in the cause of visual loss (Jung et al 2010).

1.10 Q3G, QUERCETIN AND BREAST CANCER

Various pathways are involved in cancer formation and progression. Most of them affect the normal cell cycle and suppress the cell apoptosis. Key factors in cancer prognosis and treatment are the activation or inactivation of cellular regulatory proteins. Thus phytochemicals that can target these proteins are considered to be possible chemotherapy agents. Q3G and quercetin are potential chemopreventive compounds that can affect cell viability and induce apoptosis in breast cancer cells. The mechanisms have been studied for the past few years, and several pathways and key proteins have been identified.

1.10.1 Regulation on Estrogen Receptors

Two types of breast cancer cells are widely used in research: the estrogen-receptor-negative cell lines including HCC-38 and MDA, and estrogen-receptor-positive cell lines including MCF-7 and T47D (Maggiolini et al 2001, Woude et al 2005).

The biphasic modulation of cell proliferation by quercetin was observed in several cell lines including MCF-7 (Woude et al 2003). Cell viability and proliferation were

assessed in cells exposed to quercetin by LDH-leakage method, ELISA and BrdU kit. The inhibitory effect on cell proliferation was only observed at a relatively high concentration of quercetin, 30μM for HCT-116 and 80μM for HT29 cells. When cells were exposed to a relatively low concentration of quercetin, a significant increase in cell proliferation of up to 20% for colon cell lines was observed, while this number increased to 100% for MCF-7. These results suggested that quercetin regulates cell proliferation in a biphasic manner (Woude et al 2003).

Regulation of quercetin on estrogen receptor α in MCF-7 cells was studied. Proliferation assay was used to assess the effect of quercetin on regulating cells (Maggiolini et al 2001). Ligand binding assay for ERs, evaluation of gene expression and immunoblotting against ERα were performed in the study. The biphasic effect of quercetin on proliferation was observed and consistent with previous study. Endogenous ERα was activated by 10-fold in MCF7SH cells. The maximal stimulation was achieved at 1um. Quercetin also served as the agonists of the C-terminal HBD-associated AF2 in ERα and the mutated AF2 was not responding to quercetin. ERα and ERβ were trans-activated by quercetin. ERα mRNA levels and ERα protein content were down-regulated by quercetin in a dose-dependent manner. Their results suggested that quercetin was the ligand of ERα, thus showed the possibility of regulating cell proliferation through ER (Maggiolini et al 2001).

Woude et al conducted the study on estrogen receptor as well (Woude et al 2005). ER-positive MCF-7 and T47D cells, and ER-negative HCC-38 and MDA-MB231 cells

were used. Cell proliferation was measured by ELISA, BrdU kit after 24 h treatment. The reporter gene assay was conducted in U2-OS cells transfected with ERα or ERβ. ER-ERE-mediated ERα and ERβ expressions remained no change, and then increased rapidly at concentrations higher than 10 μM. The maximum induction of ERα reached 1.7 times higher than the induction of 17β-estradiol (E2), the commonly used ligand for ER, while the maximum induction of ERβ reached 4.5 times higher. Other results showed that at lower concentration, quercetin simulated proliferation of ER-positive cells only. Both T47D and MCF-7 cells proliferation was enhanced approximately 50%. Inhibition occurred at concentrations higher than 45 or 55μM for the two cell lines, respectively. They suggested the effect to be ER-dependent (Woude et al 2005).

Then the effect of quercetin specifically on ERβ in breast cancer cells was studied (Cappelletti et al 2006). Contradictory results were obtained. Hormone-sensitive T47D cell line and hormone-insensitive BT20 cell line were used in this study. Total RNA was extracted for RT-PCR in the analysis of mRNA expression. T47D cell line expressed a 2.5 fold higher levels of total ERβ compared with BT20 cells. The β2 isoforms accounted for up to 50% of the total ERβ in T47D cells and β5 isoforms accounted to up to 84% in BT20 cells. T47D cells were exposed to 1, 5 and 10μMquercetin. A slight increase in total ERβ levels was observed. The β2 isoform was up-regulated by 52% ($p < 0.05$) compared with control. No change in total ERβ or isoform mRNA was observed in BT20 cell line. At the highest concentration, ERα levels were slightly lowered by quercetin in T47D cell line but remained same in BT20

cells (Cappelletti et al 2006). These results suggested that ERβ and ERβ mRNA isoforms might be regulated by natural hormone or by weaker estrogen agonists like genistein. Quercetin might active ER by another mechanism.

The hypothesis that the ERα/ERβ ratio might be affecting T47D breast cancer cell proliferation was brought up and then tested. T47D cells were cultured and cell proliferation was measured (Sotoca et al 2008). The ERα and ERβ specific U2OS reporter gene assay was performed. Western blotting was used to quantitatively analysis of protein contents. Quercetin EC_{50} values of binding to ERα-Luc and ERβ-Luc in U2OS cell lines were 6.5μM and 9μM respectively. But the maximal ERE-Luc activity increased to 166% and 598% when compared with E2 induced cells. The maximal proliferation level induced by quercetin was achieved at 1μM, and then continued to drop at concentrations up to 100μM. T47D were then transfected with ERβ and an Enhanced Green Fluorescence Protein (EGFP) gene as a co-expressed reporter. The ERβ expression in transfected cells can be regulated by tetracycline, thus created different ERα/ERβ ratios. Quercetin stimulated transfected cell proliferation within the concentration range from 5 to 50μM, and then proliferation dropped to values that were lower than control cells. In the presence of 50% and 100% of the maximal expression of ERβ, quercetin induced full inhibition of cell proliferation at all concentrations tested. These results showed that ERα/ERβ ratio can affect the inhibitory effect of quercetin on proliferation of T47D cells (Sotocaet al 2008).

Quercetin might be able to function through the type-II estrogen-binding site

(type-II EBS)(Scambia et al 1993). It has drawn much attention in studying the estrogen receptors involved in breast cancer cell regulation. The effect of quercetin of type-II EBS was first investigated in 1993. In this study, both MDA-MB231 and MCF-7 cell lines were cultured and exposed to quercetin. Specific binding of type-II EBS was measured by the binding of radioactive [^{3}H]-estradiol and liquid scintillation spectrometer. The increase of type-II EBS was dose dependent in both cells lines, and reached the maximal value at 10μMquercetin. The induction is significantly different from untreated cells ($P< 0.05$). The induction was correlated with the increased sensitivity of cells to the inhibitory effect of low quercetin concentrations. Therefore the conclusion was drawn that quercetin might regulated both cell line through type-II EBS rather than ER (Scambia et al 1993).

The downstream regulation of ERs has been widely studied. The induced activation of ERKs and JNKs in breast carcinoma cells was observed in cells treated with IGF-I and 17β-estradiol (E2), and led to cell proliferation. Quercetin showed effective inhibitory activities against E2/IGF-I induced proliferation. In this study, DNA synthesis indicating proliferation was measured by [3H]-thymidine incorporation. Western blot analysis was used to detect the protein levels. Intracellular ROS production was monitored by flow cytometry using an oxidant-sensitive DCHF-DA probe. Increasing cell numbers and [3H]-thymidine intensity in DNA in MCF-7 cells indicated a significantly induced proliferation by E2. When ER was inhibited, the induction was suppressed, suggesting E2 induced proliferation by activation of ER.

The phosphorylated ERα level increased in E2/IGF-I treated cells. No change in phosphorylated p38 was observed. E2 alone slightly induced ERKs, and the IFG-I addition significantly stimulated ERKS, IRS-I and JNKs. The expression of c-Jun protein, downstream of ERK and JNK was also induced. Incubation of MCF-7 cells with ERK or JNK inhibitor significantly reduced the proliferation. Results also showed that ROS production was simulated by E2/IGF-I. Pre-incubation with antioxidants inhibited the E2/IGF-I induced proteins, suggesting E2/IGF-I-induced proliferation might be initiated by ROS production. In quercetin treated cells, E2/IGF-I induced proliferation was inhibited, and c-Jun expression and peroxide production was also inhibited (Lin et al 2007). These results showed that cell proliferation can be induced through estrogen receptor and insulin-like growth factor receptor. The downstream MAPKs (ERKs and JNKs) were then stimulated and resulted in cell proliferation in MCF-7 cells. Quercetin can sufficiently inhibit this pathway.

1.10.2 Inducing Apoptosis Through p53 Dependent Pathway

Protein p53 has been widely studied for the past decades. It is proved to be involved in the regulation of apoptosis and proliferation in various cancer cell lines. The regulation of p53 in human breast cancer cell lines was first investigated in MDA-MB-468 cells in 1994(Avila et al 1994). Cell cycle was analyzed by flow cytometry. mRNA level measurements, immunoblotting and immunoprecipitation were

performed to assess the effect of quercetin on p53 expression. A dose-dependent inhibition on cell growth was observed in quercetin treated cells. Cell viability decreased to 62, 38, 27 and 20% when exposed to 5, 10, 15 and 30 μg/ml quercetin, respectively. G2/M phase cell cycle arrest was observed in cells treated with 7 or 30 μg/ml of quercetin. G2/M cells represented 46% and 61% of the total population at day 6 respectively. Level of p53 decreased both dose and time dependently when exposed to quercetin. 75 μg/ml quercetin inhibited p53 level to nearly undetectable. Changes in p53 levels were detected 2 h after addition of 50μg/ml quercetin and p53 level continued to drop during the following 6 hours. These changes were not subject to overall protein synthesis inhibition or cell cycle. mRNA transcription was not suppressed by p53, but newly synthesized p53 levels were reduced in quercetin treated group, suggesting the inhibitory effect of quercetin on p53 synthesis (Avila et al 1994). These results for first time showed that quercetin can regulate breast cancer cells via a p53 dependent pathway.

Up-regulation of phosphorylated p53 was confirmed to be associated with cell apoptosis (Seo et al 2011). Non-malignant MCF-10A and malignant MDA-MB-231 breast cells were treated with quercetin. Cell proliferation was determined. Cell cycle was analyzed by flow cytometry. Western blotting was used for related protein analysis. Both cell lines were strongly inhibited when exposed to high concentrations as of 100μM, but not effect was observed at low concentrations tested (1 to 10μM). Sub G0/G1 phase cells were decreased and the cell population in G2/M phase were induced

in both cell lines, but the effect was more significant in MDA cells. Western blot results showed that after 24 h quercetin treatment, increased level of phosphorylated p53 was found in both MCF-10A and MDA-MB-231 cells. But the total p53 level was not altered. p21, the p53 target gene that function as inhibitor of CDKs, was also significantly increased. Cyclin B1 and Bcl-xL were suppressed in both cell lines, indicating enhanced apoptosis and cell cycle arrest (Seo et al 2011). Results from this study provided more information on p53 regulation and downstream protein regulations. Also the fact that ERα negative cell line MDA was responding to quercetin and showed apoptosis and cell cycle arrest confirmed the hypothesis that quercetin could affect cells through mechanisms other that ER.

Downstream proteins including Bax, Bcl-2, caspase-3, caspase-9 and PARP are proteins involved in cell apoptosis that regulated by p53. Their expression and cleavage can induce apoptosis. Studies showed that quercetin induced cell cycle arrest and apoptosis in MDA-MB-453 cells (Choi et al 2008). Cell proliferation was inhibited by quercetin in a time- and dose- dependent manner. The first significant difference in cells occurred at 10μM after 3 h, and 1μM after 24 h. G2/M phase cells increased to 39.5 % when compared with 10.8% of the controls, and the G1 phase cells decreased to 37.42% when compared with 60.81% of the controls. DNA flow cytometry revealed that 19.0% of cells contained small DNA fragments, which were indicated to be apoptotic cells. In order to study the pathway involved, immunoblotting assay was performed. In cells exposed to quercetin for 24 h, p53 expression was elevated, along

with an increased expression of Bax and decreased expression of Bcl-2. Then the downstream protein cytochrome c that regulated by Bax was released from mitochondria, and induced the cleavage of caspase-3 and PARP, resulting in cell apoptosis (Choi et al 2008). These results suggested that quercetin can induce cell cycle arrest and apoptosis in MDA-MB453 cells through p53 dependent pathway.

1.10.3 Inducing Apoptosis Through Caspase-3 dependent pathway

Activated cleavage of caspase-3 is usually required in apoptosis (Choi et al 2001). Thus the regulation on caspase-3 and its downstream proteins is crucial in investigating the apoptotic effect. One study was conducted to investigate the effect of quercetin on apoptosis pathway (Chien et al 2009).Cell cycle and apoptosis of MDA-MB-231 cells were measured by flow cytometry. Apoptosis was also verified by DNA fragmentation assay. Western blotting was used to analyze the cleavage of caspase-3, -8, and -9, and the actual activity were measured by activity assays. Significantly inhibited cell viability was observed in quercetin treated cells in both dose- and time-dependent manner when compared with control cells. 50% inhibition at 24 h was reached at 278μM. G2/M phase cell cycle arrest was seen at 200μM quercetin. DNA damage and DNA fragmentation in MDA cells that induced by quercetin were shown at all concentrations tested. Mitochondrial membrane potential was significantly lowered by quercetin resulting in decreased levels of pro-caspases, PARP and cyclin A and E. In

contrast, protein levels of Fas, caspase-3, Bax and cytochrome c and activities of caspase-3, -8, and -9 were clearly up-regulated. The reduce in cyclins might be the reason of induced cell cycle arrest, and increased levels of Bax, caspases and decreased levels of PARP might led to apoptosis. However, p53 protein levels were not affected by quercetin, indicating a p53 independent apoptosis pathway (Chien et al 2009).

The mitochondria related apoptosis pathway was further investigated by Chou et al using MCF-7 cells (Chou et al 2010). Cell cycle was analyzed by flow cytometry, while apoptosis was assessed by DAPI staining and DNA fragmentation. Mitochondrial membrane potential and Ca^{2+} concentrations were determined. Western blotting was used to analyze the apoptotic associated proteins. When cells were treated with quercetin for 24 and 48 h with various doses of quercetin (10-175μM), cell viability was significantly inhibited in time- and dose-dependent manner. The highest inhibition was approximately 90.25%. S phase MCF-7 cells increased significantly after being exposed to quercetin. Both DAPI staining and DNA fragmentation showed induced apoptosis by quercetin in cells. No intracellular ROS production was increased in treated cells with DCFH-DA, which was inconsistent with previous ER study. But the Ca^{2+} concentration significantly increased from 3 h up to 36 h following treatment. Loss of membrane potential of mitochondria was also observed. Protein levels change was consistent with the previous reported Chien study. Caspase-6, -8, -9 activation was enhanced by quercetin. The observed decrease in cyclin A and E might result in cell cycle arrest, while the increase in Fas, caspase-8, -9 and decrease in Bcl-2 and PARP

were shown to be related to cell apoptosis (Chou et al 2010).

1.10.4 Suppressing PI3K-Akt Signaling Pathway

Increased activity of enzymes and elevated signal transduction were remarkable in cancer cells. As early as 1995, study results showed that quercetin can down-regulate signal transduction in human breast carcinoma cells MDA-MB-435(Singhal et al 1995). When cells were growing in the log phase, PI kinase and PIP kinase activities increased 95.8- and 15.5- fold when compared with normal human breast cells. The steady-state activities also increase 7.3- and 2.3- fold respectively. MDA cells were treated with quercetin, then collected and analyzed for enzyme activity. Cell growth was inhibited dose-dependently by quercetin and completely stopped when treated with 150μM quercetin. Various concentrations of quercetin were added to cell extracts, and PI kinase activity was inhibited by 75-80% at the concentration of 100μM in vitro. Quercetin also inhibited PI and PIP kinase activity dose and time-dependently. When cells were incubated with 10, 50, and 100μM quercetin for 60min, PI kinase activity reduced 47%, 51% and 80% respectively when compared with control. But PIP kinase activity only decreased significantly at the highest concentration of 100μM. When cells were incubated with quercetin for 30 and 60 min, PI kinase and PIP kinase activity dropped to 8% and 70% and then to 5% and 63%, respectively. These data suggested the quercetin inhibited breast cancer cell proliferation and suppressed the activities of

enzymes that involved in tumor formation and progression (Singhal et al 1995).

Quercetin also regulates proliferation via PI3K-Akt/PKB pathway (Gulati et al 2006). PI3K and its downstream Akt/PKB are protein kinases involved in cell proliferation and survival in response to growth factors. The PTEN gene produces PIP3, which is the upstream of the Akt/PKB pathway. Loss of PTEN function is common in the activation of PI3K-Akt/PKB pathway. The commercially available PI3K inhibitor, LY294002 (LY), is structurally very similar to quercetin, and it's actually synthesized using quercetin as a model. Therefore, the effect of quercetin on PI3K-Akt/PKB pathway was studied. Western blot analysis was used to detect the phosphorylation of related proteins. HCC1937 cells with homozygous deletion of PTEN gene and constitutively activated Akt/PKB, and T47D cells lacking constitutively activated Akt/PKB were used in order to determine whether quercetin inhibits Akt/PKB. The quercetin treated cells showed a 50% decrease in cell proliferation at 24 h. Treatments with quercetin (25μM) or LY (10μM) completely inhibited Akt/PKB activity when compared to control. Phosphorylated-Akt was undetectable in both treatments. EGF treatment can induced the phosphorylated-Akt in T47D cell line. But this induction did not happen in cells pre-treated with LY or quercetin (Gulati et al 2006). This study showed results of the downstream regulation of the Singhal study, and clearly showed that quercetin suppresses cell proliferation via Akt/PKB pathway.

PI3K-Akt pathway can also be activated by Her-2/*neu* (ErbB2), the transmembrane tyrosine kinase that acts as a co-receptor for EGFR family members. Quercetin can

suppress Her-2/*neu*and down-regulates PI3K-Akt pathway (Jeong et al 2008). Protein analysis, flow cytometry and northern blot were performed on quercetin treated SK-Br3 cells. Her-2/*neu* expression was measured by RT-PCR, and its activity was assessed by the in vitro Her-2/*neu* tyrosine kinase assay. Human breast carcino SK-Br3 cells were constituently overexpressing Her-2/*neu.* The expression is inhibited by quercetin in a time- and dose-dependent manner. Dephosphorylation of PI3K and Akt occurred starting from day 1 when treated with 100 or 200μM quercetin. These regulations were also seen in MCF-7 cells. The RT-PCR results revealed that the transcription level of Her-2/*neu*was not affected by quercetin, indicating a translational machinery or protein stability regulation on Her-2/*neu* by quercetin. Then it was confirmed that ubiquitination of Her-2/*neu* was also induced in a dose- and time-dependent manner, suggesting an ubiquitin-dependent proteasome degradation pathway of Her-2/*neu.* Quercetin caused Hsp90 dissociation from Her-2/*neu* protein complex, and resulted in the destabilization and ubiquitination of Her-2/*neu.* The Her-2/*neu* kinase activity was also inhibited by quercetin since the phosphorylation level of Her-2/*neu* was decreased. Overall, they suggested that quercetin modulated PI3K-Akt pathway through regulating Her-2/*neu* protein in SK-Br3 cells, which provided a second inhibition mechanism of PI3K-Akt pathway by quercetin (Jeong et al 2008).

1.10.5 Inhibiting Hypoxia-Inducible Factor-1α

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcriptional factor composed of α and β subunits. HIF-1 is overexpressed in many human cancers, and the level of its activity in cells is associated with tumorigenicity and angiogenesis (Maxwell et al 1997, Zhong et al 1999). The α subunit is induced by hypoxia, a growth factor, and oncogenes. Normally, HIF-1α is ubiquitinated and degraded subsequently via the 26S proteasome via an oxygen-dependent way. Under hypoxic conditions, HIF-1α protein translocates to the nucleus and forms an active complex with β subunits. It regulates vascular endothelial growth factor (VEGF) at a transcriptional level. VGEF is required in the formation of new blood vessels and is crucial for tumor growth and metastasis (Ferrara et al 1997). Therefore, an agent targeting on HIF-1α/VEGF is promising for the treatment of cancers. A few studies investigated the effect of quercetin of HIF-1α/VEGF in breast cancer cells (Lee et al 2008).

Breast cancer cell line SkBr3 was cultured in a hypoxic chamber with a 94:5:1 mixture of $N_2/CO_2/O_2$ (Lee et al 2008). Thus the hypoxic environment was created. The cells were treated with different concentrations of quercetin. Cell viability was determined. Immunosorbent assay was used to detect VEGF. Protein synthesis was assessed by radiolabeled amino acids. Immunoblot analysis was performed to measure protein contents. Interestingly, cell viability was not affected by quercetin under hypoxic conditions, and PARP cleavage and caspase activation were not observed. The

HIF-1α accumulation was reduced by quercetin in a dose-dependent manner in hypoxic conditions. The up-regulated VEGF production during hypoxic conditions was inhibited by quercetin treatment. In order to rule out the possibility of PI3K/Akt regulation on HIF-1α expression, the PI3K inhibitor LY was added in the treatment. It was shown that LY could inhibit quercetin induced HIF-1α accumulation, which is consistent with the previous study that quercetin could function through PI3K/Akt pathway. But the HIF-1α accumulation induced by hypoxia was not affected by LY, suggesting that quercetin did not suppress HIF-1α accumulation through PI3K/Akt pathway. The proteasomal inhibitor MG-132 did not affect the inhibition of HIF-1α accumulation by quercetin under hypoxic conditions, indicating the inhibitory effect of quercetin is not mediated by HIF-1α degradation. Finally, a 94% inhibition of protein synthesis was observed in quercetin treated cells compared to 48% in control cells under hypoxic condition. The inhibition was also dose-dependent. In conclusion, quercetin inhibited HIF-1α under hypoxic conditions through inhibiting protein synthesis (Lee et al 2008).

The effect of quercetin on doxorubicin (DOX)-induced cytotoxicity was investigated in 4T1 breast cancer cells. Results were consistent with the previous study, but showed a quercetin induced increase in HIF-1α accumulation in normal cells. Doxorubicin was a successful antibiotic in treating various types of cancers, but the efficacy was very restricted by its cumulative cardiotoxicity and common side effects. Since quercetin exhibits cardioprotective and hepatoprotective activities, the effect of quercetin on

improving therapeutic index of DOX was assessed. MTT assay was used to measure cell proliferation. Apoptosis was assessed by DNA fragmentation assay and flow cytometry. HIF-1α level was measured by western blot. Results showed that quercetin exhibited a dose-dependent antiproliferative activity under hypoxia. But the combined treatment of quercetin and DOX only showed a slight induction on cell viability. Quercetin at 50 μM promoted DOX-induced apoptosis in 4T1 cells under hypoxia, but the cytotoxicity of DOX was decreased by quercetin under normoxia and IC_{50} of DOX increase by 3-4 folds when co-treated with 10 or 25μM quercetin. Mice treated with quercetin 100 mg/kg day had significantly smaller tumors that untreated mice. The combined treatment showed even greater effect. The median survival time were 55 days in quercetin-treated mice compared with 38 days of untreated mice. HIF-1α level in tissue was examined by ELISA. Results showed that HIF-1α level was decreased in quercetin treated tumor cells, but increased in normal cells. VEGF exhibited the same change (Du et al 2010). These above mentioned research results showed that quercetin could suppress tumor cells and protect normal cells simultaneously by its opposing effects on HIF-1α. Thus it could be considered as an anticancer agent.

1.10.6 Inhibition on COX-2 Activity

Cyclooxygenase-2 (COX-2) can catalyze conversion of arachidonic acid. It's essential in carcinogenesis and inflammation. Overexpression of COX-2 was found in

various cancer cell lines including breast and colon cancer. The inhibition of COX-2 can effectively inhibit cell proliferation and angiogenesis, and induce apoptosis.

AMP-activated protein kinase (AMPK) is a member of a serine/threonine protein kinase family that can be found in all eukaryotes. It can directly sense cellular energy status, thus it becomes a popular target for ensuring cell proliferation when cells have adequate amount of metabolic resources. AMPK activation is linked to apoptosis through pathways including p53 and p21, caspase activation. It can also regulate cell proliferation via COX2, Akt and mTOR. The regulation of quercetin on COX-2 and AMPK was investigated (Lee et al 2009). MCF-7 breast cancer cells were cultured. Quercetin at concentrations of 50, 100, 200μM induced the inhibition of cell growth and cell cycle arrest in a dose-dependent manner. Protein levels of p53 and p21 were elevated, and VGEF was inhibited. Apoptotic cell death was promoted by 100μM quercetin. AMPK was activated by quercetin in a dose-dependent manner as well. When AMPK inhibitor was added to the treatment, the activation of AMPK by quercetin was weakened. These results indicated that quercetin can activate AMPK in MCF-7 cells. Then the ROS production was examined since it's an upstream protein candidate of AMPK. The addition of antioxidant N-acetyl-cysteine markedly abrogated quercetin–induced AMPK activation, suggesting the activation was through ROS production. The dose-dependently inhibited COX-2 expression was also observed in quercetin-treated cells. But the AMPK inhibitor can eliminate COX-2 expression in treated cells. In the cox-2 dominant negative fibroblast cells, the activation of AMPK

was still observed after quercetin treatment, indicating COX-2 was regulated by AMPK. In conclusion, quercetin can inhibit COX-2 expression in MCF-7 cells through ROS induced AMPK activation (Lee et al 2009).

The transcriptional regulation of COX-2 was widely studied. COX-2 gene promoter activity can be regulated by cyclic AMP, CCAAT enhancer and NF-κB. The simultaneous binding of multiple transactivators to promoter will result in COX-2 overexpression. P300 has a COX-2 promoter chromatin structure, and is able to enhance the binding activity. Since quercetin has been linked to the regulation of NF-κB, its effect on COX-2 was also assessed (Xiao et al 2011). MDA-MB-231 cells were used in the study. High expression of COX-2 protein and mRNA level were observed in untreated cells. Western blotting and RT-PCR confirmed that quercetin treatment resulted in reduced COX-2 expression in a dose-dependent manner. COX-2 promoter activity was measured by luciferase expression vector containing COX-2 5-flanking fragment. The down-regulation by quercetin was observed. PGE2, the down-stream product of COX-2 enzyme leading to angiogenesis, was also inhibited by quercetin. The effect of quercetin on binding activities of different transactivator was assessed. The biotin-streptavidin complex containing COX-2 promoter and transactivators (including CREB-2, C-Fox, C/EBPβ and NF-κB) was pulled down after quercetin treatment. Western blot results showed that the binding of multiple transactivators was inhibited by quercetin, but their expression levels were not affected. Then p300, the transcription co-activator, was studied. The streptavidin-agarose bead

pulldown assay and immunoprecipitation results showed that quercetin inhibited p300 recruitment to the transactivators-COX-2 promoter complex in MDA cells. But the expression level was not changed. P300 was either purified after quercetin treatment, or followed by the treatment. HAT activity of p300 was determined. The dose-dependent inhibition by quercetin was observed both *in vivo* and *in vitro*. The conclusion is that quercetin can inhibit COX-2 expression by regulating p300 HAT activity (Xiao et al 2011). These results showed another possible mechanism in transcriptional level that how COX-2 is regulated by quercetin.

1.10.7 Regulation on Matrix Metalloproteinas

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases and could be divided into four subgroups based on the substrate. Activation of MMPs is crucial in tumor invasion and metastasis. It destroys extracellular matrix components in the surrounding tissues. Tumor cells could then invade through basement membrane of blood vessels and spread to other organs (Duffy et al 2000).

Among the 24 kinds of MMPs that have been identified, MMP-2 and MMP-9 are the most important mediators (Duffy et al 2000). The expressions of these two proteins are highly correlated with the metastatic potential. While MMP-2 is constitutively overexpressed in highly metastatic tumors, MMP-9 can be stimulated by TPA. MAPK and PI3K are the important cascades affecting MMP-9 expression. Transcription

factors including activator protein-1 (AP-1), NF-κB and Sp-1 were also related to MMP-9 regulation. The regulatory effect of quercetin on MMP-9 expression was investigated (Lin et al 2008). Wound-healing assay was used to assess MCF-7 cell migration. In vitro invasion assay was performed to examine tumor invasion. RT-PCR and western blotting were carried out for MMP-9 expression analysis. The enzyme activity of MMP-2 and -9 was measured by gelatin zymography and fluorimetric kit. Results showed that quercetin can prevent the cell invasion and colony formation that induced by TPA. RT-PCR revealed that mRNA level, protein expression and enzyme activity of MMP-9, but not MMP-2, was blocked by quercetin treatment. TPA was also correlated with PKC activation. It was shown that PKCδ translocated to membrane in TPA-treated cells, but the translacation was blocked by quercetin. Phosphorylation of ERK and JNK, but not p38 or Akt, was induced by TPA, and then blocked by quercetin treatment. Furthermore, ERK inhibitor could abrogate MMP-9 expression as well as ERK activation and cell migration, suggesting quercetin suppressed ERK through PKCδ, and consequently suppressed MMP-9 expression. The binding site of AP-1 and NF-κB located on MMP-9 gene promoter was involved in MMP-9 activation. Pretreatment of quercetin showed inhibition on AP-1 promoter activity and TPA-induced c-Jun expression. These results suggested that quercetin inhibits MMP-9 activity through PKCδ/ERK/AP-1 cascades (Lin et al 2008). Therefore quercetin has the potential to be a therapeutic agent for breast cancer metastasis by suppressing MMP-9.

Then in MDA-MB-231 human highly invasive breast cancer cell line, the inhibition of MMP-3 activity by quercetin was demonstrated (Phromnoi et al 2009). MMP-3 level in MDA-MB-231 cell was 4-fold higher than MCF-7, indicating the involvement of MMP-3 in cell migration. Cell invasion was measured by modified Boyden chamber assay. MMP-3 activity was measured by casein zymography. Western blot and ELISA were performed to analysis MMP-3 secretion. A dose-dependent inhibition of cell proliferation was observed in MDA cells treated with quercetin. Cell invasion was significantly inhibited by quercetin with IC_{50} value of 27μM. MMP-3 activity was reduced by quercetin with IC_{50} value of 30μM, but the MMP-3 secretion was not affected by quercetin (Phromnoi et al 2009). Therefore, quercetin can regulate cancer cell invasion and migration through regulating MMP-3 in addition to MMP-9.

1.10.8 Regulation on Other Related Pathway

The F-box protein S phase kinase-associated protein 2 (Skp2) was identified to be associated with CDK2, the cyclin A-cyclin-dependent kinase 2. As cells exit the cell cycle, Skp2 level decreases, and as cells re-enter the cycle, Skp2 level increases. The expression of Skp2 is cell cycle regulated. The accumulation occurs during S/G2 phase, and degradation occurs during M phase. It is overexpressed in a number of human cancer cells including breast cancer, prostate cancer and small cell lung carcinoma. High expression of Skp2 is also associated with poor prognosis. It can also mediate the

degradation of cyclin E, p21, c-Myc.

The effect of quercetin on Skp2 gene expression was examined in multiple breast cancer cell lines. Cells were treated with quercetin, and cell viability was assessed by MTT assay. Immunoblotting was performed for proteins involved. The highest expression of Skp2 was found in MDA-MB-231 and BT474. The lowest expression of p27 was observed in these two cell lines as well, indicating the inverse correlation between Skp2 and p27. After the quercetin treatment, cell growth was inhibited by a time- and dose-dependent manner. G2/M phase arrest was induced in MDA cells, while G1 phase arrest occurred in BT474 cells. Up-regulated p27 protein level and down-regulated Skp2 level were confirmed in MDA cell, but no significant change was shown in BT474. Skp2 siRNA knocked down the protein level of Skp2 in MDA cells with increased p27 level as expected. But no decrease in Skp2 mRNA level was found, indicating a post-transcriptional regulation by quercetin. Then the decreased Skp2 level after quercetin treatment was observed by western blot without changes in cyclins and CDKs, suggesting quercetin induced cell cycle arrest through regulating Skp2 (Huang et al 2011).

In conclusion, food pattern has been linked to various diseases including cardiovascular diseases, hypertension, diabetes and cancers. Study results showed that a diet containing a large amount of fruits and vegetables could lower the risk of certain diseases. The functional compounds are thought to be the phytochemicals in plant

foods. Among all phenolics, flavonoids is the most widely studied compound family. Quercetin, Q3G and EMIQ are three flavonoids. The first two can be found in apples, onions, etc. EMIQ can be synthesized artificially, and has a significantly higher bioavailability than Q3G and quercetin due to the additional sugar chain.

These three compounds are related to diseases as well. They function as natural antioxidants as well as regulatory compounds. Epidemiological studies showed that the disease incidence as well as the symptoms can be reduced by consuming these compounds or the crude extracts containing these compounds. Animal studies confirmed that the tumor formation was delayed by the treatment of either quercetin or Q3G. Other experimental results provided insights into the reduction of oxidative stress and induction of cell cycle arrest and apoptosis by Q3G and quercetin in breast cancer cells. The potential mechanisms include the regulation of enzymes that involved in ROS production, and the regulation on cell signaling pathways that involved in cell proliferation and apoptosis. These results indicate that these phytochemicals are of potential value for chemoprevention of breast cancer, and can serve as the co-treatment with other chemopreventive agents.

OBJECTIVES

Although various studies investigated the quercetin-induced regulations on cell

signaling pathways, the mechanisms of action of Q3G still remains unclear. Since the existing studies mainly focused on the inhibitory effect of Q3G in ROS production in various cell lines, and little were conducted to investigate the signal transduction pathways, the objective of my study is to demonstrate the mechanism of action of Q3G induced apoptosis and inhibited cell proliferation in human breast cancer MCF-7 cells.

CHAPTER TWO

POTENTIAL MECHANISM OF ACTIONS OF QUERCETIN-3-GLUCOSIDE (Q3G) IN REGULATING CELL PROLIFERATION AND APOPTOSIS IN HUMAN BREAST CANCER MCF-7CELLS THROUGH P38/MAPK SIGNAL TRANSDUCTION PATHWAY

2.1 INTRODUCTION

Cancer accounts for 22.9% of total deaths in United States, exceeded only by heart disease (25.0%) (SEER Cancer Statistics Review 1975-2008). Among all sites of cancers, breast cancer is the second most common malignance among women. Nearly every 1 in 3 cancers diagnosed among women is breast cancer in the United States. It's also the second leading cause of among all cancers in women. Estimated number of new breast cancer cases in U.S. in 2011 is 57,650 and 230,480 for in situ cases and invasive cases, respectively. Total deaths number is estimated to be 39,520 in women (DeSantis et al 2011). Commonly used therapies include lumpectomy or mastectomy, radiation, chemotherapy, hormone therapy and target therapy. Patients usually suffered from these therapies both mentally and physically (American Cancer Society 2011). Therefore, novel alternative treatments and preventive strategies for human breast

cancer are in great need. In searching for new chemopreventive and treatment agents for breast cancer, natural products including various fruits and vegetables are studied and used as a source of anticancer agents or dietary supplements for women. Recent epidemiology studies consistently showed that a diet rich in fruits and vegetables can reduce the risk of various chronic diseases including hypertension, cancer, coronary heart diseases and cardiovascular diseases (Wang et al 2011; Anderson et al 2011; Gorlova et al 2011; Ibiebele et al 2011).

As is shown in several studies, apples have significant positive effects on human health. It has been linked to a lowered risk of cancer, coronary heart disease, asthma and type II diabetes. The antioxidant status in human body can be boosted after apple juice consumption (Yuan et al 2011). Animal studies showed that apple extracts can prevent mammary tumors in rats by suppress proliferation and induce apoptosis (Liu et al 2009; Liu et al 2005). Querceting-3-glucoside (Q3G) is one of the most important and abundant bioactive compounds in apple. Previously reported research results showed that Q3G exhibits *in vitro* antioxidant and antitumor activity on both estrogen-dependent and estrogen-independent human breast cancer cells by modulating cell cycle in cells (Yang et al 2009; Sun et al 2008)

However, little published literatures addressed the mechanism of action of Q3G in human breast cancer cells. In the present study, to investigate the anticancer activity of Q3G, we studied the inhibition of cell growth of human breast MCF-7 cells by the modified methylene blue assay (Felice et al 2009). To study the anticancer activity of

Q3G, we also tested the regulation of protein expression in induced apoptosis and cell cycle arrest. Further investigation on the mechanism of action of cell antiproliferation and apoptosis that induced by Q3G was also conducted in human breast cancer MCF-7 cells.

2.2 MATERIAL AND METHODS

2.2.1 Chemicals and Antibodies.

Quercetin-3-glucoside was purchased from Sigma Chemical Co. (St. Louis, MO). Minimum Essential Medium Alpha Medium (MEM), epidermal growth factor, fetal bovine serum (FBS), gentamicin, Hepes, insulin, penicillin, streptomycin and other cell culture reagents were purchased from GIBCO (Life Technologies, Grand Island, NY). Butanol, methanol, xylene, phosphate-buffered saline (PBS), and sodium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA). Folin-Ciocalteu reagent, hyrdrochloric acid, aprotinin, leupeptin, pepstain, phenylmethanesulfonyl fluoride (PMSF), sodiumorthovanadate and sodium fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Ultrapure Tris (base) and Tris (acid) were obtained from J. T. Baker (Phillipsburg, NJ). All other reagents used in the study were of analytical grade.

Rabbit polyclonal anti-TRAF2 was obtained from Cell Signaling Technology, Inc

(Beverly, MA); rabbit polyclonal antibodies to ASK1, phospho-p53, caspase-3, mouse monoclonal antibodies to phospho-p38, p21, Cyclin-D1, CDK4, Bax, Bcl-2 were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA); mouse monoclonal anti-β actin, anti-α Tublin, rabbit polyclonal anti-Caspase 9 were obtained from Sigma Chemical Co. (St. Louis, MO); mouse monoclonal anti-PCNA was obtained from Oncogene; secondary antibodies include anti-mouse IgG and anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, MO); anti-rabbit IgG-HRP conjugated antibody was obtained from Santa Cruz Biotechnology Co. (Santa Cruz, CA).

2.2.2 Measurement of Antiproliferative Activity toward Human Breast Cancer Cells

The antiproliferative activity of Q3G was measured by the methylene blue assay. Q3G was dissolved in DMSO with the final concentration of 40mM. Human breast cancer MCF-7 cells (American Type Culture Collection, ATCC, Rockville, MD) were incubated at 37 ℃ with 5% CO2 in MEM containing 10 μg/mL insulin,10 mMHepes, 50 μg/mL streptomycin, 50 units/ mL penicillin, 100 μg/mL gentamicin, and 10% fetal bovine serum (Gibco, Life Technologies). MCF-7 cells in growth media were then seeded in 96-well flat-bottom plates at the density of 2.5×10^4 cells/well. After 8 hours of incubation at 37 ℃ with 5% CO2, the growth medium was removed and cells were treated with media containing different concentrations of Q3G. Control cultures

include MEM and 0.6% DMSO only. After 72 h of incubation, treatment medium was removed, and the cells were washed with PBS once. A volume of 50 μL methylene blue staining solution (98% HBSS, 1.25% glutaraldehyde, 0.6% methylene blue) was applied to each well, and the plate was incubated at 37 ℃ for 1 h. After removal of the dye, the plate was rinsed by fresh deionized water three times, or until the water was clear. The water was tapped out of the wells, and the plate was allowed to air-dry briefly before applying 100 μL of elution solution (49% PBS, 50% ethanol, 1% acetic acid) to each well. The plate was shaken for 30 min to allow uniform elution. The absorbance was read at 570 nm using the MRX II DYNEX spectrophotometer (DYNEX Inc., Chantilly, VA).

2.2.3 Measurement of Cytotoxicity toward Human Breast Cancer Cells

The cytotoxicity of Q3G was measured by methylene blue assay. MCF-7 cells were plated in a 96-well plate at a density of 4×10^4 cells/well in 100 μL of MEMα medium and incubated at 37 ℃ in 5% CO_2. After 24 h incubation, the medium was removed, and the cells were treated with different concentration of Q3G in 100 μL medium. Then the plate was incubated at 37 ℃ for another 24 h. Cell cytotoxicity was then determined by the methylene blue assay described above. The absorbance readings at 570 nm for each concentration compared to the control were used to determine cell cytotoxicity (percent). At least three replications for each concentration were analyzed for the

cytotoxicity.

2.2.4 Protein Extraction and Western Blot Analyses

Cells were seeded at a density of 0.5×10^6 cells/well in 6-well plates. Six hours after seeding, MCF-7 cells were treated with different concentrations of Q3G and were then harvested after 16, 24, 44 h exposures. Each treatment was in triplicate, and the cells receiving the same concentration of treatment were combined together for protein extraction and further Western blot Analysis. Prior to analysis of proteins, cell culture media were removed from the treatment plates and each well was rinsed twice with PBS. Cells were then scraped off in PBS and centrifuged at 1000rpm at 4 ℃ for 5 minutes. After discarding PBS, 100 μL of lysis buffer (50 mMTris, pH 7.4; 1% Igepal; 150 mM sodium chloride; 1 mM EDTA) with protease inhibitors (1 μg/mL aprotinin; 1 μg/mL leupeptin; 1 μg/mL pepstain; 1 mM PMSF; 1 mM sodiumorthovanadate; 1 mM sodium fluoride) was added to each sample and vortexed. Cell lysates were kept on ice and vortexed periodically for 20 min to facilitate protein extraction. Lysates were then centrifuged at 12000g for 15 min at 4 ℃, and protein concentrations of the lysates were determined using a Sigma Diagnostics Micro Protein Determination Kit and a DynexMicroplate Reader (Dynex Technologies). Electrophoresis was carried out on SDS-PAGE gel, followed by the transfer to PVDF membranes. The membranes were blocked in 5% nonfat dry milk in TBST (Tris Buffered Saline with 1% Tween 20) for 1

h at room temperature. After blocking, the membranes were incubated with primary antibodies for 24h at 4 ℃. Membranes were then incubated with the corresponding secondary antibody in TBST with 1% nonfat dry milk powder for 2 h under agitation at room temperature. The expression of human β-actin and α-Tublin were used as an internal standard. Protein was visualized by the Enhanced Chemiluminescence kit (Cell Signaling Technology, Inc., Beverly, MA) according to the manufacturer's instruction. The densities of the specific protein bands were quantified by optical densitometry using ImageJ. All results were expressed as mean ± SD for three independent replications.

2.2.5 Determination of cell apoptosis

The cell apoptosis was determined in situ using the ApopTag@PlusPeroidaseIn Situ Apoptosis Detection Kit (Serologicals Corporation, Norcross, GA) based on the terminal deoxynucleotidyl-transferase mediated dUTP nick end labeling (TUNEL) assay. Briefly, cells were seeded on a Falcon 8-chamber culture slide (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 2.5×10^5 cells/well. After reaching 60–70% confluence, the cells were incubated in serum-free medium for 4h. The cells were then exposed to different concentrations of Q3G for 4h. Then, the cells were washed with PBS, fixed in 1% paraformaldehyde for 20 min at room temperature, and post-fixed in pre-cooled ethanol:acetic acid (2:1, v:v) at -20 ℃ for another 5 min. Endogenous

peroxidase was quenched for 15 min using 3% H_2O_2 at room temperature. DNA fragments in cell were labeled with peroxidase, which then reacted with the peroxidase substrate, 3, 3'-diaminobenzidine (DAB), to obtain a permanent, localized brown-color stain. Then cells were counterstained with methyl green to further differentiate apoptotic cells from normal cells. Finally, 2000 cells were randomly selected for observation for each treatment and the apoptosis were expressed as the percentage of apoptotic cells to total cells as described previously.

2.2.6 Statistical analysis

Data were analyzed using SPSS software (IBM SPSS Statistics 17.0) and presented as mean ± standard deviation (SD) for at least three replicates. Significance was determined at p value of < 0.05 or < 0.01 or < 0.001 by analysis of variance (ANOVA) followed by Duncan's multiple comparison tests.

2.3 RESULTS

2.3.1 Antiproliferative activity and cytotoxicity of Q3G towards human breast cells

The study in Figure 2.1 was designed to determine the antiproliferative activity and cytotoxicity of Q3G towards human breast cell line MCF-7. In order to confirm Q3G

inhibits MCF-7 cell proliferation without inducing cytotoxicity in the cells, the cytotoxicity of Q3G was first tested by the cytotoxicity assay. Results showed that no cytotoxicity was observed for Q3G at the concentrations lower than 75 μM (Figure 2.1).

Then the proliferation of Q3G treated MCF-7 cells compared to control cells was analyzed using the antiproliferation assay. Results showed that Q3G exhibited potent antiproliferative activity against MCF-7 cell growth in a dose-dependent manner. As the concentration of Q3G increased, the proliferation of MCF-7 cells decreased. The cell proliferation was inhibited by 74.3% at the concentration of 60μM (Figure 2.1). The EC_{50} value of antiproliferative activity of Q3G towards MCF-7 cells was 29.63 μM. These results confirmed that Q3G inhibits MCF-7 cell proliferation without inducing cytotoxicity.

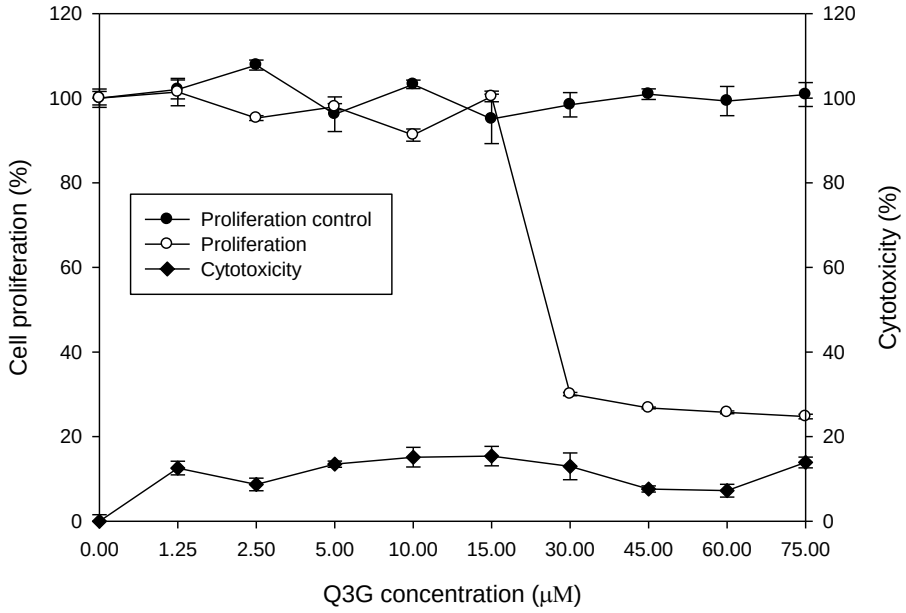

FIGURE 2.1 Effects of Q3G on cell proliferation and cytotoxicity in human breast cancer MCF-7 cells. Each value represents the mean ± SD with triplicates.

2.3.2 Effects of Q3G on expressions of proteins involved in the proliferation and cell cycle in MCF-7 cells

To confirm the antiproliferative activity of Q3G towards MCF-7 cells, we determined the expression of proliferating cell nuclear antigen (PCNA), the marker of cell proliferation. As is shown in Figure 2.2A, PCNA expression was significantly decreased in MCF-7 cells treated with Q3G in a dose-dependent manner. The PCNA expression in the MCF-7 cells treated with Q3G was inhibited by 33.8% and 42.3% at the concentration of 30 and 60μM, respectively, indicating a direct antiproliferative effect of Q3G during the DNA synthesis phase of the cell cycle in MCF-7 cells (Figure 2.2 A).

To further investigate the regulation of other cell cycle related proteins, we also examined the expression of p21, Cyclin D1 and Cdk4 in MCF-7 cells following Q3G treatment (Figure 2.2 B-D). p21 expression significantly increased by 57.0 % and 85.3% at the concentration of 30 and 60μM when compared to that of control group ($P<0.01$ and $P<0.001$, respectively). The expression of Cyclin D1 was reduced by 23.0% and 49.5% at the concentration of 30 and 60μM, respectively. Cdk4 expression was also significantly down-regulated by 36.4% and 69.0% at the concentration of 30 and 60μM respectively with the treatment of Q3G (Figure 2.2 C-D). The expression of all these proteins is regulated in a dose-dependent manner.

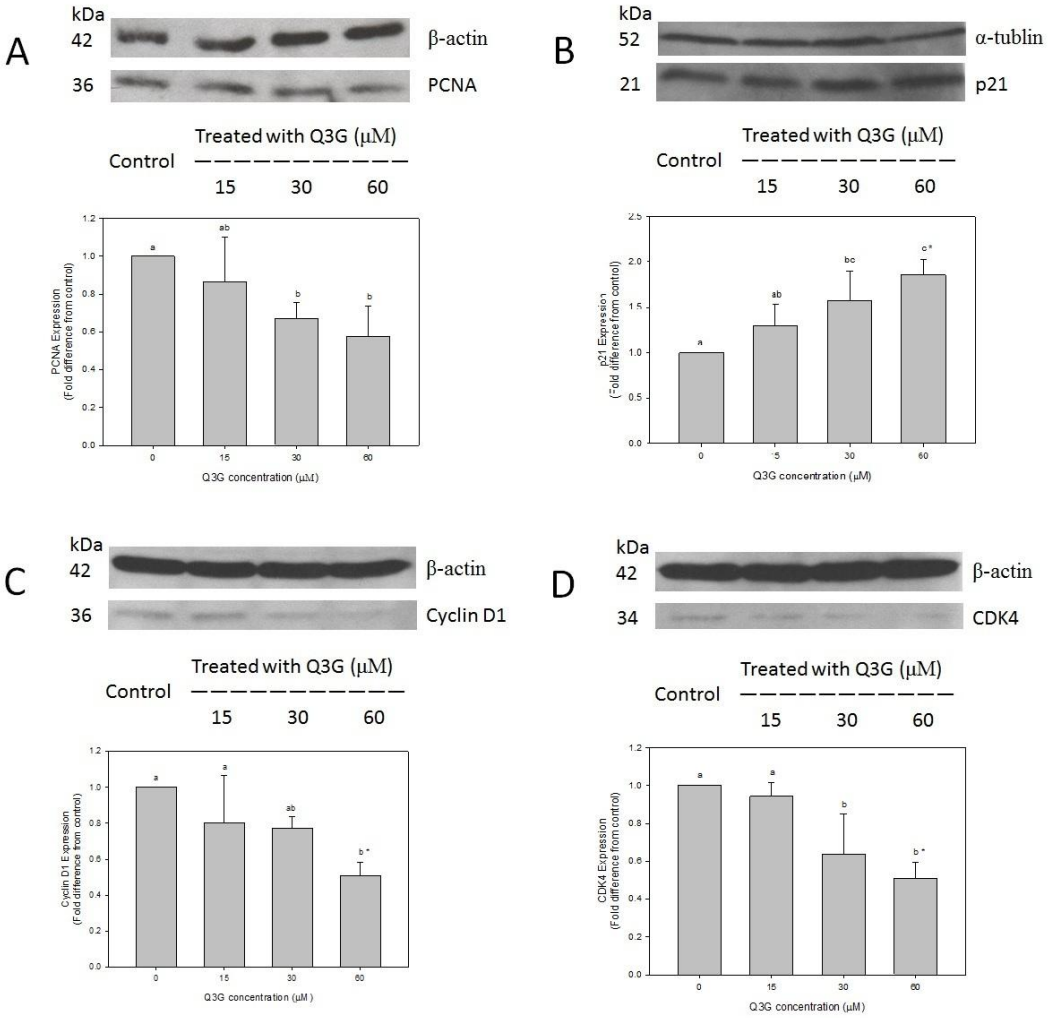

FIGURE 2.2 Effects of Q3G on expression of PCNA (*A*), p21 (*B*), cyclin D1 (*C*) and CDK4 (*D*) in human breast cancer MCF-7 cells. Bars with no letters in common in each panel are significantly different ($p< 0.05$). An asterisk (*) indicates $p< 0.01$ when compared to the control. Each value represents the mean ± SD with triplicates.

2.3.3 Effects of Q3G on expressions of Bcl-2, Bax, Caspase-3, Caspase-9, and apoptosis in human breast cancer MCF-7 cells.

It was reported that the defective apoptosis may result from abnormal expression of Bcl-2 and increased expression of caspase-3 (Marsden, O'Connor, O'Reilly, Silke, Metcalf, Ekert, et al., 2002; Okada & Mak, 2004). We therefore investigated the expression levels of apoptosis related proteins, including Bcl-2, Bax, Caspase-9 and Caspase-3 (Fig. 2.3). When compared with the control, treatment of Q3G resulted in a significantly decreased Bcl-2 expression and increased Bax expression in a dose-dependent manner in MCF-7 cells.The Bax expression was up-regulated by 33.4% ($p< 0.01$) and the Bcl-2 expression decreased by 23.9% ($p< 0.01$), and the Bax/Bcl-2 ratio increased 75.8% ($P<0.001$) when treated with Q3G at the concentration of 60μM.

In order to identify if Q3G can regulate cell apoptosis by inducing Caspase-3 expression and cleavage, we tested the pro-cleaved and cleaved casepase-3 levels in MCF-7 cells treated by Q3G at the concentration of 0, 15, 30 and 60μM. We found that caspase-3 expression was stimulated by 73.8% with the treatment of Q3G at the concentration of 15μM. A 29.5% decrease in the pro-cleaved caspase-3 expression was observed with a treatment of 60μM Q3G when compared to the treatment of 15μM, and the cleaved caspase-3 (19 kDa) was increased by 58.5%, 73.5% and 57.9% at the concentration of 15, 30 and 60μM, respectively.

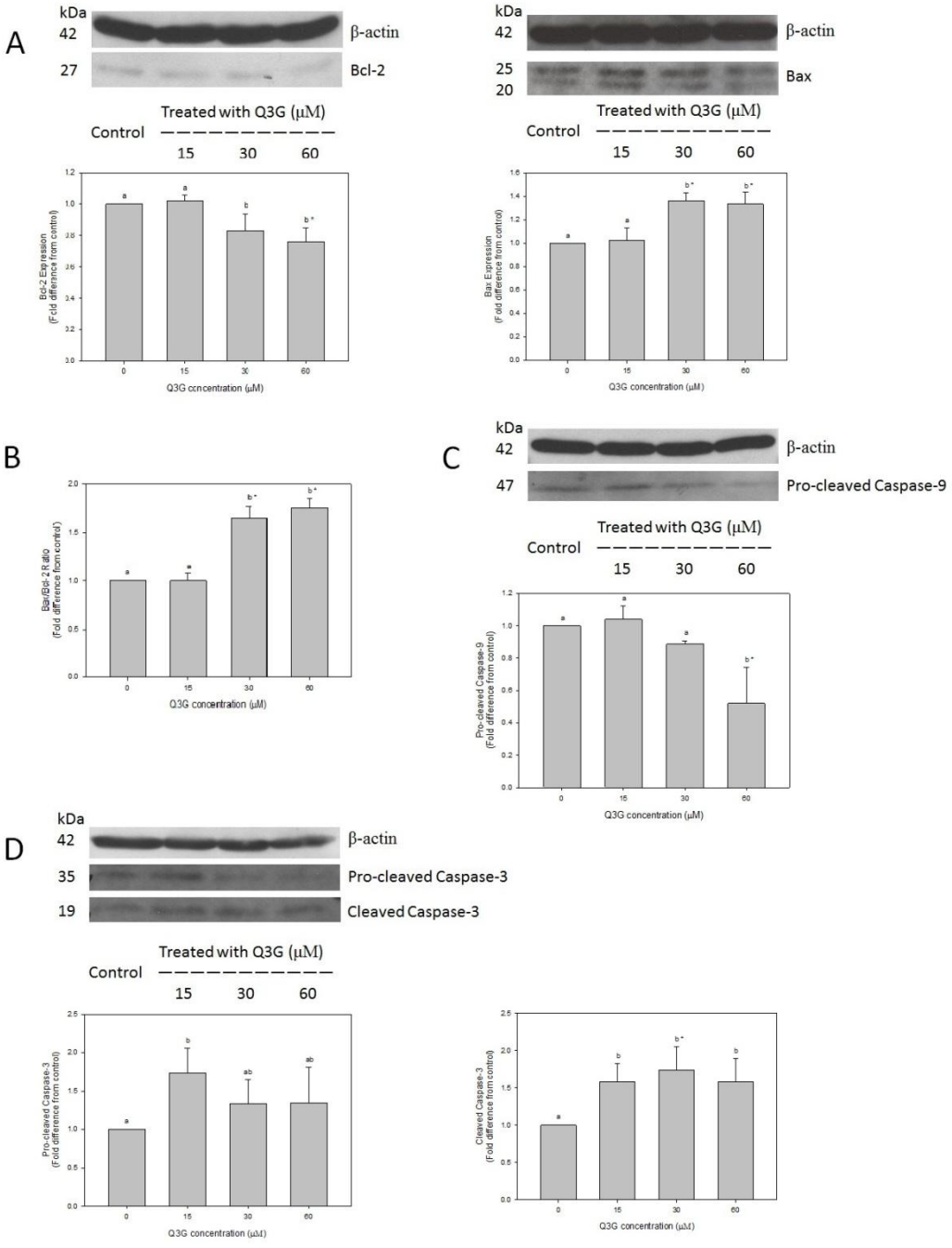

FIGURE 2.3 The effect of Q3G on expression of Bcl-2 and Bax (*A*), Bax/Bcl-2 ratio (*B*), Caspase-9 (*C*) and Caspase-3 (*D*) in human breast cancer MCF-7 cells. Bars with no letters in common in each panel are significantly different ($p< 0.05$). An asterisk (*) indicates $p< 0.01$ when compared to the control. Each value represents the mean ± SD with triplicates.

When MCF-7 cells were treated with Q3G at concentrations of 0, 15 and 30μM , cleaved caspase-3 level increased in a dose-dependent manner when compared to the control.It then decreased at the highest concentration. We also investigated the upstream caspase, caspase-9. As is shown in Figure3C, there was no significant difference in caspase-9 cleavage between the treated cells with 15μM Q3G and the none-treated cells.However, there was a dramatic increase in caspase-9 cleavage in the MCF-7 cells treated with 30 and 60μM of Q3G in a dose-dependent manner ($p<0.05$) when compared to the control, resulting in the decrease level of pro-cleaved caspase-9. The cleavage of caspase-9 was activated by 48.0% at the concentration of 60μM of Q3G when compared to the control ($p<0.01$).

We also confirmed Q3G induced apoptosis in human breast cancer MCF-7 cells by the TUNEL assay (Figure 2.4). The background apoptosis of MCF-7 cells was 0.7 apoptotic cells per 100 cells (Figure 2.4A). Treatment of Q3G significantly induced apoptosis in MCF-7 cells in a dose-dependent manner. The number of apoptotic cells were increased to 3.2 ($p < 0.05$), 6.8 ($p<0.01$), and 11.4 ($p<0.01$) per 100 cells in MCF-7 cells treated with 15, 30, and 60μM, respectively.

2.3.4 Regulation of proteins involved in the P38/MAPK pathway.

We first investigated whether the apoptosis and cell cycle arrest of Q3G treated MCF-7 cells were activated via the p53-dependent pathway (Figure 2.5A).

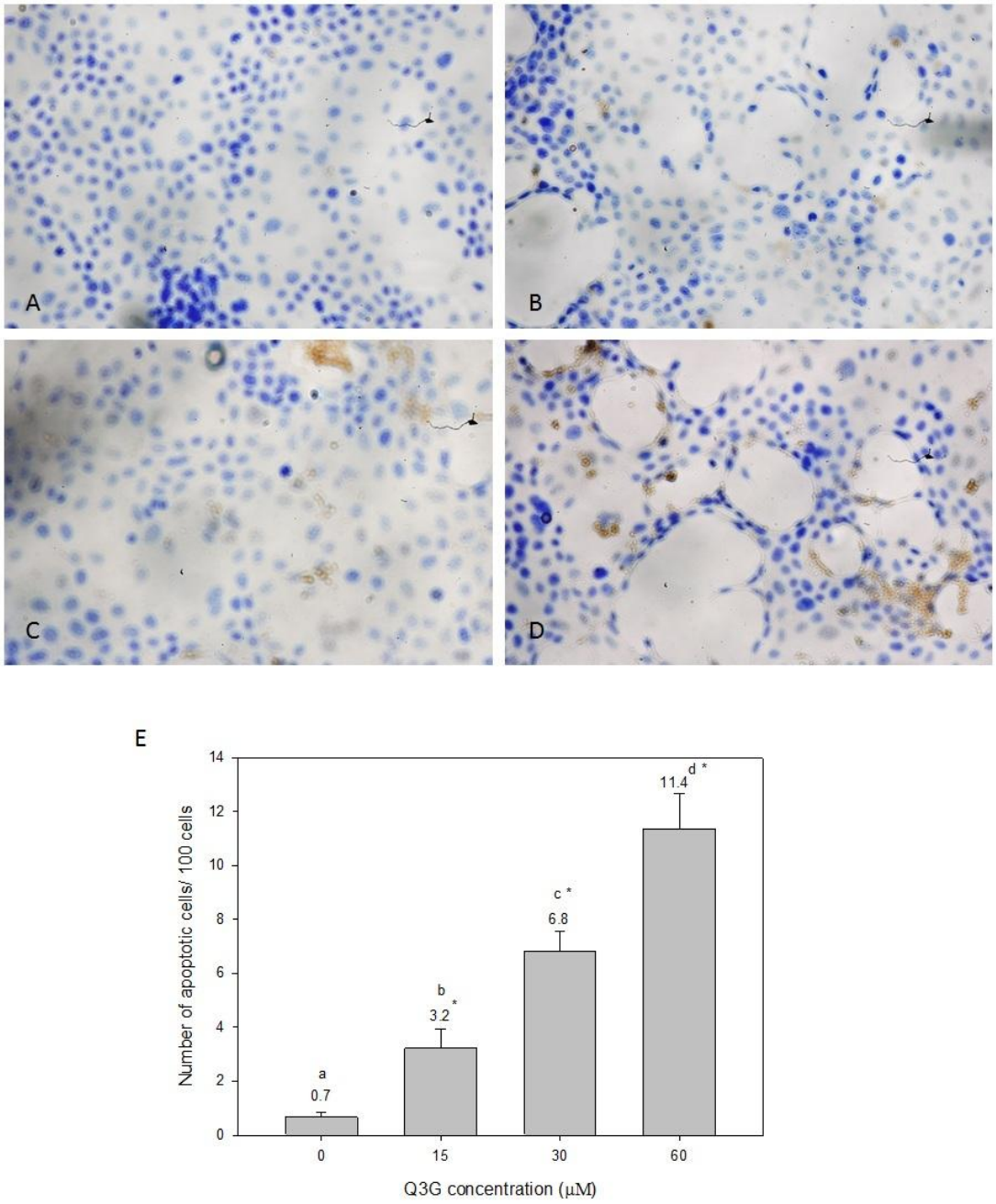

FIGURE 2.4 Effect of Q3G on apoptosis of human breast cancer MCF-7 cells at the concentrations of 0 (*A*), 15 (*B*), 30 (*C*) and 60 (*D*) µM. Bars with no letters in common in each panel are significantly different ($p< 0.05$). An asterisk (*) indicates $p< 0.01$ when compared to the control. Each value represents the mean ± SD with triplicates.

Q3G significantly increased the phosphorylation of p53 in a dose-dependent manner in MCF-7 cells when compared to the control. The phosphorylated p53 increased by 29.1%, 23.9% and 16.2% at the concentration of 15, 30, 60μM, respectively. Level of phosphor-ERK was not altered by any of the concentrations tested (data not shown), while the expression of phosphor-p38 increased by 75.0% at the concentration of 60μM, indicating that p38, not ERK, was involved in Q3G-induced antiproliferative activity and induction of apoptosis in MCF-7 cells (Figure 2.5B).

To further investigate the upstream proteins of p38/MAPK signal transduction pathway, we tested the protein expression of ASK1 and TRAF2 (Figures 2.5C and 2.5D). Compared to control, Q3G at the concentration of 30μM significantly up-regulated the expression of ASK1 by 53.6% and 22.2% in MCF-7 cells (Figure 2.5C) at the concentration of 30 and 60μM, respectively. The dose-response study on the expression of upstream protein TRAF2 in human breast cancer MCF-7 cells was performed with Q3G (Figure 2.5D). Q3G at all concentrations tested did not change the expression level of TRAF2 in MCF-7 cells when compared to the control.

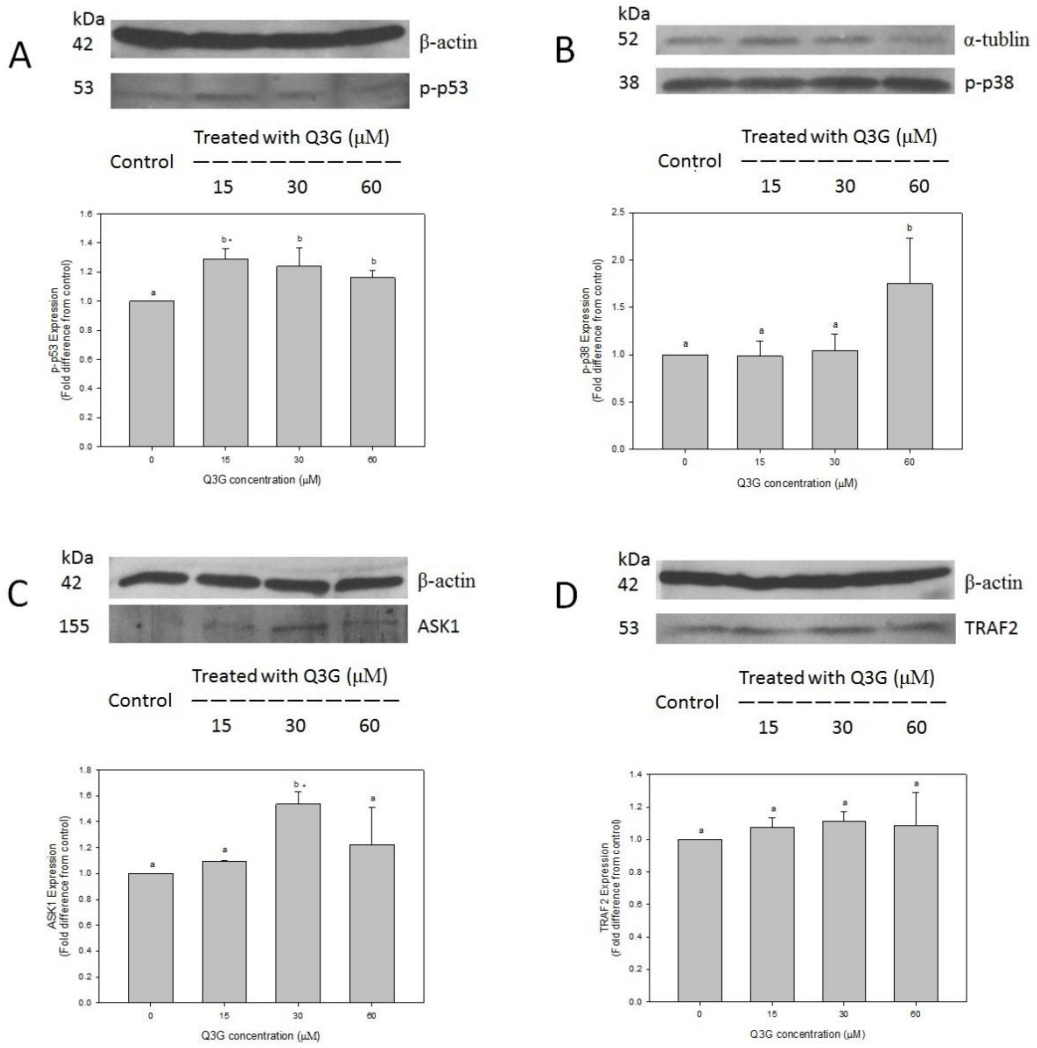

FIGURE 2.5 Effect of Q3G on expression of p-p53 (*A*), p-p38 (*B*), ASK1 (*C*) and TRAF2 (*D*) in human breast cancer MCF-7 cells. Bars with no letters in common in each panel are significantly different ($p< 0.05$). An asterisk (*) indicates $p< 0.01$ when compared to the control. Each value represents the mean ± SD with triplicates.

2.4 DISCUSSION

It has been reported that the incidence rate and mortality rate of cancers varies. The long-term incidence rate of breast cancer increased during 2004-2008, but the mortality rate decrease during 1998-2007. The 5-year survival rate is around 85% for all races during 2001-2007. The high survival rate is associated with optimized therapies and dietary factors (DeSantis et al 2011). The increased consumption of fruits and vegetables has been proved to be associated with a reduced risk of developing breast cancer (Liu et al 2009). Apple, the most commonly consumed fruit, is a good source of antioxidants (Wolfe et al 2008). In recent years, several studies indicated that apple extract can inhibit the growth of several cancer cell lines including breast cancer cells MCF-7 and MDA-MB-231 (Sun et al 2008; He et al 2008; Yang et al 2009). Quercetin-3-glucoside, one of the flavonoids in apple, is also proved to exhibit anticancer effect on breast cancer cells (Yang et al 2009). However, the underlying mechanism of Q3G induced antiproliferation and apoptosis remains unclear.

2.4.1 Antiproliferative activity of Q3G towards human breast cancer MCF-7 cells

In the present study, we tested the antiproliferative activity of Q3G using MCF-7 cells. Cell growth was inhibited by 74.3% at 60μM Q3G. The proteins that might be involved in the related signaling transduction pathways were then analyzed. p21 is a

member of the Cip/Kip family of CDK inhibitors. It can bind to and inhibit the activity of a broad range of cyclin/Cdk complexes, inducing cell cycle arrest (Gartel et al 2002). It can also bind to PCNA and inhibit the PCNA mediated DNA replication (Gohring et al 2001). In the present study, the increased level of p21, decreased levels of cyclin D1, Cdk4 and PCNA were observed in Q3G treated MCF-7 cells.

The p21 expression can be modulated by a p53-dependent pathway. The p53 protein is a proapoptotic protein. The enhanced phosphorylation level of p53 and the induced p21 expression were found in cancer cells treated by chemopreventive agents (Gartel et al 2002; Jung et al 2010; Baldi et al 2011; Nakatsuka et al 2011). In this study, the increased level of phosphorylated p53 was observed in Q3G treated MCF-7 cells. These data suggests that Q3G induces cell cycle arrest and inhibits proliferation through p53-dependent pathway.

Total p53 expression and phosphorylation are altered in cancer cells. Several studies showed the enhanced phosphorylation of p53 and caspase-3 cleavage followed by the activation of mitogen-activated protein kinase (MAPK). The phosphorylation of p38, one of the MAPKs, can significantly elevate the Ser^{33} and Ser^{46} phosphorylation on p53 protein and results in the negative regulation of cell cycle progression (Wu 2004; Bulavin et al 2004). p38 can be activated by both apoptosis signal-regulating kinase 1 (ASK1) or the environmental stress (Takeda et al 2003; Matsukawa et al 2004; Nagai et al 2007). ASK1 is a member of the MAP kinase kinasekinase family (MAP3K). Experimental results from ASK1-deficient mice have shown that ASK1 is required for

apoptosis that induced by oxidative stress, TNF and endoplasmic reticulum stress (Matsuzawa et al 2002; Takeda et al 2003). TNFα can regulate ASK1 through TNFα signaling activation. Then the TNFR-associated factor 3 (TRAF3) is ubiquitinated and degraded by proteasome. TRAF3 degradation leads to TRAF2 degradation, and the subsequent activation of MAP3Ks and MAPKs as well as NF-κB pathway (Wajant et al 2001; Bradley et al 2001; Karin et al 2009). In this study, the elevated phosphorylation of p38 and increased total expression of ASK1 were observed. But no degradation of TRAF2 was found, indicating that Q3G induces p38/MAPK activation and the subsequent cell cycle arrest and anti-proliferation via targeting ASK1.

2.4.2 Potential apoptosis induced by Q3G towards human breast cancer MCF-7 cells

Apoptosis is the programmed cell death. The regular apoptosis was always inhibited in cancer cells. Thus the induction of cancer cell apoptosis is important in cancer treatment.

Apart from proliferation, p53 protein is also associated with cell apoptosis. The p53-dependent apoptosis is mediated by pro-apoptotic proteins including Bax and Bcl-2 (Schuler et al 2001). Bcl-2 and its pro-apoptotic homolog Bax are two proteins in the Bcl-2 family. It has been reported that Bcl-2 is overexpressed and Bax is under-expressed in tumorigenesis (Wong et al 2008; Hockenbery 2010). Inhibition of Bcl-2 and induction of Bax expressions could be induced by anti-apoptotic drugs

(Hockenbery 2010). The high ratio of Bax to Bcl-2 causes the loss of mitochondrial membrane potential, thus stimulates the release of Apaf-1, procaspase-9, cytochrome c and other proteins (Yang et al 1997). In the present study, we found that Q3G reduced the expression of Bcl-2 and induced the expression of Bax in MCF-7 cells.

In order to further investigate the molecular mechanism involved in the Q3G induced apoptosis, the expressions of caspase proteins were analyzed. Our results showed increased level of cleaved caspase-9 in Q3G treated MCF-7 cells. Moreover, the increased cleavage of caspase-9 led to further activation of the downstream caspase-3, the apoptotic executioner. Both the decreased level of pro-caspase-3 and cleaved caspase3 (19 kD) were observed. The induced apoptosis by Q3G was also confirmed by TUNEL assay. The early apoptosis was increased to about 11% by 60μM Q3G treatment at 4 h. These data suggests that apoptosis in MCF-7 cells might be induced by p38/MAPK activation followed by the corresponding regulations on Bax, Bcl-2, caspase-9 and -3.

As is shown in Figure 2.6, we described for the first time, to our knowledge, quercetin-3-glucoside can specifically inhibit cell proliferation and induce apoptosis in human breast cancer estrogen-dependent MCF-7 cells.

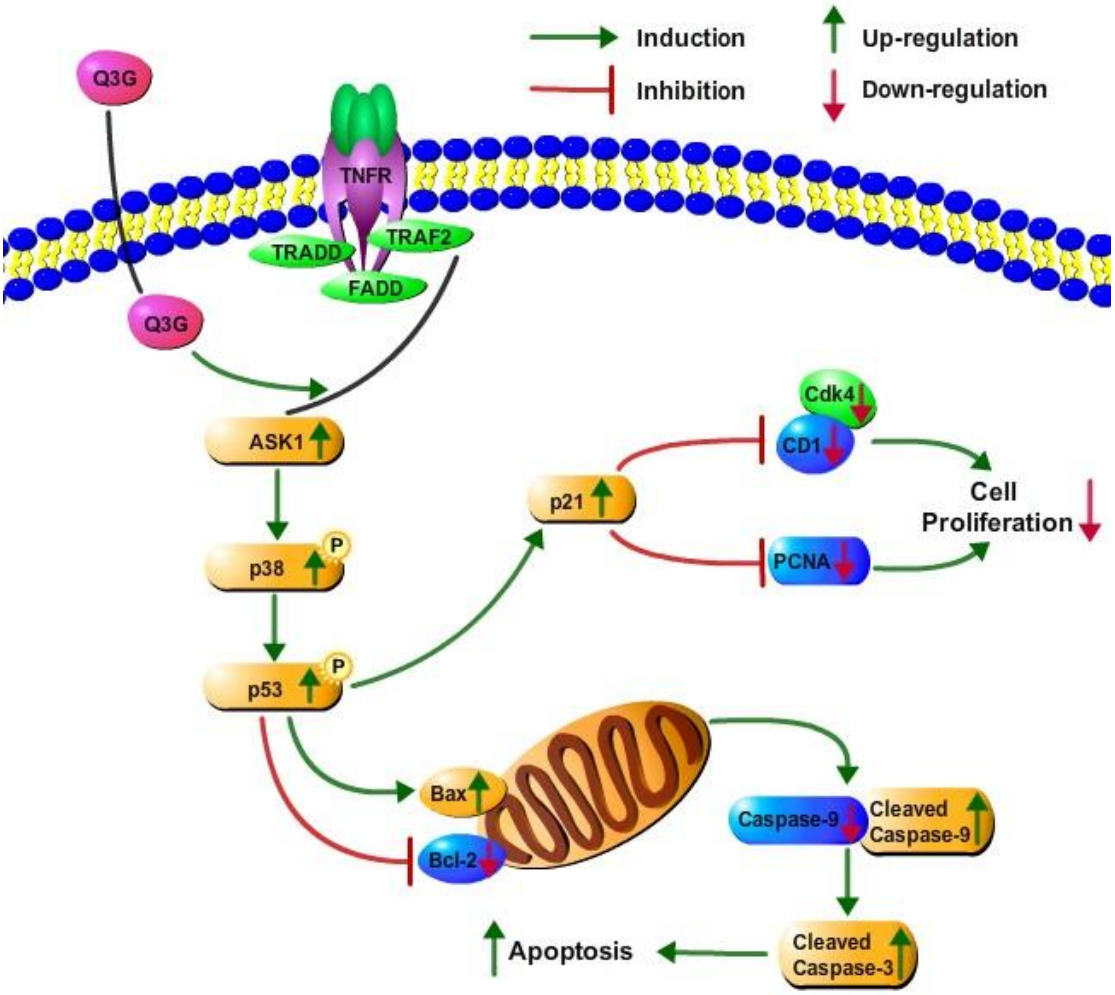

FIGURE 2.6 Potential mechanism of action of Q3G in regulating cell proliferation and apoptosis in human breast cancer MCF-7 cells through the p38/MAPK signal transduction pathway.

The Q3G treatment significantly up-regulated ASK1 expression without affecting TRAF2 protein level, and activated the phosphorylation of p38 and p53 proteins, indicating the activation of p38/MAPK pathway through targeting ASK1. The activated p38/MAPK pathway could then up-regulate p21 expression, and lead to the blockage of cyclin D1, Cdk4 and PCNA, resulting in the disrupted cell cycle and DNA replication. In addition, the activated p38/MAPK pathway can also result in down-regulated Bcl-2 level and up-regulated Bax level, and consequently lead to the increased cleavage of caspase-9 and caspase-3, resulting in the enhanced cell apoptosis.

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