URSOLIC ACID INHIBITED PROLIFERATION AND INVASION OF MDA-MB-231 HUMAN BREAST CANCER CELLS VIA REGULATING CELLULAR SIGNAL TRANSDUCTION PATHWAYS

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Breast cancer is the most common cancer and the second leading cause of cancer death among American women. Increased intake of fruits and vegetables has been suggested to be one of major dietary factors reducing the risk of breast cancer. The health benefits were largely attributed to phytochemicals in fruits and vegetables. Ursolic acid (UA), a widely-distributed triterpenoid in fruits, vegetables, herbs, and spices, has been reported to have anti-cancer activities. However, its mechanism of actions against breast cancer remain unclear.

The hypothesis of this study is that UA inhibits proliferation and invasion of MDA-MB-231 human breast cancer cells via regulating cellular signal transduction pathways. Specific objectives are designed as: a) to investigate anti-proliferation and anti-invasion effects of UA in MDA-MB-231 human breast cancer cells; b) To determine specific molecular targets of UA on cellular signal transduction pathways in MDA-MB-231 human breast cancer cells; and c) to investigate potential synergistic effects of combining ursolic acid with paclitaxel toward breast cancer proliferation.

UA significantly inhibited proliferation of MDA-MDB-231 human breast cancer cells in a dose-dependent manner at the concentrations without cytotoxicity. The EC_{50} value of anti-proliferative activity was 30.67 μM. UA at concentrations of 20, 30, and 40 μM significantly inhibited cell invasion. Additional tests associated anti-invasion activity of UA with antagonizing the stimulation of EGF.

UA affected 8 targeted proteins in cellular signaling pathway in primary signaling screening. Akt, mTOR and MAPK signaling pathways were involved. Western blots indicated UA significantly downregulated EGF-induced EGFR phosphorylation, which was correlated with reported inhibitory effects of UA on EGF-induced invasion. UA inhibited JAK/STAT3 and Akt activation, and downregulated NF-κB expression and activation.

UA, paclitaxel and their combination significantly inhibited proliferation of MDA-MB-231 and MCF7 breast cancer cells in a dose-dependent manner. Synergistic effect was observed at 95% inhibition rate in MDA-MB-231 breast cancer cells and at 50% inhibition rate in MCF7 cells.

We demonstrated that UA exhibited inhibitory activity toward proliferation and invasion of MDA-MB-231 cells via regulating cell signaling pathways. These data shed light on understanding the protective activity of plant foods against breast cancer.

BIOGRAPHICAL SKETCH

Hongyu Chen is a Ph.D. candidate in Department of Food Science, Cornell University. She majors in food science, and her minors are nutrition and toxicology sciences. Her research interest lies in food nutrition and functional foods.

Hongyu received her B.S. degree in Hebei Agriculture University, China, majoring in Food Quality and Safety. She had been working in the food nutrition lab since sophomore year, and won the Extracurricular Academic Work Competition of University. She won Academic Scholarships and received The Award of Merit Student for multiple times. Her graduation thesis was mentioned as Excellence in Graduation Thesis.

Hongyu received her M.S. degree in Hebei Agriculture University, China, majoring in Food Science. Her research covered food nutrition and toxicology. She won the Academic Scholarships, received the Award of Graduate Student and was mentioned as Outstanding Graduate of the Year in 2013.

Her current research focuses on anticancer activities of dietary phytochemicals. She received national funding from the CSC, as well as Cornell funds for her Ph.D. studies. She won the 3rd place in IFT Graduate Student Research Paper Poster Competition in 2016 and the 2nd place in TOMATO-FSN product development competition in 2016.

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LIST OF ABBREVIATIONS

AhR, aryl hydrocarbon receptor

Akt, protein kinase B

α-MEM, α-minimum essential medium ()

ARE, antioxidant-responsive element

AMPK, 5' AMP-activated protein kinase

Cdc, cell-division cycle proteins

CDK, cyclin-dependent kinases

Chemokines, chemotactic cytokines

Chk, checkpoint kinase

CI, combination index

CXCR4, chemokine receptor type 4

CYP450, cytochrome P450

DLT, dose-limiting toxicity

DMSO, Dimethyl sulfoxide

ECM, extracellular matrix

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

ELAM-1, endothelial-leukocyte adhesion molecule 1

ER, endoplasmic reticulum

ER, estrogen receptor

ERK, extracellular signal-regulated kinase

FBS, fetal bovine serum

Fen1, Flap endonuclease 1

HER2, human epidermal growth factor receptor

GCL, glutamate cysteine ligase

γ-GCS, gamma-glutamylcysteine synthetase

GLUT, glucose transporter

GPx, glutathione peroxidase

GSK-3, glycogen synthase kinase 3

GRO-α, growth-related oncogene-α

GST, glutathione S-transferase

HIF-1, hypoxia-inducible factor-1

HO-1, heme oxygenase-1

ICAM-1, Intercellular adhesion molecule 1

IFN-γ, interferon gamma; IL, interleukin

IκB, inhibitor of Kb

IKK, IκB kinase

JAK, Janus kinase

IP-10, interferon gamma (IFN-γ)-induced protein 10

JNKs, c-Jun amino-terminal kinases

KEAP1, Kelch-like-ECH-associated protein 1

LC3, microtubule-associated protein 1 light chain 3

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MTD, maximum tolerated dose

miRNA, microRNA

MDC, monocyte-derived chemokine

MMPs, matrix metalloproteinase

mTOR, mammalian target of rapamycin

NCI, national cancer institute

NQO, NAD(P)H quinone oxidoreductase

Nrf2, nuclear factor erythroid 2-related factor

PBS, phosphate-buffered saline

PCNA, proliferating cell nuclear antigen

PARP 1, Poly [ADP-ribose] polymerase 1

PI3K, phosphoinositide 3-kinase

PMSF, phenylmethylsulfonyl fluoride

PR, progesterone receptor

PTEN, phosphatase and tensin homolog

PVDF, Polyvinylidene fluoride

Q3G, quercetin 3-β-D-glucoside

RTK, receptor tyrosine kinases

RPTK, transmembrane receptors with intrinsic protein tyrosine kinase activity

ROS, reactive oxygen species

SD, standard deviation

SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SFM, serum free medium

SMAD, mothers against decapentaplegic homolog

SOD, superoxide dismutase

STAT, signal transducer and activator of transcription

TGF, transforming growth factor

TLR9, Toll-like receptor 9

TNFα, tumor necrosis factor alpha

TRAMP, transgenic adenocarcinoma of the mouse prostate

UGT, uridine diphosphate-glucuronosyl transferases

UA, Ursolic acid

uPA, urokinase plasminogen activator

VCAM-1, vascular cell adhesion molecule 1

WHO, world health organization

XRE, xenobiotic responsive element

Chapter 1 Introduction

1.1 Breast Cancer

Cancer is a severe health problem that significantly undermines life span and quality. According to the *World Cancer Report* by WHO (world health organization), cancer is viewed as a major contributor to morbidity and mortality, with approximately 14 million new cases and 8 million cancer-related deaths in 2012 (Stewart and Wild, 2014). Among men, the three most common cancers were the lung (16.7% of the total), prostate (15.0%), and colorectal cancers as diagnosed in 2012 (10.0%); while lung cancer (23.6% of the total), liver cancer (11.2%) and stomach cancer (10.1%) were the most common causes of cancer death (Stewart and Wild, 2014). Among women, the three most common diagnosed cancers were the breast (25.2% of the total), colorectal (9.2%), and lung (8.7%) cancers in 2012; they also represented the most common causes of cancer death in woman (14.7% for breast cancer, 13.8% for lung cancer and 9.0% for colorectal cancer) (Stewart and Wild, 2014). In both sexes combined, the five most common cancers were the lung (13.0% of the total), breast (11.9%), colorectum (9.7%), prostate (7.9%), and stomach (6.8%); they constitute half of the overall global cancer burden (Stewart and Wild, 2014). The *World Cancer Report* concluded that breast cancer had a substantially higher incidence (43.3 per 100 000) than any other cancer in woman and had the highest mortality rate of all cancers in women (12.9 per 100 000) (Stewart and Wild, 2014).

Each year, the American Cancer Society reports the current statistics of cancer incidence, mortality, and survival, as well as estimates the probability of new cancer cases and deaths in the United States (Siegel et al., 2017). According to NCI's report (National Cancer Institute), breast cancer is the most common type of cancer and second leading cause of cancer death among American women (Siegel et al., 2017). As estimated by American Cancer Society, in 2017, approximately 252,710 new cases of invasive breast cancer would be diagnosed in women and 2,470 in men; meanwhile estimated 41,070 breast cancer deaths (40,610 women, 460 men) are expected (Siegel et al., 2017)

Most breast cancers are invasive, or infiltrating, and they break through the walls of the glands or ducts where they are originated and then grow into surrounding breast tissues and migrate to distant organs such as bone, brain, liver, and lung (Kim and Baek, 2010; Simpson et al., 2005). Breast cancer is usually treated with surgery, and most patients with breast cancer will be offered adjuvant treatment such as radiotherapy, hormones, chemotherapy and biological agents (Davies, 2016). Chemotherapy is one of the key treatment for breast cancer: for slow progression breast cancers, monotherapy is enough, such as using anthracycline or taxane; for rapid progression breast cancers, combination chemotherapy is needed such as paclitaxel with gemcitabine, docetaxel with gemcitabine, docetaxel with capecitabine, anthracycline with taxane, and anthracycline with cyclophosphamide (Harbeck and Gnant, 2017).

Triple-negative breast cancers are defined as tumors that lack expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), which highly increase treatment difficulty. Those three receptors are frequent targets of chemotherapies (Foulkes et al., 2010; Hulka and Moorman, 2001). These tumors account for approximately 15% of invasive breast cancers, with a higher histologic grade, a worse prognosis, and more common distant recurrences in the brain and visceral metastases than in hormone receptor-positive tumors (Cleator et al., 2007; Foulkes et al., 2010).

Triple-negative cancers often require combination therapies (Davies, 2016; Harbeck and Gnant, 2017; Hulka and Moorman, 2001). There is no preferred standard form of chemotherapy for triple-negative breast cancers, and their treatment is currently selected as it is for other cancer subtypes; however, hormone therapy and drugs that target estrogen, progesterone, and HER-2 are ineffective in triple-negative breast cancers (Foulkes et al., 2010; Harbeck and Gnant, 2017). Available therapies for triple negative breast cancer include surgery with adjuvant chemotherapy, radiotherapy, and various neoadjuvant (Chacon and Costanzo, 2010; Cleator et al., 2007). Specifically, for chemotherapy, some agents are cytotoxic that cause DNA inter-strand breaks or inhibition of DNA repair, while some others have specified targets, such as: PARP1 (poly ADP-ribose polymerase 1, enzyme responsible for DNA repair), surface receptors (e.g. EGFR, the epidermal growth factor receptor), mitogen activated protein (MAP)-kinase pathway, Wnt pathway, the protein kinase B (Akt) pathway and mTOR (mammalian target of rapamycin) pathway (Chacon and Costanzo, 2010; Cleator et

al., 2007; Foulkes et al., 2010; Harbeck and Gnant, 2017).

In this study, a triple-negative breast cancer cell line MDA-MB-231 will be employed to test anti-proliferation and anti-invasion effects of ursolic acid and its regulation on cellular signal transduction.

1.2 Diet and Cancer

Diet is a bidirectional risk factor that actively affects cancer development. Several dietary factors, such as non-excessive calories, increased intake of whole grains, fruits and vegetables, dietary fibers, reduced intake of red meat, and less sugar consumption reduced risk of developing cancers (Bingham and Riboli, 2004; Donaldson, 2004; Kotecha et al., 2016; Surh, 2003). Protective components in a cancer prevention diet include phytochemicals, selenium, folic acid, vitamin B-12, vitamin D, chlorophyll, antioxidants (such as the carotenoids), dietary fibers, and prebiotics (Bingham and Riboli, 2004; Donaldson, 2004; Kotecha et al., 2016; Melino et al., 2011; Miller and Snyder, 2012). These components have higher distribution in whole fruits, vegetables and whole grains. (Cotterchio et al., 2008; Haminiuk et al., 2012; Lei et al., 2016; Miller and Snyder, 2012).

A wide array of population-based studies has highlighted plant-based diet in cancer prevention (Liu, 2013b; Marchand, 2002; Surh, 2003). Vegetables, fruits and whole grains are all significant sources of beneficial bioactive compounds. National Cancer Institute (NCI) promotes the ‘Five-A-Day for Better Health’ program to encourage

people in the United States to eat at least five servings a day of fruits and vegetables, to reduce the risk of cancer and other chronic diseases. Epidemiological studies suggest that, high intake of fruit and vegetables (especially cruciferous vegetables) have a moderately reduced risk of cancer at several sites, especially for oral cavity and the upper gastrointestinal tract cancers (Aune et al., 2012; Turati et al., 2015; Vieira et al., 2015). Whole grains, abundant in dietary phytochemicals and fibers, are associated with reduced risk of gastrointestinal and pancreatic cancers (Lei et al., 2016; Makarem et al., 2016; Okarter and Liu, 2010). The association between plant-based diet and reduced risk of other common cancers, such as breast cancer, prostate cancer, liver cancer and bladder cancer is not conclusive (Aune et al., 2012; Liu, 2013a; Makarem et al., 2016; Miller and Snyder, 2012; Vieira et al., 2015). However, it is recommended to increase intake of vegetables, whole fruits and whole grains with a wide variety for cancer and other chronic disease prevention, especially for those people who have original low consumption (HHS and USDA, 2015; Liu, 2013b; Okarter and Liu, 2010).

As for breast cancers, some but not all epidemiological studies have linked increased intake of fruits and vegetables with reduced breast cancer risk (Mourouti et al., 2015; Thomson, 2012; Willett, 2001). Meta-analysis of prospective studies suggested high intake of fruits, and fruits and vegetables combined, but not vegetables, was associated with a weak reduction in risk of breast cancer: random effects models suggested that summary relative risk (RR) for the highest versus the lowest intake was 0.89 (95% CI: 0.80-0.99) for fruits and vegetables combined, 0.92 (95% CI: 0.86-0.98) for fruits, and 0.99 (95% CI: 0.92-1.06) for vegetables (Aune et al., 2012). However, in this meta-

analysis study, even the pooled result suggested reduced risk of breast cancer was associated with the increased intake of fruits and vegetables, not all the individual studies had significant difference (Aune et al., 2012). Another cohort study for triple negative cancer indicated that vegetable consumption was inversely associated with risk of ER- breast cancer in pooled analyses among 993 466 women followed for 11 to 20 years in 20 cohort studies, while no association between total fruit and vegetable intake and risk of overall breast cancer was observed (Jung et al., 2013).

Dietary approach helps provide preventive, safe and economical anti-cancer strategies (Kotecha et al., 2016; Surh, 2003). The increase in the cost of health care and drug prices and potential side effects promotes researches on alternative modes of anti-cancer approaches (HemaIswarya and Doble, 2006). It has been reported that 10 ~ 70% (average 35%) of human cancer mortality is diet-related, and more than two-thirds of human cancers could be prevented by healthier lifestyle (Doll and Peto, 1981; Surh, 2003). Dietary guidance, with the support of epidemiologic evidence, recommends consumption of whole grains, whole fruits and vegetables for prevention of chronic disease including cancer; and plant-derived foods are significant sources of dietary fiber, resistant starch, and oligosaccharides, fermentable carbohydrates, trace minerals, and phenolic compounds (Services and Agriculture, 2015; Slavin, 2000; Surh, 2003). Whole natural products and derived bioactive compounds showed anti-cancer activities in various studies. They are consumed in low doses and may have best outcomes for cancer prevention among people without cancer (Donaldson, 2004; Hussain et al., 2016; Kim et al., 2010; Martins et al., 2011). Advantages of anti-cancer

strategies by dietary approach are majorly due to additive and synergistic effects and low toxicity of dietary agents (HemaIswarya and Doble, 2006; Liu, 2004; Liu, 2013a).

Encouraging in-vitro studies have associated some non-toxic or low-toxic cocktails of food components (such as phytochemicals) with anti-cancer activities and even synergistic effects in human cancer cells (Chen et al., 2009; Huang et al., 2010; Singh et al., 2014; Sukumari-Ramesh et al., 2011; Yang and Liu, 2009). Apple extracts and quercetin 3-β-D-glucoside (Q3G) showed synergistic effects in antiproliferative activity in MCF-7 human breast cancer cells at the concentrations without cytotoxicity: the EC_{50} values of apple extracts and Q3G were 2- and 4-fold lower, respectively, when used in combination than applied alone; the combination index (CI) values at 50 and 95% inhibition rates were 0.76 and 0.42; the dose-reduction index (DRI) values of the apple extracts and Q3G at 50% inhibition rate were reduced by 2.03 and 4.28 (Yang and Liu, 2009). Ellagic acid and resveratrol synergistically reduced cell viability after treated for 48 hours and caspase-3 activity in human leukemia cells (Mertens-Talcott and Percival, 2005). The mechanism behind the synergism effects of bioactive compounds may related to regulation on more than one signal transduction pathway, stabilization of the compounds, or increasing the bioavailability of compounds (HemaIswarya and Doble, 2006; Yang and Liu, 2009). Even challenge still exists since it is difficult to characterize the dietary factors in clinical trials, it is useful to understand how dietary agents interact with cancer cells, the immune system, and anti-oxidant system, and hence offer effective, safe and affordable solutions in cancer prevention for human beings (HemaIswarya and Doble, 2006; Kotecha et al., 2016;

Liu, 2013b; Miller and Snyder, 2012).

1.3 Dietary phytochemicals

Anti-cancer benefits of plant-based foods are largely attributed to phytochemicals. Phytochemicals are chemical compounds that occur naturally in plants after secondary metabolism, where phyto refers to "plant" in Greek. Based on chemical structure, phytochemicals can be generally classified into carotenoids, polyphenols, nitrogen-containing compounds and organosulfur compounds. As special benefits of phytochemicals intake through food, additive and synergistic effects of dietary phytochemicals are consistently observed in many studies (Liu, 2013b).

1.3.1 Major group of phytochemicals

There are thousands of phytochemicals have been found to be naturally produced in plants, their function is highly related to chemical structure and functional groups. Phytochemicals have great chemical diversity, majorly include carotenoids, polyphenols, nitrogen-containing compounds and organosulfur compounds (Liu, 2004; Liu, 2013b). Phenolic compounds is the largest phytochemical group. They are a class of chemical compounds consisting of a hydroxyl group bonded directly to an aromatic hydrocarbon group and can be categorized to subgroups by their backbone, including flavonoids, phenolic acids, lignanes, coumarins, chromones, antraquinones, and stilbenes (Bahramsoltani et al., 2015; Boudet, 2007; Crozier et al., 2010; Haminiuk et al., 2012). Flavonoids are further classified to anthoxathins (flavones and flavonols), flavanones, flavanonols, flavans (flavanols, thearubigin and proanthocyanidins),

anthocyanins, and isoflavonoids (Crozier et al., 2010; Liu, 2004) . Alkaloids are another big class of phytochemicals containing containing at least one nitrogen atom, the major groups include pyrrolidine derivatives, tropane derivatives, pyrrolizidine derivatives, piperidine derivatives, quinolizidine derivatives, indolizidine derivatives, isoquinoline derivatives, pyridine derivatives, oxazole derivatives, thiazole derivatives, purine derivatives, indole derivatives, acridine derivatives, quinoline derivatives, muscarine derivatives, diterpenes and steroids (Bahramsoltani et al., 2015; Michael, 2008b; Robertson and Stevens, 2014).

1.3.2 Plant foods and major phytochemicals

Dietary phytochemicals are majorly derived from plant foods, such as whole fruits, vegetables and whole grains. Phenolic compounds, naturally produced by plants and microorganisms, have a wide distribution in fruits, vegetables, grains, spices and herbs, either in their free or bound forms (Boudet, 2007; Karakaya, 2004; Liu, 2004; Martins et al., 2011; Soto et al., 2011). Flavonoids from the phenolic group are reported to be heat stable, and exhibited relatively low losses after cooking or frying (Hertog et al., 1993). They have a general structure consists of two phenyl rings and a heterocyclic ring, and are widely distributed in plant diet and considered as a functional component of herbal medicine. For instance, phenolic compounds in green tea products are predominately composed of catechin derivatives from flavanol subgroup of flavonoids, such as catechin, gallocatechin, catechin 3-gallate, gallocatechin 3-gallate, epicatechins, epigallocatechin, epicatechin 3-gallate, and epigallocatechin 3-gallate; while flavonols and phenolic acids have a lower occurance (Amirdivani and Baba,

2015; Balentine et al., 1997; Hertog et al., 1993). Isoflavonoids (such as genistein, daidzein, and pelargonidin) are produced through phenylpropanoid pathway for flavonoid synthesis in vascular plants; their typical dietary sources are legumes (such as soybean, peas) and legume-based products (Cotterchio et al., 2008; Veitch, 2009). Anthocyanins, such ascyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin, are water-soluble flavonoids; these natural pigments have higher distribution in fruits and vegetables with dark red, blue, purple dark orange and dark green colors (Haminiuk et al., 2012; Karakaya, 2004). Stilbenes, another group of phenolics, are precursors of natural dyes that have heterogeneous distribution in plants, especially grape plants (Cassidy et al., 2000; Riviere et al., 2012; Waldeck, 1991). Resveratrol, a bioactive stilbenoid, has higher occurance in peanuts and skins of fruits such as red grapes, mulberries, blue berries and raspberries (Sanders et al., 2000; Shrikanta et al., 2015; Walle, 2011). Organosulfur compounds are organic compounds containing sulfur. They typically have foul odor and can be widely found in spices, fruit, nuts, vegetables and fungi, for example, diallyl sulfide, S-allyl cysteine, allicin from garlic and green onions, and sulforophane from allium, broccoli, sprouts and cruciferous vegetables (Chu et al., 2017; Donaldson, 2004; Melino et al., 2011). Alkaloids are traditionally purified by acid-base extraction from herbal medicine plants. They usually evoke a bitter taste and has showed potential various pharmacological activities including anti-inflammation, anti-bacterial, psychotropic and stimulant activities. Those nitrogen-containing compounds have various distributions, e.g. capsaicin from red chilli, piperine from black pepper, indole-3-carbinol from cruciferous vegetables, diosgenin and other steroids derivatives from

fenugreek (Bahramsoltani et al., 2015; Michael, 2008a). Other samples for plant-derived phytochemicals include: carotenoids, such as beta-carotene from carrots and lycopene from tomatoes; ursolic acid, a pentacyclic triterpenoid from apple, cranberries, prunes, and many other fruits or herbs; anethol from anise, camphor, and fennel; limonene from citrus fruits; curcumin, a diarylheptanoid from turmeric, etc. (Liu, 2013a; Surh, 2003; Yeh et al., 2010).

1.4 Phytochemicals, carcinogenesis and cell signaling transduction

1.4.1 Carcinogenesis and cellular regulation

Cancer formation (i.e. carcinogenesis) is a multistep process whereby normal cells are reprogrammed to undergo uncontrolled cell division and transformed into cancer cells, as latterly form a malignant mass (tumor) (Barrett, 1993; Hanahan and Weinberg, 2000; Tomasetti et al., 2017). Changes at cellular, genetic and epigenetic levels would all contribute to this progression. The classic cancer theory recognizes the carcinogenesis process as briefly three steps: tumor initiation, promotion and progression (Barrett, 1993; Berenblum and Shubik, 1947; Land et al., 1983). Initiation is the rapid and trigger process that involves the exposure to a carcinogen, for example the initial uptake of carcinogenic agent and its distribution to organs and tissues where metabolic activation or detoxification can occur, and the covalent interactions between reactive species and targeted DNA, resulted in genotoxic damage and cell transformation (Cohen and Arnold, 2011; Land et al., 1983; Surh, 2003). Different from initiation, tumor promotion is a relatively lengthy and reversible evolution process that allows preneoplastic cells to accumulate through expanded survival and

proliferation. The promotion process is typically regulated by cell signaling transduction pathways with participators of receptors, kinases, regulatory proteins, transcriptional factors and cyclins (Arends, 2013; Barret, 1993). Progression, the final stage of neoplastic transformation, is marked by cell behaviors of fast-speed growth and high invasive and metastatic potential (Cohen and Arnold, 2011; Land et al., 1983). After progression, the cancer cells invade and migrate to secondary sites through lymph system (metastasis) (Stacker et al., 2002; van Zijl et al., 2011). The bidirectional communication between cells and their microenvironment is critical for cancer development and metastasis and is a promising therapeutic target for reduced risk of resistance and tumor recurrence (Quail and Joyce, 2013).

One typical mechanism of action of anti-cancer phytochemicals is that they regulate adaptive cellular stress response pathways. The whole picture of the molecule cross-talk for cancer cell fate is still not totally clear. However, despite in such conditions that some molecules directly work on nuclei DNA, cellular regulation is carried out by signal transduction, which normally begins with interactions between extracellular substances and cell membrane receptors, followed by cascades consist of regulatory proteins and/or transcription factors, thus affects expression of oncogenes and defensive proteins, resulting in different cell fates (e.g. apoptosis, cell-cycle arrest, proliferation, or invasion and reflective feedbacks to upstream(Barret, 1993; Dhillon et al., 2007; Koury et al., 2017; Lee et al., 2003; Sung et al., 2012; Surh, 2003). Most evidence supports that phytochemicals intervene the signaling pathways and finally regulate on downstream genes/proteins related with specific cell behavior: for instance,

Bax, Bcl-2 family proteins and caspases are related with apoptosis, Myc is related with cell cycle arrest, cyclins and CDK (cyclin-dependent kinases) are related with cell proliferation, and MMPs (matrix metalloproteinase) are related with invasion (Barret, 1993; Haldar et al., 1997; Overall and Lopez-Otin, 2002; van Zijl et al., 2011). The participators along signaling pathways can be regulated either via activation (phosphorylation and cleavage) or expression (at genetic, epigenetic or translational levels) (Cohen and Arnold, 2011; Land et al., 1983; Surh, 2003).

1.4.2 Molecular targets of phytochemicals in cellular signal transduction pathways

Phytochemicals not only act as antioxidants affecting DNA integrity, most of them participate in anti-cancer signaling cascades that are related to cell survival, proliferation and invasion, at transcriptional and post-transcriptional levels. (Liu et al., 2015b; Schroeder et al., 2011; Singh et al., 2014; Surh, 2003). Cell signaling is the communication and interaction process that governs cells activations, include cell cycle arrest, proliferation, apoptosis, autophagy, and migration. The molecular targets in cell signaling transduction pathways for phytochemicals include membrane receptors, kinases, downstream cancerous or tumor-suppressor proteins, transcriptional factors, miRNAs, cyclins and caspases (Koury et al., 2017; Reya and Clevers, 2005; Sung et al., 2012). Sometimes the phytochemicals work on activation (e.g. cleavage, release or phosphorylation) of the proteins, while other times the expression of certain proteins are directly downregulated. The cell signaling pathways exhibit lots of crosstalk, the most reported cancer-related signaling pathways include

MAPK, PI3K/Akt and Wnt/β-catenin pathways, while new researches further reported more epigenetic level regulations and interactions between phytochemicals and related miRNAs (Cao et al., 2017; Koury et al., 2017; Mundi et al., 2016; Tilghman et al., 2013). Some molecular targets (e.g. p53 tumor suppressor protein, STAT3 transcriptional factor) are central players, while some markers and downstream proteins are more specifically linked to certain cell behaviors, for example, cyclins are related to cell cycle regulation and proliferation, caspases are related to apoptosis and NF-κB transcriptional factor is more related to inflammation (Arends, 2013; Quail and Joyce, 2013).

1.4.2.1 Effects of dietary phytochemicals on cancer initiation

Initiation of a cancer requires exposure to carcinogen, DNA damage and activation of oncogene (Cohen and Arnold, 2011; Land et al., 1983). Carcinogens are typically transformed by phase I metabolizing enzymes to more DNA-binding reactive forms, but those metabolites can be detoxified through conjugation catalyzed by phase Ⅱ metabolizing enzymes into water-soluble forms which can then be eliminated from the body; where both enzymes can be targets for drug intervention (Hassan et al., 2015; Talalay, 1989). Early research related singlet oxygen and other free radicals with DNA damage and tumor promotion (Land et al., 1983; Talalay, 1989). Several mechanisms, especially those associated with DNA damage and repair, e.g. oxidative stress and antioxidant profile, phase I and phase II enzyme action are highly related to cancer initiation. Antioxidant phytochemicals are typically capable of capturing free radicals, hence influence antioxidant response, DNA damage/repair, and tumor promotion

(Forester and Lambert, 2011; Owen et al., 2000; Roleira et al., 2015). Phytochemicals showed their impact on bio-activation or detoxification of carcinogens through affecting phase Ⅰ and phase Ⅱ enzyme activities, including affecting cytochrome P450 and antioxidant enzymes (Bowen-Forbes et al., 2010; Davenport and Wargovich, 2005; Wang et al., 2012b).

Phase I metabolism reactions, such as hydrolysis, oxidation, reduction and deamination, play vital role in metabolic activation for carcinogens and some bioactive compounds. Various studies have indicated that the superfamily of enzymes, as specific cytochrome P450 form (CYP450), activate a wide range of known carcinogens via phase I metabolism. (Gelboin et al., 1995; Mittal et al., 2015). On the other hand, CYP450- catalyzed oxidative metabolism of drugs and bioactive phytochemicals could be considered as detoxification process (Moon et al., 2006; Omura, 1999). CYP450, a subfamily of hemoproteins, are the terminal oxidase enzymes in electron transfer chains, while the term P450 was derived from the spectrophotometric peak at the maximum absorption wavelength of 450 nm when discovered (Omura, 1999). Genes encoding CYP enzymes are named following standard nomenclature with group serial number, subfamily code and gene serial number (For example, human CYP1A1 gene encodes cytochrome P450 1A1 enzyme). Phytochemicals have shown impacts on CYP450 through a host of CYP genes. Dietary flavonoids has been found to modulate the CYP450 system through induction, activation and inhibition of specific CYP isozymes (Moon et al., 2006). Earlier researches have showed that many flavonoids, flavone, naringenin, tangeretin and tea

flavonoids, inhibited 450 2B-, 2E1- and 3A-dependent activities, while flavone, naringenin, tangeretin also inhibited CYP1A2 dependent metabolism (Fuhr et al., 1993; Obermeier et al., 1995). Recent point mutation research of CYP2A6 showed that a polymorphic variant CYP2A6.25 conferred new substrate specificity toward 7-ethoxycoumarin, coumarin, flavone, α-naphthoflavone, flavanone and hydroxyflavanone, while CYP2A6 affects flavonoid hydroxylation metabolism and CYP2A6 mutations may suppress anti-cancer activities of flavonoids and result in tumor growth (Uno et al., 2015).

The phase Ⅱ enzyme induction system crucially acts in cellular stress response to eliminate a broad range of electrophilic and oxidative toxicants before they damage the DNA (Hassan et al., 2015; Itoh et al., 2010; Talalay, 1989). Antioxidant phytochemicals protect cell components not only by scavenging free radicals, but also by inducing de novo expression of genes that encode defensive proteins, e.g. phase Ⅱ enzymes and/or antioxidant enzymes. Those enzymes catalyze conjugation and neutralize electrophilic chemicals by sulfation, glucuronidation, glutathioylation, acetylation, methylation, etc., the representatives include glutathione peroxidase (GPx), glutamate cysteine ligase (GCL), gamma-glutamylcysteine synthetase (γ-GCS), glutathione S-transferase (GST), UGT (uridine diphosphate-glucuronosyl transferases), superoxide dismutase (SOD), NAD(P)H quinone oxidoreductase (NQO) and heme oxygenase-1 (HO-1) (Hassan et al., 2015; Talalay, 1989). Early researches have linked green tea polyphenols to increased activities of glutathione peroxidase, catalase, and quinone reductases in various target organs and potential cancer chemopreventive

effects (Khan et al., 1992). Then increased researches are focusing on the underlying mechanisms through cell signaling (Furfaro et al., 2016; Hu et al., 2006; Liu et al., 2015a; Su et al., 2013; Zhang et al., 2013).

Two underlying mechanisms are majorly responsible for phase Ⅱ enzyme induction: Nrf2–ARE (nuclear factor erythroid 2-related factor-antioxidant-responsive element) signaling and AhR–XRE (aryl hydrocarbon receptor–xenobiotic-responsive element) signaling (Fukunaga et al., 1995; Itoh et al., 1997; Menegon et al., 2016). The 5'-flanking regions of certain stress-response genes contain a common antioxidant-responsive cis-element, known as the ARE, which is bound and regulated by leucine zipper (bZIP) transcription factors, such as Nrf, Jun, Fra, Fos, Maf and Ah receptor (Fukunaga et al., 1995; Tkachev et al., 2011). Nrf2 is a basic helix–loop–helix transcription factor that involves in antagonizing cancer initiation, it regulates the expression of a host phase Ⅱ enzymes, hence assists carcinogens detoxification and oxidative stress prevention (Itoh et al., 1997). A cytoplasmic actin-bound inhibitor of Nrf2, the Kelch-like-ECH-associated protein 1 (KEAP1), blocks its translocation to the nucleus; oxidants can oxidize and phase Ⅱ enzyme can covalently modify the cysteine residues of KEAP1 and dislocate Nrf2 (Itoh et al., 2010; Itoh et al., 1999). Certain kinases (Also see 3.3) e.g. PI3K, ERK, JNK and protein kinase C (PKC) phosphorylate Nrf2 at serine (S) and/or threonine (T) residues, hence help release Nrf2 from KEAP1 and subsequently translocate to the nucleus, triggering protective antioxidant responses (Liu et al., 2015a; Tkachev et al., 2011; Zipper and Mulcahy, 2003). Earlier research showed sulforaphane, carnosol, curcumin and green tea

polyphenols, induced activation of phase Ⅱ detoxifying enzymes in vitro via Nrf2-ARE regulation, which are related to adaptive pathways (e.g. Akt and MAPK pathways) (Balogun et al., 2003; Hu et al., 2006; Martin et al., 2004; Wu et al., 2006). A recent study reports dietary cocoa be protective against colitis-associated cancer through Nrf2/Keap1 pathway activation, with increased levels of enzymatic antioxidants and decreased levels of inflammatory promotors (Pandurangan et al., 2015). Curcumin inhibited proliferation of breast cancer cells through Nrf2-mediated downregulation of Fen1 expression, where Fen1 (Flap endonuclease 1) is a DNA repair-specific nuclease and its overexpression is related to breast cancer development (Chen et al., 2014). Sulforaphane was suggested to enhance Nrf2 expression in prostate cancer cells through DNA demethylation and histone modifications in epigenetic regulations (Zhang et al., 2013). Z-Ligustilide, a phytochemical from widely used herb Radix *Angelicae Sinensis* in Asia, epigenetically restored Nrf2 gene expression in prostate cancer cells through demethylation the Nrf2 promoter CpGs, and hence upregulated gene expression of Nrf2 downstream detoxification enzymes HO-1 and NQO-1 (Su et al., 2013).

However, even traditionally Nrf2 has been considered as a tumor suppressor due to anti-oxidant and cytoprotective activities, recent studies indicate that hyperactivation of the Nrf2 favors the survival of malignant cells, which make Nrf2 play dual roles in cancer (Menegon et al., 2016). On the other hand, since Nrf2 and HO-1 are frequently upregulated in different types of tumors, which correlate with tumor aggressiveness and resistance to therapy, the cellular defense response of Nrf2/HO-1 is also a

promising target for overcoming resistance to cancer therapies (Furfaro et al., 2016).

1.4.2.2 Effects of dietary phytochemicals on cancer promotion signaling: regulation of cell proliferation, apoptosis, cell cycle, and autophagy

Once oncogenes get activated and carcinogenesis gets initiated, cells start self-defense actions which can be promoted by certain phytochemicals (Kotecha et al., 2016; Michl et al., 2016; Sukumari-Ramesh et al., 2011; Surh, 2003). Carcinogenesis signal transduction is an interactive process among extracellular matrix, cell membrane, plasma and nucleus via multiple regulators. After signal transduction, cells give reflective responses and take actions on deciding cell fate, such as cell proliferation, apoptosis, and cell cycle arrest (Barrett, 1993; Dhillon et al., 2007; Sukumari-Ramesh et al., 2011). The final targets of cell signaling pathways majorly include: apoptosis regulators, such as Bax, Bad, Bcl-2 family proteins and caspases; cell cycle regulators, regulators, such as myc and Chks (Checkpoint kinase); and proliferation regulators, such as cyclins, Cdcs (cell-division cycle proteins) and CDKs (cyclin-dependent kinases) (Barret, 1993; Cohen and Arnold, 2011; Haldar et al., 1997; Overall and Lopez-Otin, 2002; van Zijl et al., 2011). The major signaling channels is shown in Figure 1 and discussed in following paragraphs.

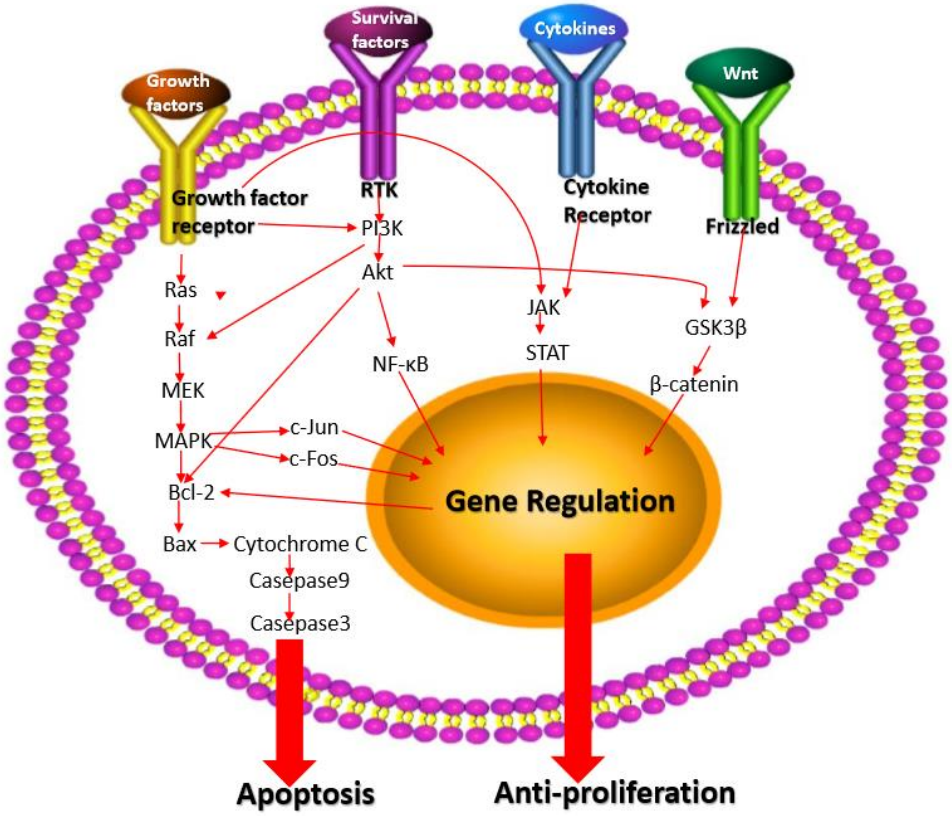

Figure 1. Major signaling pathways involved in cancer progression (Bonni et al., 1999; Burotto et al., 2014; Koury et al., 2017; Mundi et al., 2016; Rawlings et al., 2004; Reya and Clevers, 2005).

1.4.2.2.1 MAPK pathways

MAPK/ ERK pathway (MAPK for mitogen-activated protein kinase, ERK for extracellular signal-regulated kinase) is an essential route in cell survival and growth regulation, which can be targeted by phytochemical for pro-apoptosis, anti-invasion and anti-proliferation effects in various cancers (Bonni et al., 1999; Burotto et al., 2014; Lee et al., 2003). It is one of the main downstream effectors of membrane receptors, e.g. ER (estrogen receptor), EGFR (epidermal growth factor receptor) and TNFα (tumor necrosis factor alpha) (Gallo and Johnson, 2002; Lee et al., 2003). This

route can also be described as Ras-Raf-MEK-ERK transduction cascade, where Ras is an activator and Raf-MEK-ERK is a crucial link that comprised of three protein kinases: a MAPK kinase kinase (MAPKKK, for instance Raf), a MAPK kinase (MAPKK, for instance MEK) and a MAPK, typical terminal MAPKs include ERKs, JNKs (c-Jun amino-terminal kinases, also known as SAPKs), and p38 kinases (Bonni et al., 1999; Dhillon et al., 2007). JNKs, for instance, are reported to phosphorylate Bcl-2 and BH3-only proteins thus to encourage apoptosis (Chen et al., 1996; Gallo and Johnson, 2002). Many phytochemicals, e.g. resveratrol, ursolic acid, kampferol, gingerol, genistein, sulforaphane, and isothiocyanates, have all exhibited anti-cancer effects (especially pro-apoptosis) in various cancers through MAPK pathways (Adachi et al., 2009; Burotto et al., 2014; Cao et al., 2016; Ecker et al., 2009; Huang et al., 2010; Jin et al., 2009; Li et al., 2014b; Ryu and Chung, 2015). As adaptive pathways activated by stress, MAPK pathway can both function as tumor suppressor or pro-oncogenic signal in different malignant transformation situations, understanding the divergent nature of MAPK signaling activation is critical in investigating chemopreventive effects of phytochemicals (Burotto et al., 2014; Dhillon et al., 2007). Ursolic acid was reported to trigger caspase-dependent apoptosis in human osteosarcoma cells via the activation of ERK1/2 MAPK pathway, while UA-induced apoptosis was significantly abolished under ERK1/2 inhibitor treatment (Wu et al., 2016). Fisetin, a dietary flavonoid, induced apoptosis and ER stress in human non-small cell lung cancer cells through upregulation of ERK, JNK and p38 MAPK; while transfection tests with specific MAPK siRNAs confirmed contribution of MAPK signaling in regulating ER stress-induced apoptotic cell death (Kang et al., 2016).

Resveratrol inhibited migration and invasion of hyperglycemia-driven ROS-induced pancreatic cancer cells via ERK and p38 MAPK pathways, while in that case resveratrol antagonized the hyperglycemia-induced migration of cancer cells and activation of MAPKs (Cao et al., 2016).

1.4.2.2.2 Akt signaling pathways

Another primary signaling channel, the PI3K/Akt (PI3K for phosphoinositide 3-kinase, Akt for protein kinase B) pathway, was also prevailingly targeted in anti-cancer research (Karar and Maity, 2011; Porta et al., 2014). This prototypic survival pathway also receives extracellular signals from multiple membrane receptors (e.g. EGFR), and ubiquitously participated in regulation of cell survival as well as angiogenesis (Campbell et al., 2001; Mundi et al., 2016). The PI3K pathway largely contributes to the glycolytic phenotype of cancer cells via the serine/threonine kinase Akt, while this glycolytic phenotype is observed in major cancers (Elstrom et al., 2004; Schmidt et al., 2010). Its signaling mechanisms include engagement of receptor tyrosine kinases (RTKs) and activation of PI3K: RTK activation results in PI(3,4,5)P3 and PI(3,4)P2 production by PI3Ks (a lipid kinase family) at the inner side of the plasma membrane, while Akt interacts with these phospholipids by inducing its translocation to the inner membrane after phosphorylation and activation by PDK1 and PDK2 (Fresno Vara et al., 2004; Yang et al., 2004). Akt is highly sensitive to levels of EGF and regulates a series of transcription factors, e.g. NF-κB, further activation of Akt phosphorylation leads to the promotion of cell proliferation and resistance to apoptosis, while downstream effectors e.g. Bcl-2, caspases, GSK3, endothelial nitric oxide synthase,

and mTOR (Campbell et al., 2001; Ho et al., 2010; Karar and Maity, 2011). A wide array of phytochemicals, luteolin, resveratrol, curcumin, proanthocyanidin, diallyl disulfide and quercetin has been reported to inhibit Akt/PI3K signaling and result in anti-cancer activities toward anti-proliferation, pro-apoptosis and anti-invasion (Park et al., 2011; Prasad et al., 2012; Roy et al., 2009; Shin et al., 2012; Sun et al., 2010; Zanotto-Filho et al., 2012). Flavone, apigenin and luteolin were reported to induce cell cycle arrest and apoptosis in breast cancer cells via suppression of PI3K/Akt at Ser473 and activation of FOXO3a, which subsequently increased the expression levels of cyclin-dependent kinase inhibitor p27 and p21 and decreased expression levels of cyclin B and cyclin D (Lin et al., 2015). Sulforaphane inhibited growth of phenotypically different breast cancer cells via ErbB2/ER-PI3K-Akt-mTOR-S6K1 signaling pathway: sulforaphane suppressed viability and downregulated key PI3K-Akt-mTOR-S6K1 in all cells which differ in the expression pattern of growth factor or estrogen receptors and PTEN suppressor, suggesting both ErbB2 and ER serve as upstream receptors for sulforaphane-induced PI3K/Akt inhibition (Pawlik et al., 2013). Apigenin, a widely distributed plant biflavonoid, reduced tumorigenesis by suppressing activation of PI3K/Akt/FoxO pathways in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, while inhibition of Akt (Ser473) and FoxO3a (Ser253) phosphorylation resulted in decreased binding and increased nuclear retention of FoxO3a (Shukla et al., 2014).

1.4.2.2.3 Wnt/β-catenin signaling pathways

Wnt/β-catenin signaling is another frequent signaling abnormalities that has been

discovered in major human cancers, including liver, lung gastric, ovarian, breast, colon, leukemia, endometrial, brain, melanoma and thyroid (Reya and Clevers, 2005; Wang et al., 2012a). This pathway is highly involved in process of cell survival, proliferation, migration, and epithelial-mesenchymal interactions (Driehuis and Clevers, 2017; Reya and Clevers, 2005; Smalley and Dale, 1999). Its activation requires the binding of Wnt-protein ligand to Frizzled family transmembrane receptors and results in β-catenin accumulation in the nucleus, thus interacts with transcriptional factors and activates target genes like s c-myc, cyclin D, c-jun, Met, Snail and VEGF(Reya and Clevers, 2005; Taipale and Beachy, 2001). Avenanthramide 2p, a polyphenol from oats, exhibited chemopreventive effects via promoting β-catenin degradation, suppressing of β-catenin nuclear localization and downregulating of downstream oncogenic targets of Wnt/β-catenin pathway (Wang et al., 2012a). In most cases, glycogen synthase kinase 3 (GSK-3) negatively regulates β-catenin stability via phosphorylation and degradation of the transcription coactivator β-catenin, while GSK-3 can further be regulated by upstream kinases from Akt and MAPK pathways(Tejeda-Munoz and Robles-Flores, 2015). 3,5,4-trimethoxystilbene (a natural analog of resveratrol), antagonized breast cancer invasion via Wnt signaling and upstream Akt/GSK-3β regulation, accompanied with reduced the expression and nuclear translocation of β-catenin (Tsai et al., 2013). However, EGCG, the major green tea phenol, inhibited Wnt/β-catenin signaling by promoting GSK-3β-independent β -catenin phosphorylation/degradation in colon cancer cells, and suppressed β-catenin dependent genes cyclin D1 and myc (Oh et al., 2014).

1.4.2.2.4 JAK/STAT signaling pathways

JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway, in response to growth factors and cytokines, is a signaling cascade regulates gene expression related to various cellular functions including proliferation, growth and migration(Abell and Watson, 2005; Yu et al., 2014). JAK, associated with cytoplasmic domains of membrane receptor subunits, is activated after ligand binding-induced multimerization of receptor subunits (Rawlings et al., 2004; Schindler and Darnell, 1995). Activated JAKs subsequently phosphorylate STATs, then STATs translocate to nucleus and transcriptionally regulates on a wide array of genes, including: apoptosis/survival-related Bcl-2, p53 and survivin, growth-related PCNA, cyclin D and myc, and premetastatic MMP, IL-6 and CXCL3 (Bromberg and Wang, 2009; Yu and Jove, 2004; Yu et al., 2014). Sulforaphane and moringin (an isothiocyanates from cruciferous vegetables) reversed the survival and growth advantage mediated by oncogenic STAT5 and triggered cell death, the study identified STAT5 and to a lesser extent STAT1/STAT2 as novel targets of moringin (Michl et al., 2016). Carnosic acid induced apoptosis via JAK2/STAT3 signaling pathway in human colon cancer cells: it inhibited DNA binding and the reporter gene activity of STAT3, attenuated the expression of STAT3 target gene products (such as survivin and cyclin Ds), and activated apoptosis cascade via induction of p53 and Bax, downregulation of Bcl-2 family, and initiation of caspase cascade (Kim et al., 2016). Astaxanthin, a keto-carotenoid, suppressed cell proliferation, invasion and angiogenesis in a hamster model of oral cancer via regulating JAK2/STAT3 pathway: its supplementation inhibited STAT3 phosphorylation, nuclear translocation and target gene expression

(Kowshik et al., 2014).

1.4.2.2.5 The tumor suppressor p53

The tumor suppressor protein p53 is central to defensive actions against carcinogenesis(Gottlieb et al., 2002; Muller and Vousden, 2013; Rueda-Rincon et al., 2015; Wu, 2004). As the guardian of the genome, p53 protein binds to DNA and facilitates multiple anti-cancer progresses especially via activating apoptosis cascade and can be targeted by various phytochemicals (Toshiyuki and Reed, 1995). The p53 signaling pathway is activated in response to stress signals, loss of p53 function (either via mutation or disturbed upstream signaling) is a common feature in major human cancers (Muller and Vousden, 2013; Toshiyuki and Reed, 1995). Quercetin, resveratrol, EGCG and piceatannol activated p53 signaling via increasing both expression and phosphorylation in MDA-MB-231 breast cancer cells, the activation was reported to be calcium-dependent (van Ginkel et al., 2015). In the same study, increase in mRNA encoding p53 was not observed, the authors suggested p53 expression upregulation was likely due to alterations in protein stability and proteasomal processing rate (van Ginkel et al., 2015). Quercetin enhanced apoptosis induced by 5-fluorouracil (anti-cancer drug) in colorectal cancer cells through p53 modulation, the dependence of p53 was confirmed by small interference RNA (siRNA) in and p53 knockout cells (Xavier et al., 2011). The p53 protein signaling has crosslinks with major pathways, e.g Akt and MAPK. Akt activation induced phosphorylation in MDM2 protein (a key p53 function regulator) was reported to inactivate p53 (Gottlieb et al., 2002), whereas p53 attenuated Akt signaling through modulating membrane phospholipid composition

(Rueda-Rincon et al., 2015). Various dietary phytochemicals include curcumin, eupatilin, oleanolic acid, wedelolactone, dibenzoylmethane, andrographolide, were linked to downregulated Akt, upregulated p53, and increased cell cycle rest and apoptotic cancer cell death (Cho et al., 2011; Sukumari-Ramesh et al., 2011; Wang et al., 2013a). The p53 protein also functionally interacts with MAPK pathways, MAP kinases phosphorylate and activate p53 under stress stimuli, while p53 actively serves as an upstream regulator for MAPK signaling via transcriptional activation of dual specificity phosphatases (Wu, 2004). Resveratrol and black tea polyphenol synergistically suppressed mouse skin tumor growth with decreased tumor volume and number, the combination displayed significant inhibitive activity toward MAPKs (ERK, JUNK and p38) and p53 (George et al., 2011). ECG induced cell cycle arrest and apoptosis associated with the stimulation of p-p53, p53, and MAPKs in colon cancer cells, the critical role of p53 was confirmed when ECG-induced apoptosis was blocked by p53 with restored cell viability and impaired caspase-3 and pro-apoptotic protein activity (Cordero-Herrera et al., 2013). ECG also stabilized p53 by inhibiting p53 protein and RNA degradation, JNK and p38 (upon activation by p53) were identified as necessary for ECG-induced apoptosis in the same study (Cordero-Herrera et al., 2013).

1.4.2.2.6 Energy regulation and Autophagy pathways

Autophagy is the adaptive response and survival mechanism in cells, where cell shut down and undergo degradation of unnecessary cellular processes or dysfunctional cell components through lysosome action (Lemasters et al., 1998). Even tumor cells adapt

well to poor nutrient and hypoxic conditions, energetic failure via blocking glucose uptake from mitochondrial respiratory chain triggers autophagy (Mathew et al., 2007; White, 2015). Since cell division and growth highly rely on energy state, intervention through ATP synthesis or mitochondrial oxidative phosphorylation chain serves as potential pathways for stimulating cancer cell death (White, 2015). In cancer cells, metabolic stress induces autophagy through mTOR (mammalian target of rapamycin pathways) signaling, the role of autophagy in that case is divergent from its role in serving as survival mechanism in normal cells (Hippert et al., 2006; Mathew et al., 2007). Paradoxically, both autophagy inhibitors and promoters are related to chemoprevention mechanisms: many cancer drugs and ionizing radiation increases autophagy, autophagy limits genome damage and cancer progression; on the other hand, inhibiting autophagy under nutrition-deprived condition promotes apoptotic cancer cell death (Hippert et al., 2006; Mathew et al., 2007; Thorburn et al., 2014). Genetic inhibition of autophagy (by ATG5 or ATG7 knockout) inhibited tumor growth in the cells with wild type p53 expression, but encouraged tumor growth in RAS mutant, p53 null cells (Rosenfeldt et al., 2013). Delicaflavone induced autophagic cell death via increasing the ratio of LC3-II to LC3-I, the generation of acidic vesicular organelles, and autolysosomes formation in the human lung cancer cells; the upstream regulation of these effects was related to downregulation of Akt/mTOR/p70S6K signaling pathway (Sui et al., 2017). Curcumin markedly induced autophagic cell death in colon cancer cells with involvement of oxidative stress production: curcumin enhanced conversion of microtubule-associated protein 1 light chain 3 (LC3)-I, degradation of sequestome-1, and formation of puncta (an autophagosome marker);

curcumin-induced cell death was blocked by autophagosome lysosome fusion inhibitor and an strong antioxidant; however, reactive oxygen species (ROS) production-dependent activation of ERK and p38 MAPK were not involved in curcumin-induced autophagy (Lee et al., 2011). Rottlerin, a natural polyphenol, induced autophagy in prostate cancer stem cells via suppressing AMPK (5' AMP-activated protein kinase): Rottlerin induced the lipid modification of LC3, transition from LC3-I to LC3-II, and the formation of autophagosomes; Rottlerin induced the expression of Atg5, Atg7, and Atg12 and Beclin-1 proteins during autophagy, knockdown of Atg7 and Beclin-1 blocked Rottlerin-induced autophagy; Rottlerin induced AMPK (energy sensor) phosphorylation, inhibition of AMPK expression by shRNA blocked Rottlerin induced autophagy (Kumar et al., 2014). Interestingly, 2',3'-dimethoxyflavanone, a flavonoid family member, increased conversion of autophagy marker LC3 and ubiquitination of caspase-8 in breast cancer cells, however blocking autophagy degradation did not show any change in the degree of LC3 conversion, LC3 lipidation inhibition test suggested this flavonoid induced apoptosis via LC3 conversion-mediated activation of caspase-8 (Tran et al., 2014).

1.4.2.2.7 Oncogenic and tumor suppressor miRNAs

MicroRNAs (miRNAs) are short non-coding RNA molecules (20-22 nucleotide), they negatively regulate gene expression via silencing RNA (Lee et al., 1993). miRNAs play important role in cancer progression, cell behaviors including cell cycle arrest, proliferation, apoptosis and invasion can be affected by miRNAs via regulation on expression of oncogenic genes (e.g. Myc, Ras) and tumor suppressor genes p53 (Croce

and Calin, 2005; Farazi et al., 2011). A study measuring miRNA expression in tissue samples from 264 lung cancer cases suggested that, high quercetin-rich food intake was linked to expression of tumor suppressor miRNA-let-7 family, while other carcinogenesis-related miRNA families (miR-146, miR-26, and miR-17) also exhibited significant difference between high and low quercetin consumption (Lam et al., 2012). Sulforaphane, quercetin and catechins treatments alone or in combination suppressed pancreatic cancer cell viability and growth through induction of miR-let-7 expression and inhibition of KRAS expression, as miR-let-7 was demonstrated to be a direct regulator of Ras oncogene, and hence regulate downstream cell cycle or apoptosis-related proteins, including CDKs, Bcl-2 family and caspases (Appari et al., 2014). EGCG suppressed lung cancer cell proliferation rate and anchorage-independent growth via upregulated expression of miR-210, which is a participator in hypoxia pathway (Wang et al., 2011). The same study also reported EGCG's upregulation on miR-210 was achieved via stabilization of HA-tagged HIF-1α but not the P402A/P564A-mutated HIF-1α, suggesting EGCG targets the oxygen-dependent degradation domain of HIF-1α (Wang et al., 2011). Curcumin suppressed MCF-7 breast cancer cell proliferation via miR-19/PTEN/AKT/p53 axis: curcumin antagonized Bisphenol A-induced MCF-7 oncogenic miR-19 overexpression and cancer cell proliferation; curcumin upregulated PTEN, a tumor suppressor gene and direct effector of miR-19; cucurmin downregulated downstream p-AKT, p-MDM2, p53, and PCNA (proliferating cell nuclear antigen) (Li et al., 2014a).

1.4.2.3 Effects of dietary phytochemicals on tumor progression: regulation of

inflammation, invasion, angiogenesis and metastasis

1.4.2.3.1 Anti-inflammation

Inflammation, a protective response under harmful stimuli involving immune cells, blood vessels, and molecular mediators in normal tissues, is yet recognized as a risk factor in certain cancers due to carcinogenic actions of inflammatory cells: stimulating DNA damage and transformation, releasing survival and growth factors, encouraging angiogenesis and lymphangiogenesis, antagonizing host defense, and facilitating invasion via protease overexpression, ECM remodeling and cancer cell coating for disseminating cells via lymphatics and capillaries (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Growth factors and cytokines also both work as messengers for downstream inflammatory signaling. Some growth factors, for instance, epidermal growth factor (EGF) and transforming growth factor (TGF) participate in regulation of cell proliferation and invasion during inflammation, in both tissue repair process for a normal tissue and tumor cell growth process for a tumor (Signore et al., 2003; Sporn and Roberts, 1986). Interleukins and tumor necrosis factors (TNF) are two major messenger cytokines, their interaction or independent activities activate regulatory proteins or transcription factors in downstream inflammation pathways, e.g. JAK/STAT or NF-κB signaling pathway (Barton, 2001; Macarthur et al., 2004). These pro-inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) also work as mediators for NO, hence are also involved in angiogenesis signaling (Candido and Hagemann, 2013; Moldawer and Copeland, 1997). kaempferol significantly suppressed oncogene growth-related oncogene-α (GRO-α) and inhibited the lipopolysaccharide (LPS)-

induced production of inflammatory Chemokines (chemotactic cytokines), monocyte-derived chemokine (MDC), interferon gamma (IFN-γ)-induced protein 10 (IP-10) and interleukin-8 (IL-8) in THP-1 human monocytic cells; the regulation was associated with MAPK signaling pathway (Huang et al., 2010). Resveratrol suppressed IL-6-induced ICAM-1 (Intercellular adhesion molecule 1, namely CD54) expression via attenuating STAT3 phosphorylation and hence interfering with Rac-mediated pathways, the study suggested resveratrol benefits endothelial responses to cytokines during inflammation (Wung et al., 2005). EGCG suppresses proinflammatory cytokines and chemokines prostate cancer cells: Toll-like receptor 9 (TLR-9) agonist was employed to mimic microbial pathogen-induced inflammation via stimulation of DU145, PC3, or LnCap expression, while proliferative inflammatory atrophy was frequently linked to prostate cancer development; EGCG suppressed cell migration, cytokine and chemokine gene induction, and MMP activities in the above model; the above protective effects of EGCG against inflammation in the prostate cancer were reported to be independent of the androgen receptor and p53 status (Mukherjee et al., 2014).

1.4.2.3.2 Anti-invasion

Cancer invasion is a cell- and tissue-driven process which involves tissue remodeling and allow cancer cells to invade adjacent tissues and then migrate distant sites, it is a prerequisite for metastasis (Johnsen et al., 1998; Wittekind and Neid, 2005). During spread, cancer cells must breakdown their surrounding extracellular matrix (ECM) and get access to circulation system, and matrix metalloproteinases (MMPs) are the major

enzymes taking this duty (Friedl and Alexander, 2011; Johnsen et al., 1998). kaempferol inhibited migration, adhesion and invasion as well as downregulated activity and expression of MMP-2 and MMP-9 in human breast cancer cells, the upstream signaling was linked to PKC/MAPK/AP-1 cascade (Li et al., 2015). A natural methoxylated analog of resveratrol, 3,5,4-trimethoxystilbene, exhibited anti-invasion effects in breast cancer cells including reversing the epithelial-mesenchymal transition process and restoring epithelial-like characteristics via downregulating PI3K/Akt and Wnt/β-catenin signaling, while GSK-3β specific inhibitor model indicated that stabilized GSK-3β activity negatively controlled β-catenin stability (Tsai et al., 2013). Curcumin, demethoxycurcumin and bisdemethoxycurcumin differentially (bisdemethoxycurcumin > demethoxycurcumin > curcumin) inhibited human fibrosarcoma cancer cell invasion through the downregulation of MMPs and uPA (urokinase plasminogen activator), while cell migration was not affected (Yodkeeree et al., 2009). Curcumin suppressed proliferation and invasion in non-small cell lung cancer cells through regulating MTA1-mediated Wnt/β-catenin pathway, while MTA1 (metastasis-associated protein 1) overexpression is important to cell invasion and metastasis and have been detected in a wide variety of aggressive tumors (Lu et al., 2014). Curcumin also suppressed proliferation and invasion in non-small cell lung cancer cells via IL-6/JAK/STAT3 pathway, the key role of STAT3 in invasion was confirmed when knockdown of STAT3 by siRNA showed anti-invasion effects, invasive proteins such as MMP-2, MMP-7 and ICAM-1 were also downregulated (Yang et al., 2012a). Curcumin suppressed colon cancer cell invasion via AMPK-induced inhibition of NF-κB, uPA and MMP-9, AMPK inhibitor abolished curcumin-

induced inhibition of NF-κB, uPA and MMP-9, DNA binding activity of NF-κB was also suppressed by curcumin in this study as well (Tong et al., 2016). Curcumin inhibited lung cancer A459 cell invasion and metastasis by attenuating GLUT1/MT1-MMP/MMP2 pathway, Glucose transporter 1 (GLUT) transfected A549 cells exhibited resistance to curcumin's anti-invasion effects (Liao et al., 2015).

1.4.2.3.3 Anti-angiogenesis

Tumor growth and metastasis actively adopts the process of new blood vessel formation (termed as angiogenesis), which requires both oxygen and nutrients, hence in the case of malignant tumors, hypoxia and nutrient limitation stimulate expression of pro-angiogenic factors to recruit de novo and existing vasculature throughout the tumor (Tsujii et al., 1998). Excessive angiogenesis feeds diseased tissue and impairs normal tissue in tumorigenesis. Several specific genes are labeled as switch to adjust the balance between proangiogenic and antiangiogenic molecules from cancer cells themselves or the host microenvironment, e.g. the gene coding VEGF (Martiny-Baron and Marmé, 1995; Valtola et al., 1999). Vascular endothelial growth factor (VEGF) is a principal messenger to initiate cellular angiogenesis signal transduction upon its binding to receptor, both VEGF release and expression can be regulated by phytochemicals (Carmeliet, 2005; Martiny-Baron and Marmé, 1995). Kaempferol inhibited angiogenesis and tumor growth induced by ovarian cancer cells, along with downregulation of VEGF expression at both mRNA and protein levels (Luo et al., 2009). The same study also indicated downregulation of kaempferol on VEGF was realized via both HIF1 (hypoxia-inducible factor 1)-dependent (Akt/HIF) and HIF1-

independent (ESRRA) pathways (Luo et al., 2009). Kaempferol also inhibited angiogenesis and VEGF secretion via ERK/NFκB/cMyc/p21/VEGF pathway in ovarian cancer cells: kaempferol redced ERK phosphorylation, NFκB expression and cMyc expression, but increased p21 expression; inhibitory effect of kaempferol on VEGF transcriptional activation was abolished by ERK1 plasmid transfection, cMyc plasmid transfection, and p21 knockdown by siRNA (Luo et al., 2012). Curcumin inhibited tumor growth and angiogenesis in an orthotopic mouse model of human pancreatic cancer；correspondingly it suppressed activation of transcriptional factor NF-κB in cell and tumor samples, and further downregulated NF-κB-dependent gene products (VEGF, cyclin D1, MMP-9, COX-2, IKKα, and IKKβ) in tumor tissues (Bimonte et al., 2013).

1.4.2.3.4 Anti-metastasis

As one of the hallmarks of malignant tumor, metastasis describes the immigration process within which cancer cells travel to other parts of body through blood or lymph vessels from their tissue that they original developed (Woodhouse et al., 1997). Metastasis is carried out via serial processes, such as epithelial-mesenchymal transition (EMT), invasion, anoikis, angiogenesis, transport through vessels and outgrowth of secondary tumors (Geiger and Peeper, 2009; Wittekind and Neid, 2005). Curcumin inhibited tumor growth and liver metastasis of colorectal cancer in mouse model, in vitro tests associated anti-invasion and EMT suppression effects with downregulation of Sp-1, FAK, and CD24 as well as upregulation of E-cadherin expression (Chen et al., 2013). Ursolic acid treatment inhibited metastasis of prostate

cancer to distal organs (including lung and liver) in transgenic adenocarcinoma of mouse prostate (TRAMP) model, and suppressed expression levels of CXCR4 (chemokine receptor type 4) —a cytokine receptor important for distant organ metastasis—in the prostate tissues (Shanmugam et al., 2011). 4,4 -dihydroxy-trans-stilbene (DHS), a resveratrol analogue, suppressed lung cancer invasion and metastasis in two in vivo models (mouse and zebrafish): DHS significantly inhibited tumor growth, angiogenesis and liver-metastasis in mice lung cancer invasion model; DHS suppressed cell dissemination, invasion and metastasis in zebrafish lung cancer invasion model; in vitro tests associated DHS with reduced cell migration, invasion and cell cycle arrest at G1 phase via PCNA and PARP 1 (Poly [ADP-ribose] polymerase 1) regulation (Chen et al., 2012). Whole blueberry powder, enriched in diet, inhibited metastasis of breast cancer in a xenograft mouse model with smaller tumors, less ulceration, and significantly less metastasis to the inguinal lymph nodes; whole blueberry powder dietary treatment also upregulated serum levels of anti-inflammatory cytokines including IP-10, IL-12, IL-2, and TNF-α, and downregulated pro-inflammatory cytokines IL-17, IL-10, IL-4 and VEGF; however, several pro-inflammatory cytokines including IL-5, IL-6, IL-1β, MCP-1, and FGF were also upregulated in serum samples of blue berry powder-enriched groups; in tumor samples, anti-inflammatory IP-10 and IL-12 were upregulated; these results shed lights on anti-inflammation and anti-metastasis effects of blue berry powder via cytokine driven pathways (Kanaya et al., 2014).

1.4.2.3.5 NF-κB pathway

NF-κB, is a transcription factor which regulates certain genes associated with cell proliferation, inflammation, invasion and angiogenesis, hence plays key role in cancer progression and metastasis (Baldwin, 1996; Bours et al., 1994; Ghosh et al., 1998). NF-κB is typically overexpressed in cancers; it is activated by many proinflammatory stimuli such as TNF-α, EGF, IL-1β, and it regulates a wide array of cancer-related genes, including: invasive and metastatic genes MMP, ICAM-1, ELAM-1 (endothelial-leukocyte adhesion molecule 1), and VCAM-1 (vascular cell adhesion molecule 1); angiogenetic gene VEGF; energy metabolism gene GLUT3; and survival genes Bcl-2, TRAP, p53 and Fas (Bours et al., 1994; Moretti et al., 2012; Prasad et al., 2010). NF-κB dimers are sequestered in the cytoplasm by a family of its inhibitors, called IκBs (Inhibitor of κB), and can be activated by IκB degradation via upstream kinase IKK (IκB kinase) under stimuli (Baldwin, 1996; Prasad et al., 2010). Squalene, a hydrocarbon and triterpene that can be found in amaranth seed, rice bran, wheat germ, olives and several animal oil sources, inhibited inflammatory actions in DSS-induced acute colitis mouse model; squalene downregulated COX-2, inducible nitric oxide synthase, and cytokines via suppressing p38 MAPK and NF-κB signaling pathways, where STAT3 and FOXP3 were not involved (Sanchez-Fidalgo et al., 2015). *Hibiscus sabdariffa* Leaf extract inhibited human prostate cancer cell invasion via suppressing Akt/NF-κB/MMP-9 pathway: the extract was demonstrated to be rich in polyphenols; the extract inhibited prostate cancer cell migration, and invasion under non-cytotoxic concentrations, as it also inhibited tumor growth and metastasis in vivo; the extract reduced MMP-9 expression and NF-κB DNA binding activity, which was regulated by Akt signaling pathway as validated by Akt overexpression vector,

expressions of metastatic-related were also downregulated in vivo (Chiu et al., 2015). Plumbagin, a naphthoquinone constituent of plants, suppressed the invasion of HER2-overexpressing breast cancer cells via repressing activation of NF-κB, which is typically overexpressed in a subset of HER2-positive breast cancers (Kawiak and Domachowska, 2016). The study reported plumbagin to be a negative regulator of NF-κB phosphorylation and transcriptional activity along with downstream MMP-9 expression, silencing of NF-κB p65 increased sensitivity of breast cancer cells to anti-invasion effects of plumbagin; NF-κB inhibition was associated with IKK inhibition, knockdown of IKKα increased sensitivity of breast cancer cells to plumbagin-induced decrease of NF-κB transcriptional activity and MMP-9 expression (Kawiak and Domachowska, 2016).

1.4.2.3.6 Regulation on miRNAs related with inflammation and metastasis

Large bunch of oncogenic miRNAs that causally linked to tumorigenic processes and metastasis have been identified, offering potential molecular targets to dietary phytochemicals(Chan and Wang, 2015; Croce and Calin, 2005; Farazi et al., 2011; Hagiwara et al., 2015). During inflammatory response, expression of some miRNAs (such as miR-155 and miR-21) are upregulated, they are also considered as oncogenic miRNAs due to their overexpression in several types of tumors (Song et al., 2010; Tili et al., 2009). Some miRNAs, such the miR-200 family and miR146, show impacts on epithelial-mesenchymal transition via gene expression of E-cadherin pathway proteins including ZEB, Twist, Snail, Slug and TGF-β, and cell invasion indicators such as NF-κB and MMPs (Chan and Wang, 2015). A recent screening on 139 nature products

reported three compounds - enoxolone, magnolol and palmatine chloride induced miR-200c expression in breast cancer cells, while miR-200c is a tumor suppressor miRNA via downregulating E-cadherin expression and antagonizing invasion (Hagiwara et al., 2015). Resveratrol has been shown to downregulate oncogenic miRNAs targeting genes encoding Dicer1, such as miR-155, miR-21, miR-196a, miR-25, miR-17, and miR-92a-2 in colon cancer cells (Tili et al., 2010). Those oncogenic miRNAs abolished expression of tumor suppressor PDCD4 or PTEN and negative regulators of TGF signaling via Dicer, suggesting resveratrol to have anti-cancer as well as anti-metastatic effects (Tili et al., 2010). Additional tests suggested resveratrol also suppressed TGFβ1 expression via both miR-663 upregulation and miR-663 independent approaches, and upregulated TGF signaling suppressor SMAD7 (mothers against decapentaplegic homolog 7) metastasis (Tili et al., 2010). Resveratrol also downregulated IL-6/STAT3/miR-21 pathway and hence induced prostate cancer associated transcript 29 (PCAT29) expression, which promotes tumor suppressor function and suppresses prostate cancer metastasis (Al Aameri et al., 2017). Genistein inhibited renal cancer cell invasion while downregulating TOPflash reporter activity (indicator for monitoring Wnt/β-catenin signaling pathway) and miR-1260b expression (Hirata et al., 2013). The same study also reported oncogenic miR-1260b (typically overexpressed in renal cancer cells) promoted cancer cell proliferation and invasion via abolishing gene expression of tumor suppressors and negative regulators of Wnt-signaling (e.g. (sFRP1, Dkk2, SMAD4), which can be counteracted by miR-1260 inhibitor, hence inhibition of miR-1260b and Wnt pathway by genistein antagonized by tumor progression and metastasis (Hirata et al., 2013).

1.5 Ursolic acid and its anticancer effects

1.5.1 Ursolic acid and its pharmcokinetics

Ursolic acid (UA), a natural pentacyclic triterpenoid carboxylic acid (Figure 2), is a phytochemical presenting in spices (e.g. rosemary and thyme), peels of fruits (e.g. apple, cranberries, prunes, bilberries and other various fruits, and some traditional medicine herbs (Borovkov and Belova, 1967; Cargnin and Gnoatto, 2017; He and Liu, 2007; Mezzetti et al., 1971; Yin et al., 2012). Terpenoids exist in all types of plants, where they function as metabolites with roles in respiration, photosynthesis, and regulation of growth and development (Hill and Connolly, 2012). The distribution of UA was around 57 mg/100 g in mahogan, 42 mg/100 g in balsam pear and 17 mg/100 g in basil (Yin et al., 2012). Apples contain ursolic acid in peels, which was around 50 mg per middle sized fruits with a surface area of 50 ~ 70 cm^2 (Frighetto et al., 2008). In the present study, lab-isolated ursolic acid from apple peel was studied as target compound (He and Liu, 2007).

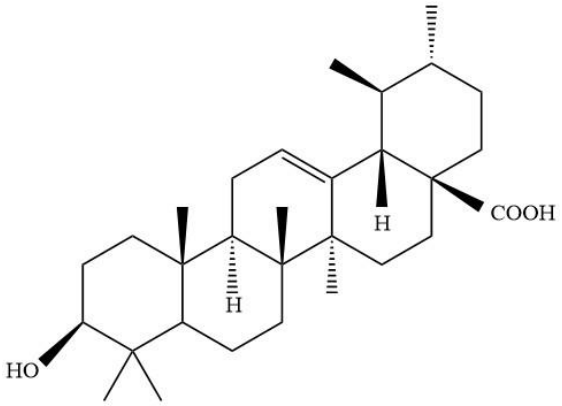

Figure 2. Chemical structure of ursolic acid.

According to a previous report, ursolic acid had rapid absorption and broad tissue distribution in rat after given to animals by oral administration (Chen et al., 2011). The pharmacokinetic parameters of UA in rat were detected by HPLC-MS: the Cmax was around 1.10 μg/mL, AUC (area under the curve) was approximately 1.45 μg/mL; Tmax of UA in plasma was about 30 min and half-life was less than 1 h, indicating it was rapidly absorbed and rapidly eliminated (Chen et al., 2011). They also found that UA was probably absorbed in the liver; concentrations of UA in rat lung, spleen, liver, heart, and cerebellum could be detected at 1 h after oral administration, suggesting UA could across the blood-brain barrier (Chen et al., 2011). Another longer-term study conducted in mice suggested that the level of UA in plasma, brain, heart, liver, kidney, colon, and bladder increased as the feeding period was increased from 4 weeks to 8 weeks after oral administration, and triterpenes were absorbed and deposited in their intact forms, which in turn exerted in vivo anti-oxidative protection (Yin et al., 2012). Liposomal ursolic acid was used by a previous study to determine the maximum tolerated dose (MTD), dose-limiting toxicity (DLT), and pharmacokinetics for application in human clinical trials: 4 patients with advanced solid tumors and 35 healthy volunteers participated in toxic study, and 24 healthy adults participated in pharmacokinetics study, who received a single-dose of ursolic acid liposomes (11, 22, 37, 56, 74, 98, and 130 mg/m^2) administered as a 4h intravenous infusion; liposomal ursolic acid had manageable toxicities with MTD of 98 mg/m^2, DLT were primarily hepatotoxicity and diarrhea, it had a linear pharmacokinetic profile (Wang et al., 2013b).

UA is a natural compound with limited water solubility, and new technologies has been applied to increase its bioavailability. Ursolic acid (UA) nanoparticles (with mean particle size ranging from 139.2 ± 19.7 to 1039.8 ± 65.2 nm) using the supercritical anti-solvent process significantly exhibited higher and extended absorption (Yang et al., 2012b). UA nanoparticles with TPGS1000 as stabilizer enhanced absorption in in vivo study: the bioavailability and Cmax of the UA nanosuspensions were about 27.5-fold and 9-fold higher than those of raw UA when given to rats by oral administration (Ge et al., 2015).

1.5.2 anti-cancer effects of ursolic acid

UA showed anti-cancer promising activities both in vivo and in vitro in various cancers and highly interacts with cell signal transduction, targeting multiple pro-inflammatory transcription factors, cell cycle proteins, apoptosis cascade participators, growth factors, kinases, cytokines, chemokines, adhesion molecules, and inflammatory enzymes (Achiwa et al., 2013; Huang et al., 2011; Ikeda et al., 2008; Shanmugam et al., 2013; Wang et al., 2012c; Wu et al., 2012; Zhang et al., 2016). The anti-cancer effects of UA majorly include anti-proliferation, pro-apoptosis anti-inflammation, and anti-invasion.

UA inhibited cell proliferation and promoted apoptosis in different cancers (such as colon cancer, bone cancer, endometrial cancer) via major cellular signaling pathways, such as MAPK pathway (Achiwa et al., 2013; Shan et al., 2009; Wu et al., 2016). UA

suppressed MAPK-cyclin D1 pathway and RING type E3 ligase in two endometrial cancer cell lines, and CD36 was noted as a cell surface receptor for UA; UA treatment also increased ubiquitinated protein level, while ubiquitin-mediated proteolysis decides cytoplasmic degradation of cyclin D1 and cyclin D1 levels (Achiwa et al., 2013). UA induced apoptosis in myelogenous leukemia cells, which is related to increase of PTEN gene expression and inactivation of the PI3K/Akt pathway, since PTEN gene expression upregulation inhibits Akt kinase activity, changes mitochondrial transmembrane potential and undermines the release of cytochrome c and the activity of caspases (Wu et al., 2012). UA repressed proliferation and induced apoptosis by inactivating Wnt/β-catenin signaling in human osteosarcoma cells： luciferase reporter results suggested Wnt/β-catenin signaling was inhibited by UA, correspondingly expression level and nuclear translocation of β-catenin was reduced; UA also increased tumor suppressor p53 protein, inhibition of p53 partly reversed the UA-induced downregulation of β-catenin and targets of Wnt/β-catenin signaling, such as c-Myc and cyclin D1(Zhang et al., 2016). Inhibition of proliferation and induction of apoptosis by UA also involved miRNA participation. UA exhibited anti-proliferation and pro-apoptosis effects in human glioblastoma cell lines U251 by suppressing TGF-β1/miR-21/PDCD4 pathway, where PDCD4 (programmed cell death 4) is a miR-21 targeting pro-apoptotic gene (Wang et al., 2012c). Ursolic acid promoted cancer cell death by inducing Atg5-dependent autophagy in cervical cancer cells: UA increased punctate staining of LC3, the autophagy marker in TC-1 cells, while apoptosis was not affected; application of an inhibitor of autophagy and a siRNA confirmed the regulation was Atg5 dependent (Leng et al., 2013).

UA exhibited anti-migratory and anti-invasive effects in non-small cell lung cancer cells with decrease in VEGF, ICAM-1 and MMP expression, UA also undermined cell viability and Na^+-K^+-ATPase activity (Huang et al., 2011). UA suppressed epithelial-mesenchymal transition via downregulating astrocyte-elevated gene-1 in human non-small lung cancer cells (Liu et al., 2013). UA inhibited the invasive phenotype of SNU-484 human gastric cancer cells and significantly reduced MMP-2 expression; they reported among the MMPs, MMP-2 expression was associated with the invasive phenotype of gastric cancer cells (Kim and Moon, 2015).

For breast cancer, UA triggered apoptosis the via intrinsic mitochondrial pathway, and activated the PARP cleavage and downregulated Bcl-2 via in MCF-7 breast cancer cells; UA bound to glucocorticoid receptor, and translocated it into nucleus (Kassi et al., 2009). Ursolic acid induced apoptotic cell death via both mitochondrial death pathway and extrinsic death receptor pathway in MDA-MB-231 cells: UA induced the appearance of Fas receptor and cleavage of caspase-8, -3 and PARP; UA upregulated Bax, downregulated Bcl-2 down-regulation, and suppressed release of cytochrome C to the cytosol from mitochondria; UA cleaved caspase-9 and decreased mitochondrial membrane potential (Kim et al., 2011). Ursolic acid promoted apoptosis by suppressing the expression of FoxM1 in MCF-7 human breast cancer cells, accompanied by a significant decrease in CyclinD1/CDK4 expression, which can be regulated by FoxM1 (Wang et al., 2012d). UA-mediated changes in glycolytic pathway promoted cytotoxic autophagy and apoptosis in phenotypically different

breast cancer cells: UA-induced energy stress activated AMPK, increase in nitric oxide levels and ATM activation might also account for AMPK activation-mediated cytotoxic response；UA-induced apoptosis was associated with decreased pERK1/2 signals and the depolarization of mitochondrial membrane potential (Lewinska et al., 2017). UA was reported to suppress migration and invasion of human breast cancer cells by modulating c-JNK, Akt and mTOR signaling in MDA-MB-231 cancer cells at non-cytotoxic concentrations, and hence downregulated expression of invasion-related proteins cJun, cFos, VEGF, NF-κB, MMP-2 and uPA (Yeh et al., 2010).

Among in vivo studies, UA has been reported to inhibit tumor initiation, growth, progression and metastasis in various animal models of cancer (Ikeda et al., 2008; Shanmugam et al., 2013; Shanmugam et al., 2012; Zhang et al., 2016). UA inhibited osteosarcoma tumor growth in a mouse xenograft model, which was related to Wnt/β-catenin signaling (Zhang et al., 2016). Transgenic adenocarcinoma of mouse prostate (TRAMP) mice fed with UA in the diet enriched with 1% w/w ursolic acid for 8 weeks (weeks 4-12) exhibited delayed formation of prostate intraepithelial neoplasia, and those fed for 6 weeks (weeks 12-18) also showed inhibited progression of prostate intraepithelial neoplasia to adenocarcinoma (Shanmugam et al., 2012). The same study also reported UA reduced activation of various pro-inflammatory mediators such as NFκB, STAT3, Akt and IKKα/β in the dorsolateral prostate tissues which were correlated with the decrease of TNF-α and IL-6 in serum levels; cyclin D1 and COX-2 were downregulated and but caspase-3 was upregulated by UA in tumor tissue sections (Shanmugam et al., 2012). Oral administration of UA increased survival rate

of melanoma lung metastasis in C57BL/6 mice along with reduce ICAM-1 expression, which is in correspondence to its in vitro anti-adhesion, anti-invasion and anti-migration effects and regulation on ICAM-1, VCAM-1, E-selectin, P-selectin, integrin α6β1, FAK, Src, paxillin and PTEN. (Xiang et al., 2015). Nevertheless, in vivo study towards breast cancer inhibition is rarely reported.

1.6 Hypothesis and objectives

Breast cancer is the most common cancer among women worldwide and in the United States (Siegel et al., 2016; Stewart and Wild, 2014). According to reports from WHO and NCI, breast cancer is the first leading cause of cancer death in global women and second leading cause of death in American women (Siegel et al., 2016; Stewart and Wild, 2014). Ursolic acid, a pentacyclic triterpenoid carboxylic acid found in peels of fruits, spices and herbs, have showed potent anti-cancer activities in various cancers (Kassi et al., 2009; Leng et al., 2013; Liu et al., 2013; Shanmugam et al., 2013; Wu et al., 2012; Zhang et al., 2016). However, the anti-cancer activities of UA toward breast cancer and its mechanism of action remains unclear.

The hypothesis of this study is that ursolic acid inhibits proliferation and invasion of MDA-MB-231 human breast cancer cells via regulating cellular signal transduction pathways, and the combination of UA and anti-breast cancer drug paclitaxel has synergistic anti-proliferation effects.

Specific objectives proposed to test my hypothesis include: 1) to investigate anti-

proliferation and anti-invasion activity of ursolic acid toward MDA-MB-231 human breast cancer cells;2) to determine the specific molecular targets of ursolic acid in regulating cellular transduction pathways in MDA-MB-231 human breast cancer cells; and 3) to investigate potential synergistic anti-proliferation effects in breast cancer cells when combing UA with clinical anti-cancer drug paclitaxel.

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Chapter 2 Anti-proliferation and anti-invasion effects of ursolic acid in MDA-MB-231 human breast cancer cells

2.1 Introduction

Cancer development is intricate multistep process (Barrett, 1993). Most cancers share several hallmarks during tumorigenesis, which distinguish malignant tumors from benign tumors (Cohen and Arnold, 2011; Hanahan and Weinberg, 2000). Several biological activities have been recognized as hallmarks of cancer, which majorly include: uncontrolled proliferation, aborted cell death, angiogenesis induction, activation of invasion and metastasis, disruption in energy metabolism and dysfunction of immune system (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Rodriguez-Enriquez et al., 2009; Wittekind and Neid, 2005). Inhibitory effects toward proliferation and invasion are critical and promising parts of anti-cancer actions (Geiger and Peeper, 2009; Kotecha et al., 2016; Surh, 2003; van Zijl et al., 2011). Previously, a wide array of dietary bioactive compounds such as apigenin, luteolin, sulforaphane, resveratrol, quercetin, gingerol and EGCG showed anti-proliferation effects in various cancers including breast cancer (Adachi et al., 2009; Appari et al., 2014; Bahramsoltani et al., 2015; Cao et al., 2016; Jin et al., 2009; Lin et al., 2015; Miller and Snyder, 2012; Pawlik et al., 2013; Ryu and Chung, 2015; Surh, 2003). Ursolic acid (UA), a bioactive pentacyclic triterpenoid, has showed potent anti-proliferation activities in certain cancers (such as colon cancer, endometrial cancer,

brain cancer and cervical cancer), suggesting it probably have anti-cancer effects in breast cancer. (Leng et al., 2013; Lewinska et al., 2017; Wang et al., 2012; Wu et al., 2012; Zhang et al., 2016).

Breast cancer consists of heterogeneous subgroups, which are vary in morphology, pathology, and responses to therapies (Davies, 2016; Harbeck and Gnant, 2017; Hulka and Moorman, 2001). Triple negative breast cancers are those cancers lack of the three receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Chacon and Costanzo, 2010; Cleator et al., 2007). They typically exhibit more common distant recurrences in the brain and visceral metastases than in HR-positive cancers (Cleator et al., 2007; Foulkes et al., 2010; Hulka and Moorman, 2001). For triple negative breast cancers (ER^-, PR^-, $HER2^-$), treatment difficulty is increased due to ineffectiveness of hormone therapy and ER, PR, HER targeted chemotherapy (Chacon and Costanzo, 2010; Cleator et al., 2007; Foulkes et al., 2010). It is necessary to find alternative chemotherapy approach for this kind of breast cancer.

Highly invasiveness is another critical hallmark for malignant tumor (Hanahan and Weinberg, 2000). Cancer invasion, which recruits procedures such as migration, epithelial-mesenchymal transition (EMT), and tissue remodeling, is the process allows cancer cells to invade adjacent tissues and then migrate to distant sites (Friedl and Alexander, 2011; Johnsen et al., 1998). Invasion through the extracellular matrix (ECM) is an important step in tumor metastasis (Wittekind and Neid, 2005). Cancer

cell initiates invasion by adhering to and spreading along the blood vessel wall, while the microenvironment is modified and remodeled by proteolytic enzymes (e.g. MMPs) to allow cancer cell invasion (Friedl and Alexander, 2011; Johnsen et al., 1998; Wittekind and Neid, 2005). Previous studies reported some phytochemicals had anti-migration and anti-invasion effects along with MMP downregulation in different cancers including breast cancer, such as kaempferol, curcumin, resveratrol and trimethoxystilbene (Li et al., 2015; Lu et al., 2014; Tsai et al., 2013; Yodkeeree et al., 2009). Ursolic acid exhibited activity against migration and invasion in lung and gastric cancer cells (Huang et al., 2011; Kim and Moon, 2015; Liu et al., 2013).

Epidermal growth factor (EGF) is a 53-amino acid polypeptide with 3 intramolecular disulfide bonds (molecular weight around 6-kDa), stimulates cell growth, proliferation and differentiation by binding to its receptor EGFR (Carpenter and Cohen, 1990). Previously, EGF was reported to stimulate migration in various cancer cells include triple negative cancer cells, and this effect was antagonized by several chemicals such as quercetin, resveratrol and curcumin (Bhat et al., 2014; Ji et al., 2008; Lee et al., 2011).

However, the anti-invasion effects of UA and its role in cellular actions upon EGF stimulation in breast cancer is not clear yet.

2.2 Objectives

The objective of this research was to study the anti-proliferation and anti-invasion

effects of UA in MDA-MB-231 human breast cancer cells. The specific objects are designed as follow:

a) To determine anti-proliferation activity and cytotoxicity of UA in MDA-MB-231 human breast cancer cells

b) To determine the effects of UA on MDA-MB-231 human breast cancer cell migration

c) To determine the effects of UA on MDA-MB-231 human breast cancer cell invasion

d) To determine the effects of UA on MMP activities of MDA-MB-231 human breast cancer cells.

2.3 Materials and methods

2.3.1 Chemicals

Ursolic acid was isolated from apple peels in our lab as reported previously (He and Liu, 2007). UA was diluted in Dimethyl sulfoxide (DMSO) first and then dilute by 1: 100 in medium before use. Dimethyl sulfoxide (DMSO) and Epidermal Growth Factor (EGF) were purchased from VWR (Radnor, PA, USA). Phosphate-buffered saline (PBS), Anti-anti and α-minimum essential medium (α-MEM) were received from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum was received from Atlanta Biologicals (Lawrenceville, GA, USA). CHEMICON Cell Invasion Assay Kit was bought from EMD Millipore (Billerica, MA, USA).

2.3.2 Cell culture

MDA-MB-231 human breast cancer cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in α-MEM containing 10 mM Hepes, 1% antibiotic-antimycotic and 10% heat-inactivated fetal bovine serum as described previously (Jiang et al., 2016). The cells were cultured at 37 °C in a 5% CO_2 humidity atmosphere.

2.3.3 Anti-proliferation assay

The anti-proliferative activities towards MDA-MB-231 cells were assessed by lab-developed methylene blue assay as reported previously (Felice et al., 2009; Jiang et al., 2016). 100 µL fresh medium with cells was seeded in 96-well plate at the concentration of 2.5 x 10^4cells/well. After 8 hours incubation at 37 °C and 5% CO_2 to allow cells to get attached, growth medium was removed and cells was treated with 100 µL fresh medium with different concentrations of UA or Dimethyl sulfoxide (DMSO) as the control. After 72 hours incubation, cells was washed with 100 µL phosphate-buffered saline (PBS). Then 50 µL methylene blue staining buffer (98% Hanks balanced salt solution, 0.67% glutaraldehyde, 0.6% methylene blue) was added to each well, and incubated for 1 hour. After the staining, the methylene blue staining solution was removed, and the plate was immersed and rinsed in deionized water for four times. After the wells were dried, a volume of 100 µl elution buffer containing (1% acetic acid, 49% PBS, and 50% ethanol) was added to each well and the plate was shaken on a bench shaker for 15 minutes. Finally, cell numbers were referring to the absorbance at 570 nm using the MRX Microplate Reader. The anti-proliferation

activity was measured as percentage compared to the control. All measurements were conducted in triplicates.

2.3.3 Cytotoxicity assay

The cytotoxicity of UA towards MDA-MB-231 cells were assessed by lab-developed methylene blue assay as reported previously (Felice et al., 2009; Jiang et al., 2016). 100 µL fresh medium with cells was seeded in 96-well plate at the concentration of 4 x 10^4cells/well. After 24 hours incubation at 37 °C and 5% CO_2, growth medium was removed and cells was treated with 100 µL fresh medium with different concentrations of UA or Dimethyl sulfoxide (DMSO) as the control. After 24 hours incubation, cells was washed with 100 µL phosphate-buffered saline (PBS). Then 50 µL methylene blue staining buffer (98% Hanks balanced salt solution, 0.67% glutaraldehyde, 0.6% methylene blue) was added to each well, and incubated for 1 hour. After the staining, the methylene blue staining solution was removed, and the plate was immersed and rinsed in deionized water for four times. After the wells were dried, a volume of 100 µl elution buffer containing (1% acetic acid, 49% PBS, and 50% ethanol) was added to each well and the plate was shaken on a bench shaker for 15 minutes. Finally, cell numbers were referring to the absorbance at 570 nm using the MRX Microplate Reader. Cytotoxicity was measured as a percentage compared to the control. More than 10% cell number reduction was considered as cytotoxic. All measurements were conducted in triplicates.

2.3.4 Cell migration (Scratch) assay

The migration of MDA-MB-231 human breast cancer cells was determined by the scratch assay modified on the previous protocol (Liang et al., 2007). 2 mL fresh medium with MDA-MB-231 cells was plated in 6-well plates at a concentration of 5 x 10^5 cells/mL (1 x 10^6 cells/well). The plates were incubated for 24 hours at 37 °C with 5% CO_2 to allow cells grow confluent, Then, the medium was replaced with 2 mL of Serum Free Medium (SFM) to starve the cells for 12 hours. The mono cell layer was scratched using a 200 µl pipette tip before the removal of the conditioned medium. Cell debris was washed out by 37 °C pre-warmed PBS for twice, then cells was treated with either SFM or EGF (100 ng/mL) in the presence or absence of various concentrations of ursolic acid. Cells images was on three marked sites per well at 0 hour, 8 hours and 12 hours by digital camera under the inverted microscope. Cell migrations was calculated by the following formula: rate (%) = B/A x 100, where A is the area of beginning scratch, and B is the uncovered area at 12 or 18 hours. The area was analyzed by ImageJ2x software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.3.5 Cell invasion (Transwell) assay

Cell invasion (Transwell) was conducted under instructions of CHEMICON cell invasion assay kit. Before the test, invasion chamber was adjusted to room temperature in a tissue culture hood. Then 300 µL of warm serum free media was added to the interior of inserts for 1.5 hour to allow ECM layer to get rehydrated. Cell suspension containing 7.5x 10^6cells/mL in serum free media was prepared and then

added with DMSO or different concentrations of ursolic acid for upcoming use. Then lower chamber was added with 500 µL of culture media containing 10% FBS or 100ng/mL EGF as chemoattractant for different tests. For upper chamber, previous media was removed after rehydration and 300 µL of prepared cell suspension was added. After incubated for 24 hours, a cotton-tipped swab was used to remove non-invading cells as well as the ECMatrix gel from the interior of the inserts. Then 500 µL of Quick-stain staining solution was added to the unoccupied wells of the plate and invasive cells on lower surface of the membrane was stained for 30 minutes by dipping inserts in the staining solution. The stained cells were quantified by dissolving stain in an elution buffer containing 10% acetic acid (150 µL/well), and latterly solutes were transferred to 96-well plates and the absorbance was read at 560 nm using the MRX Microplate Reader. Cell invasion was measured as percentage compared to the control. All measurements were conducted in triplicates.

2.3.6 Zymography assay for MMP activity

The Zymography Assay was conducted referring to previous protocols (Toth et al., 2012; Troeberg and Nagase, 2004). Briefly, MDA-MB-231 human breast cancer cells were seeded in a 6-well plate at a density of 1 x 10^6 cells/well (5 x 10^5 cells/mL for 2mL). After 24 hours, the cells were treated with either SFM or EGF (100 ng/mL) in the presence or absence of various concentrations of ursolic acid. and incubated for 24 hours. The medium was then harvested and centrifuged for 3 minutes at 1500 RPM, the supernatants were collected and mixed with 4× loading buffer (pre-prepared 10 ml of 0.25 M Tris/HCl buffer, pH 6.8, containg 40% v/v glycerol, 8% w/v SDS, and 0.01%

w/v bromophenol blue). 25 µL samples were loaded on 10% acrylamide gel with 0.1% gelatin. After running gel for 1 hour at 150 V, the gel was washed four times with enzyme renaturing buffer (2.5% v/v Triton X-100 in deionized H_2O) for 15 minutes each time with gentle agitation at room temperature. Then the gel was transferred to developing buffer (6.055‰ w/v Tris base, 11.69 ‰ w/v NaCl, 0.0007 ‰ w/v $ZnCl_2$, 0.74 ‰ w/v $CaCl_2 \cdot 2\ H_2O$, and 0.2‰ w/v NaN_3, pH 7.5) and agitated on a shaker for 1 hour. After the gel was incubated in incubator for 16 hours at 37 °C, the developing buffer was decanted. The gel was then stained by the staining solution (0.125% w/v Coomassie brilliant blue in mixture solution of 1.25 L methanol, 0.5 L acetic acid, and 0.75 L water) for 20 minutes and then de-stained by the de-staining solution (mixture solution of 1.5 L methanol, 3.5 L water, and 50 mL formic acid) until clear bands were visible. The bands were visualized on a scanner and quantified by the ImageJ2x software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). MMP-9 activity was expressed as a percentage compared to the EGF-stimulation control. All measurements are conducted in triplicates.

2.3.7 Data analysis.

Data were analyzed and presented using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD) for at least three replicates. Statistical difference was analyzed by SPSS 17.0 software (International Business Machines Corp., NY, USA). The significance was determined at a p value of <0.05 by the analysis of variance (ANOVA) followed by Tukey's test.

2.4 Results

2.4.1 Anti-proliferation activity and cytotoxicity of UA in MDA-MB-231 human breast cancer cells

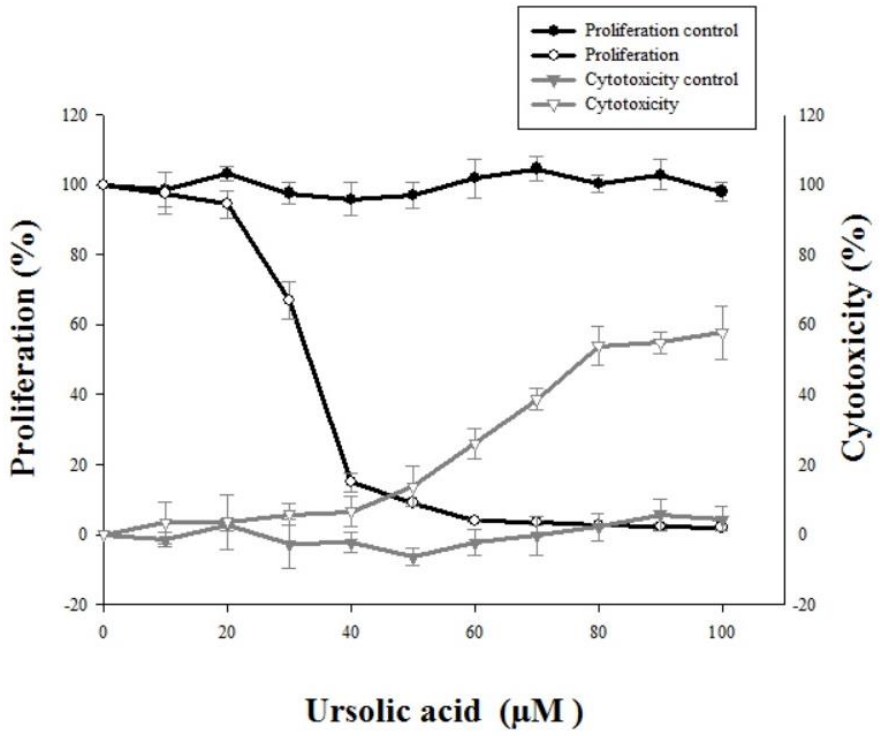


Figure 3. Effect of UA on cell proliferation and cytotoxicity in MDA-MB-231 human breast cancer cells (Mean ± SD, n=3). UA significantly inhibited proliferation of MDA-MDB-231 human breast cancer cells in a dose-dependent manner at the concentrations without cytotoxicity The EC_{50} value of anti-proliferative activity was 30.67 μM.

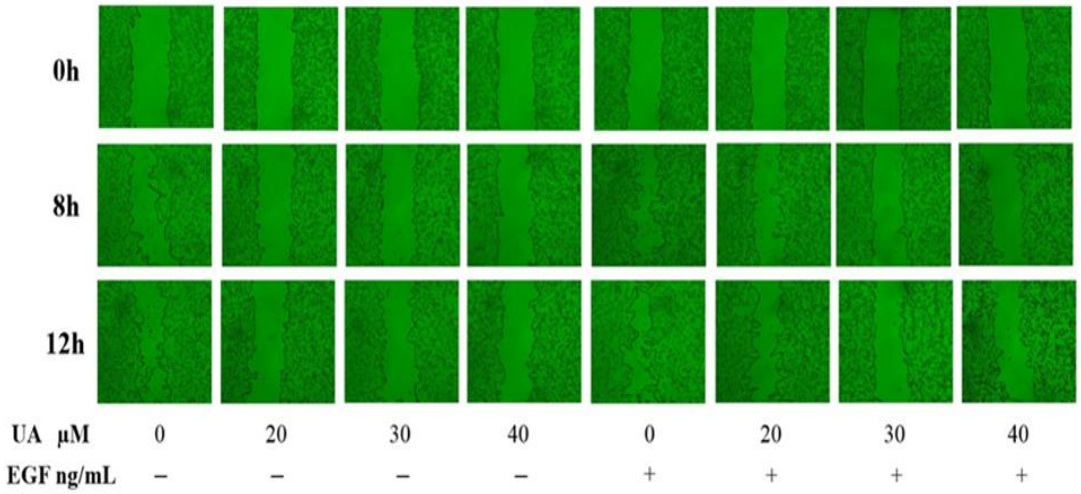
Anti-proliferation activity and cytotoxicity of UA was assessed by lab-developed assays. As shown in Figure 3, UA inhibited proliferation of MDA-MB-231 human breast cancer cells in a dose-response manner, with EC_{50} of 30.67 μM. Less than 10% cytotoxicity were observed at doses lower than 47.8 μM. Hence UA significantly

inhibited MDA-MB-231 human breast cancer cells at doses without cytotoxicity. Concentrations of 20, 30 and 40 μM were selected as doses for UA in upcoming researches.

2.4.2 Inhibitory activity of UA toward MDA-MB-231 human breast cancer cell migration

Effect of UA toward MDA-MB-231 human breast cancer cell migration is shown in Figure 4. Photos were taken at 0h, 8h and 12h. After scratch, the wound closed in time-dependent manner. UA at concentrations of 20, 30, and 40 μM significantly inhibited cell migration. As compared to blank control, cell migration was inhibited by 73.9% after 12 hours UA treatment at the concentration of 40 μM. Additional tests suggested EGF stimulated MDA-MB-231 migration, which was antagonized by UA. As compared to EGF-stimulation model control, 40 μM UA inhibited 81.4 % of EGF-induced migration.

A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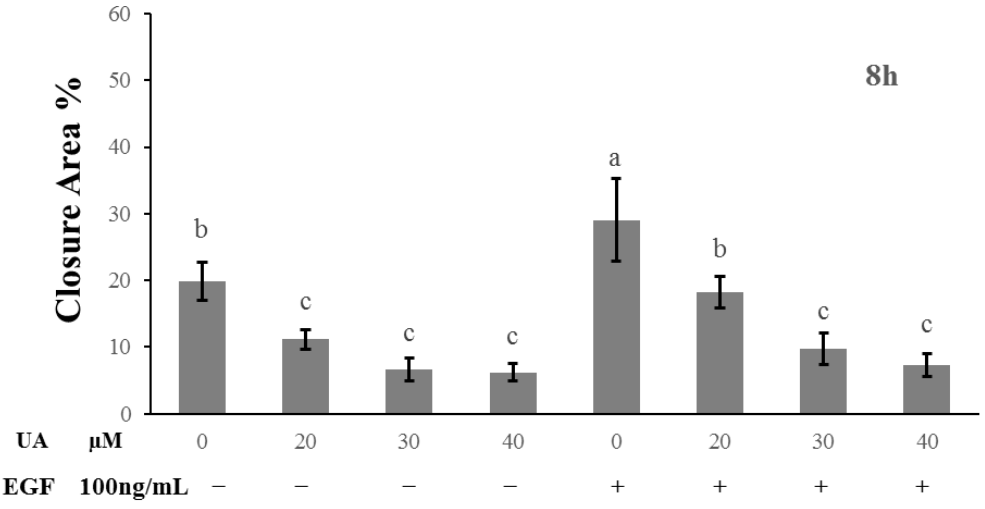
B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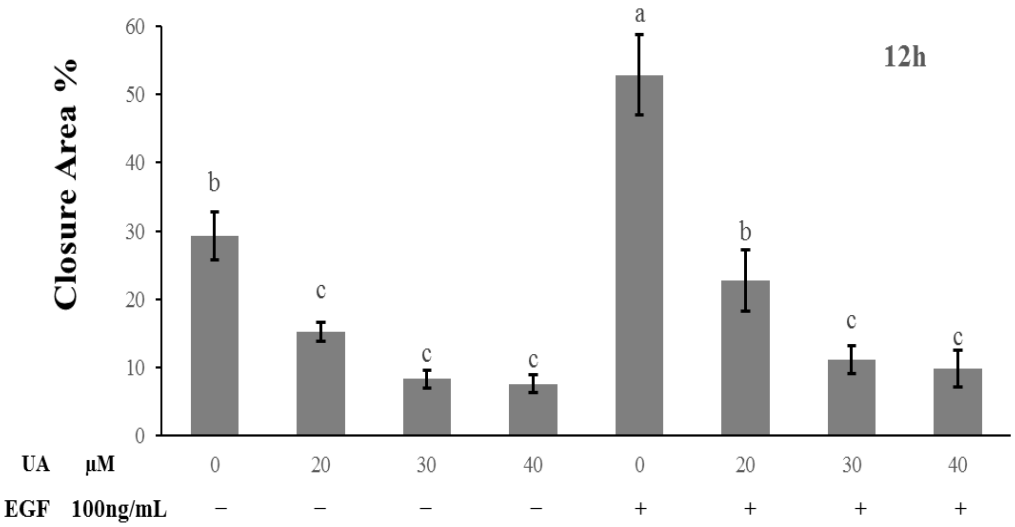
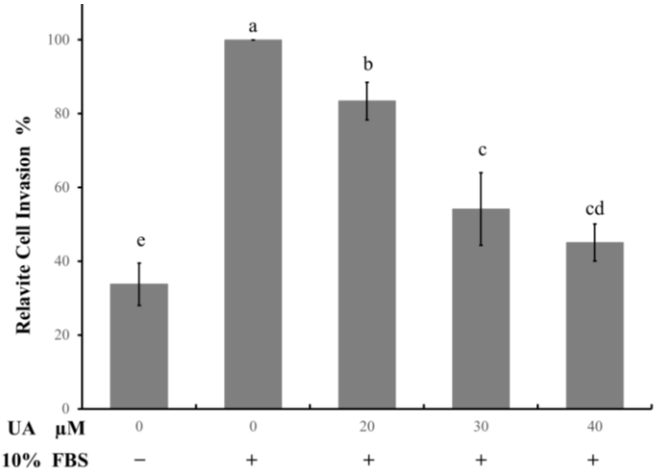


Figure 4. Effects of UA on cell motility in MDA-MB-231 human breast cancer cells. (A) Photos taken under phase control microscope in Scratch Assay at 0 h, 8h and 12h. Cells were treated with 0,20,30,40 μM ursolic acid, with or without EGF (100 ng/ml) stimulation. (B) Closure area at 8h for each group as compared to 0 h. Bars with no letters in common are significantly different ($p < 0.05$; Mean ± SD, n=3). (C) Closure area at 12h for each group as compared to 0h. Bars with no letters in common are significantly different ($p < 0.05$; Mean ± SD, n=3)).

2.4.3 Inhibitory activity of UA toward MDA-MB-231 human breast cancer cell invasion

A



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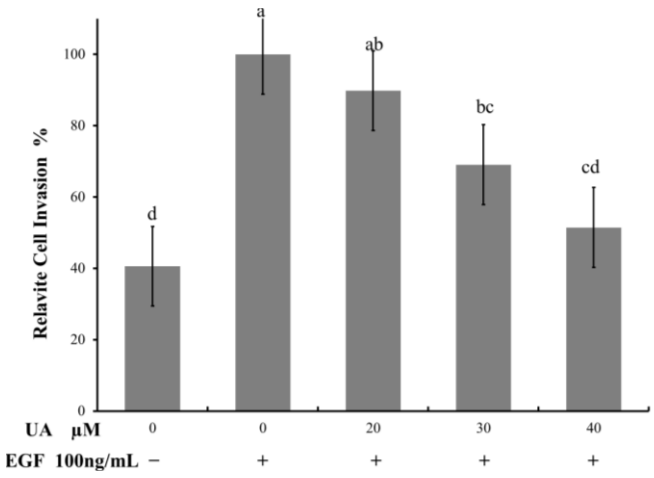

Figure 5. Effects of ursolic acid on cell invasion in MDA-MB-231 human breast cancer cells. Cells were treated with stimulation of either 10% FBS or 100ng/mL EGF and 0,20,30,40 μM ursolic acid for 24hrs, then relative cell invasion were measured by CHEMICON cell invasion assay kit. (A) Relative cell invasion% under stimulation of 10% FBS (Mean ± SD, n=3). (B) Relative cell invasion% under stimulation of 100ng/mL EGF (Mean ± SD, n=3). Bars with no letters in common are significantly different ($p < 0.05$).

The anti-invasion activities of UA in MDA-MB-231 human breast cancer cells were tested by CHEMICON cell invasion assay kit with ECM coating. General anti-invasion activity of UA was assessed first using FBS as chemoattractant. Then specific

anti-invasion activity of UA toward EGF stimulation was evaluated. The results are shown in Figure 5. UA at concentrations of 20, 30, and 40 μM significantly inhibited cell invasion in a dose-dependent manner under chemoattractant of 10% FBS (Figure 5A) or 100ng/ml EGF (Figure 5B). 40 μM UA inhibited 54.9% FBS-induced cell invasion and 48.5% EGF-induced cell invasion.

2.4.4 Effects of UA on MMP-9 activity

Zymography results evaluating UA's effect on MMP-9 activities is shown in Figure 6. EGF significantly increased MMP-9 activity in MDA-MB-231 human breast cancers. UA at concentrations of 20, 30, and 40 μM significantly inhibited EGF- promoted MMP-9 activity.

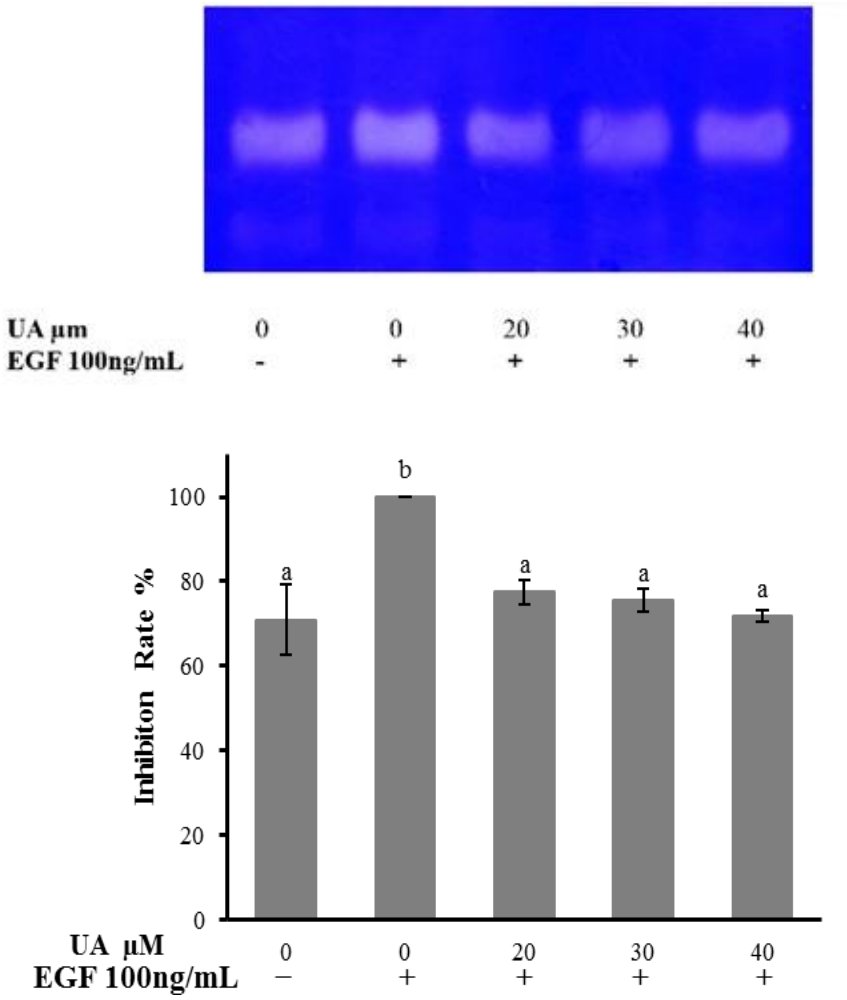

Figure 6. Effects of ursolic acid on MMP-9 activities (Mean ± SD, n=3). Cells were treated with 0,20,30,40 µM ursolic acid for 16 hrs under stimulation of 100 ng/mL EGF. Then MMP-9 activities were measured by Gelatin Zymography. Bands were visualized on a scanner. Inhibition rate were calculated as compared to model control (treatment with 0 µM UA under 100 ng/mL EGF stimulation). Bars with no letters in common are significantly different.

2.5 Discussion

Once oncogene is activated, unlimited cell growth is one of the hallmarks of cancer cells, hence inhibiting cell proliferation is a key indicator of anti-cancer activities

(Cohen and Arnold, 2011; Hanahan and Weinberg, 2000). A lot of phytochemicals, such as flavonoids, have shown protective effects in cancer development that associated with inhibition of cell proliferation and/or induction of apoptosis, hence to delete cell carrying mutations and to maintain a normal cell population (Chan et al., 2007; Kuntz et al., 1999). In our study, ursolic acid inhibited proliferation of MDA-MB-231 human breast cancer cell in a dose-response manner at the concentrations without cytotoxicity, which suggests that it is a promising phytochemical in anti-cancer research.

Cell migration is a fundamental process occurring in a wide range of physio-pathological situations, it also plays essential role in cancer invasion and metastasis (Wittekind and Neid, 2005). The migration process in cancer cells has been suggested to be similar as the wound healing process of normal tissues (Liang et al., 2007). Phytochemicals in wide categories inhibited cancer cell migration and invasion such as flavonoids, phenolic acids, stilbenes, triterpenoids and organic sulfur compounds (Li et al., 2015; Marchand, 2002; Singh et al., 2014; Yang et al., 2012). For breast cancer, curcumin, a diarylheptanoid, was found to inhibited human breast cancer cell migration and invasion via negatively regulate cell signaling transduction pathways, such as β-catenin and Akt pathways (Guan et al., 2016). Resveratrol exhibited preventive effects against breast cancer cell migration and invasion, which were related to alterations in cell surface actin structures and focal adhesion assembly (Azios and Dharmawardhane, 2005). The current study investigated anti-migration effects UA in highly invasive MDA-MB-231 human breast cancer cells. It is suggested

that UA significantly inhibited migration in non-EGF stimulation circumstances, while also significantly inhibited EGF-stimulated migration.

Invasion, as an indispensable step in cancer metastasis, is another hallmark and therapy target in carcinogenesis (Hanahan and Weinberg, 2000; van Zijl et al., 2011). Various phytochemicals showed anti-invasion effects previously (Azios and Dharmawardhane, 2005; Bao et al., 2015; Li et al., 2015; Yang et al., 2012). Invasion process requires remodeling of microenvironment and reconstruction of ECM by MMP enzymes, hence anti-invasion effects is highly associated with and frequently assessed together with MMP activity (Johnsen et al., 1998; Quail and Joyce, 2013; Wittekind and Neid, 2005). For breast cancer, Curcumin inhibited LPA-induced by inhibiting RhoA/ROCK/MMPs pathway in ER positive breast cancer cells, along with downregulation of MMP-2 and MMP-9 at both translational and transcriptional levels (Sun et al., 2016). Triple negative breast cancers have exhibited invasiveness since their distant recurrences in the brain and visceral metastases are more frequent as compared to ER positive cancers (Cleator et al., 2007; Foulkes et al., 2010). Sulforaphane inhibited tumor necrosis factor-α induced migration and invasion in estrogen receptor negative human breast cancer cells, along with downregulations of MMP-2, MMP-9, and MMP-13 protein expression, and MMP-2 and MMP-9 enzymatic activities (Bao et al., 2015). We tested anti-invasion effects of UA in MDA-MB-231 human breast cancer cells by a commercially available kit, which is based on a basement membrane model utilizing reconstituted ECM to provide the microenvironment for cancer cell invasion. Our results indicated ursolic acid inhibited

MDA-MB-231 human breast cancer cell invasion across ECM upon FBS induction. Later tests suggested UA also significantly repressed EGF-induced cell invasion. In current study, we also focused on MMP enzymatic activity, and found UA significant inhibited MMP-9 activities while antagonizing EGF stimulation. The downregulation MMP activity may further obstruct invasion of breast cancer cells through ECM.

EGF is a positive signal stimulates cell growth and differentiation for both normal and cancer cells (Carpenter and Cohen, 1990). Overexpression of its receptor EGFR is found in several cancers including triple negative breast cancer and is associated with reduced recurrence-free or overall survival rates (Masuda et al., 2012). EGFR, the ligand for EGF and an important member of RPTK family (transmembrane receptors with intrinsic protein tyrosine kinase activity), is overexpressed in approximately half of cases of triple negative breast cancer and inflammatory breast cancer (Masuda et al., 2012; Wells, 1999). Both EGF and TGF binds to EGFR, hence activates multiple cellular response via signal transduction, including cell proliferation, migration and invasion (Brachmann et al., 1989). It is important to find chemotherapy against EGF stimulation in triple negative breast cancers. Quercetin, a plant flavonoid, prevented migration and invasion induced by EGF in PC-3 prostate cancer cells, and reversed EGF-induced the transition; the effects were related to EGFR regulated cell signaling transduction (Bhat et al., 2014). Our study indicated UA antagonized EGF-stimulated migration, invasion and increase in MMP-9 activity. The contradictory effects against EGF stimulation of UA may further affect downstream pathways of EGFR.

2.6 Limitations and future directions

Cancer invasion and metastasis are intricated multistep processes. More related activities such as EMT evaluation should be assessed and finally anti-metastasis effects of UA should be tested in a metastatic animal model.

2.7 Conclusion

The objective of this chapter was to investigate anti-proliferation and anti-invasion effects of UA in MDA-MB-231 human breast cancer cells. UA significantly inhibited proliferation of MDA-MDB-231 human breast cancer cells in a dose-dependent manner at the concentrations without cytotoxicity. The EC_{50} value of anti-proliferative activity was 30.67 μM. UA at concentrations of 20, 30, and 40 μM significantly inhibited cell proliferation and migration. Additional tests indicated the potent anti-invasion activity of UA was partially due to antagonizing the stimulation of EGF. UA also neutralized EGF-induced increase in MMP-9 enzymatic activity, which help prevent MDA-MB-231 human breast cancer cells from invasion via ECM reconstruction. We demonstrated that UA exhibited anti-cancer activity through inhibiting both proliferation and invasion of MDA-MB-231 cells. These data shed light on understanding the protective activity of plant foods against breast cancer.

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Chapter 3 Effects of UA on specific molecular targets in regulating cellular signal transduction pathways in MDA-MB-231 human breast cancer cells

3.1 Introduction

Breast cancer is the second leading cause of cancer death among American women (Siegel et al., 2016). Approximately 15% of invasive breast cancers are triple negative breast cancers (ER^-, PR^-, $HER2^-$) (Cleator et al., 2007; Foulkes et al., 2010). They are highly chemosensitive yet with generally short progression-free time (Chacon and Costanzo, 2010). Current chemotherapy suggested several molecular targets are probably effective for triple negative breast cancer treatment, including: surface receptors (such as EGFR and TNFα), DNA repair enzyme PARP1, and targets from several cellular signaling pathways (such as MAPK, Akt and mTOR) (Chacon and Costanzo, 2010; Cleator et al., 2007; Harbeck and Gnant, 2017).

The epidermal growth factor receptor EGFR is overexpressed in approximately half of cases of triple negative breast cancer and inflammatory breast cancer, which makes it a promising target for anti-triple negative breast cancer therapy (Masuda et al., 2012). It can be activated via phosphorylation by both EGF or TGF (Brachmann et al., 1989; Wells, 1999). As a member of RPTK family (transmembrane receptors with intrinsic protein tyrosine kinase activity), EGFR transduces extracellular stimulation signal and directly or indirectly stimulates a wide array of downstream pathways related to cell survival and mobility, such as MAPK, Akt, JAK-STAT (Bhat et al., 2014; Lee et al., 2003; Nicholson et al., 2001; Rawlings et al., 2004; Wells, 1999). Previously, several

phytochemicals (such as resveratrol, quercetin and curcumin) have been reported to inhibit EGF-induced EGFR activation in cancer cells, hence survival and invasive proteins via regulating central pathways (Bhat et al., 2014; Ji et al., 2008; Lee et al., 2011; Nicholson et al., 2001).

A prototypical survival pathway for normal cancer cells is the PI3K/Akt (PI3K for phosphoinositide 3-kinase, Akt for protein kinase B) pathway is receive upstream signals from RTK (Receptor tyrosine kinase) activated by both cytokines and growth factors (Fresno Vara et al., 2004; Mundi et al., 2016). Akt is activated via phosphorylation when it interacts with phospholipids produced by PI3K (Campbell et al., 2001; Fresno Vara et al., 2004; Yang et al., 2004). Akt promotes cell survival and migration via directly or indirectly affecting a wide array of downstream participators including tumor suppressor p53, Bcl family protein and caspases involved in apoptosis cascade, IKKα and its downstream inflammatory transcriptional factor NF-κB, and proteins from mTOR pathway promoting growth, proliferation and protein synthesis (Fresno Vara et al., 2004; Karar and Maity, 2011; Populo et al., 2012; Sun et al., 2010). Akt pathway was broadly targeted by plant extracts (e.g. grape proanthocyanidin and curry spice extracts) and phytochemicals (such as flavones, sulforaphane, and curcumin) in anti-cancer researches (Ho et al., 2010; Lin et al., 2015; Pawlik et al., 2013; Prasad et al., 2012; Shukla et al., 2014; Tsai et al., 2013; Wang et al., 2013; Zanotto-Filho et al., 2012).

JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway, is a

relative simple signaling cascade which consists of: JAK, a kinase associated with cytoplasmic domains of membrane receptor subunits and activated after multimerization of receptor subunits; and STAT, an intracellular transcription factor, which is activated by JAK via phosphorylation at Tyr705 residue in cytoplasm and then form hetero- or homodimers and translocate to the cell nucleus (Rawlings et al., 2004; Schindler and Darnell, 1995). STATs regulate lots of survival, proliferative or migration related proteins in both normal and cancer cells, such as p53, Bcl-2 family, cyclins, myc and MMPs, hence serve as promising targets for chemotherapy (Bromberg and Wang, 2009; Yu and Jove, 2004; Yu et al., 2014). Phytochemicals such as flavonoids, carotenoids and triterpenoids showed anti-cancer activities via JAK/STAT inhibition (Kim et al., 2016; Kowshik et al., 2014; Lewinska et al., 2017; Michl et al., 2016).

As a key marker of inflammation and cell invasion, the transcription factor NF-κB (Nuclear factor-kB) is crucial in regulating cell survival, energy metabolism, proliferation, invasion and apoptosis via its effector genes, such as TRAP, GLUT3, Fas, p53, Bcl-2, ICAM-1, MMP, VEGF (Bours et al., 1994; Moretti et al., 2012; Prasad et al., 2010; Viatour et al., 2005). NF-κB is mainly activated when it is released from its inhibitors (IκB) and translocate to nucleus; IKK (IκB kinase) is the mediator that degrades inhibitory molecules of NF-κB via phosphorylation following upstream regulation such as Akt and MAPK pathway (Baldwin, 1996; Bours et al., 1994; Ghosh et al., 1998; Prasad et al., 2010). Most anti-invasive food extracts, phytochemicals and drugs downregulated NF-κB via different upstream approaches in cancer cells (Barton,

2001; Chiu et al., 2015; Huang et al., 2010; Kawiak and Domachowska, 2016; Mukherjee et al., 2014; Sanchez-Fidalgo et al., 2015; Wung et al., 2005).

However, effects of UA on cellular signal transduction in anti-breast cancer activities is not clear. In this chapter, we aimed at investigating effects of UA on specific molecular targets in regulating cellular signal transduction pathways in MDA-MB-231 human breast cancer cells.

3.2 Objectives

To investigate the effects of UA on cellular signal transduction pathways in MDA-MB-231 human breast cancer cells, the specific objectives are designed as follow:

a) To screen the potential key targets of UA in anti-cancer regulation on cellular signal transduction pathways in MDA-MB-231 human breast cancer cells
b) To investigate the effects of UA on the key protein expression of cell signaling pathways in MDA-MB-231 human breast cancer cells

3.3 Materials and methods

3.3.1 Chemicals

Ursolic acid was isolated from apple peels in our lab as reported previously (He and Liu, 2007). UA was diluted in Dimethyl sulfoxide (DMSO) first and then dilute by 1: 100 in medium before use. DMSO and Epidermal Growth Factor (EGF) were purchased from VWR (Radnor, PA, USA). Phosphate-buffered saline (PBS) and α-minimum essential medium (α-MEM) were received from Gibco BRL Life

Technologies (Grand Island, NY, USA). Fetal bovine serum was received from Atlanta Biologicals (Lawrenceville, GA, USA). PathScan intracellular signaling array kit (chemiluminescent readout) was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against EGFR, p-EGFR, JAK, p-JAK, STAT3, p-STAT3, Akt, p-Akt and NF-κB were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against anti-mouse IgG, and anti-rabbit IgG were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against β-actin and nucleolin were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

3.3.2 Cell culture

MDA-MB-231 human breast cancer cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in α-MEM containing 10 mM Hepes, 1% antibiotic-antimycotic and 10% heat-inactivated fetal bovine serum as described previously (Jiang et al., 2016). The cells were cultured at 37 °C in a 5% CO_2 humidity condition.

3.3.3 Molecular target screening assay

Target protein screening was conducted under manufacturer's instructions of PathScan intracellular signaling array kit with minor modification. Briefly, cells were cultured in described condition with DMSO or UA. After 24 hours culture media was removed and wells were washed by ice-cold PBS for 2 times. Cell lysates were prepared by offered lysis buffer with 1mM phenylmethylsulfonyl fluoride (PMSF) in ice-cold

environment for 2 minutes. Then the lysates are micro-centrifuged at 11000 rpm for 3 minutes at 4°C and the supernatants were transferred to new tubes. Lysates were stored at −80°C in single-use aliquots before use. Immediately before performing the assay, lysates were diluted to 1.0 mg/ml after protein analysis. The slide was activated following the instruction and 75 µL diluted lysate was added to each well and incubated overnight at 4°C. Then the slide is washed by wash buffer for 4 times, 5 minutes per time. Later 75 µL detection antibody cocktail was added to each well and the slide was covered with sealing tape. The slide was then incubated for 1 hour at room temperature on an orbital shaker. After being washed by wash buffer for 4 times, 5 minutes per time, 75 µL HRP-linked Streptavidin was added to each well and the slide was incubated for 30 minutes on an orbital shaker. Then the slide was washed for 4 times and added with combined LumiGLO and Peroxide reagents. Images was captured after 3 minutes exposure by UVP Bioimaging system.

3.3.4 Western blots

Key protein expression was tested by western blot assay as described by our group previously (Jiang et al., 2016). The MDA-MB-231 human breast cancer cells were treated with control, or various concentrations of ursolic acid. Cells were scraped from the wells in cold PBS and then cell suspension was centrifuged at 1000 rpm for 5 minutes at 4 °C. Cells were harvested after removal of supernatants and stored at −80°C. Protein was extracted by cell lysis using Ripa buffer (50 mM Tris, 1% Igepal, 150 mM sodium chloride, 1 mM EDTA, pH 7.4) with a cocktail of protein inhibitors (1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstain, 1 mM sodium

orthovanadate, 1 mM sodium fluoride, 1 mM PMSF). Cell lysates were centrifuged at 11000 rpm for 5 minutes at 4 °C and protein were collected as supernatants. For nuclear protein extraction, cells were lysed by lysis buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.05% Igepal, pH 7.9) with proteinase cocktails for 10 minutes at first, and then centrifuge at 4°C at 3500 rpm for 10 minutes to remove cytosolic fraction (supernatant). Nuclear protein was extracted by continued lysis using lysis buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% v/v glycerol, pH 7.9) with proteinase cocktails for 30 minutes on ice. Then nuclear protein was harvested as supernatants after centrifuge at 11,000 rpm for 20 min at 4°C. Concentrations of extracted proteins were determined by Biuret-Folin protein assay. All protein samples were distributed, mixed with 1× sample loading buffer and stored at −80°C. Extracted protein samples was then subjected to electrophoresis on 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lysate proteins after separation by running gel were transferred onto a Polyvinylidene fluoride (PVDF) transfer membrane and the membrane was blocked in 5% nonfat dry milk in TBST (Tris-base buffer solution containing of 0.1% Tween 20) at room temperature for 2 hours and then incubated with primary antibodies (diluted in 5% nonfat dry milk in TBST) overnight at 4 °C. Membranes were then rinsed three times with TBST (5 minutes each time) and incubated for 2 h at room temperature with a corresponding secondary antibody diluted in 5% nonfat dry milk in TBST. After subsequent washing with TBST, membrane-bound antibodies were visualized by the Enhanced Chemiluminescence Kit (Cell Signaling Technology, Inc., Beverly, MA, USA). Bands were quantified by ImageJ2x software (Wayne Rasband, National

Institutes of Health, Bethesda, MD, USA). The expression of β-actin or nucleolin was used as an internal standard control. All measurements were conducted in triplicates.

3.3.5 Data analysis.

The data were statistical analyzed by SPSS 17.0 software (International Business Machines Corp., NY, USA) and presented as a mean ± standard deviation (SD) for at least three replicates. The significance was determined at a p value of <0.05 by the analysis of variance (ANOVA) followed by Tukey's test.

3.4 Results

3.4.1 key targets of UA on cellular signal transduction in MDA-MB-231 human breast cancer cells.

We screened multiple targets related with signaling transduction pathways primarily by a slide-based antibody array founded upon the sandwich immunoassay principle. MDA-MB-231 human breast cancer cells were treated with DMSO or UA for 24 hours, then the signaling nodes were detected at their phosphorylated or cleaved forms at the specified residues, the results can be found in Figure 7 and Table 1.

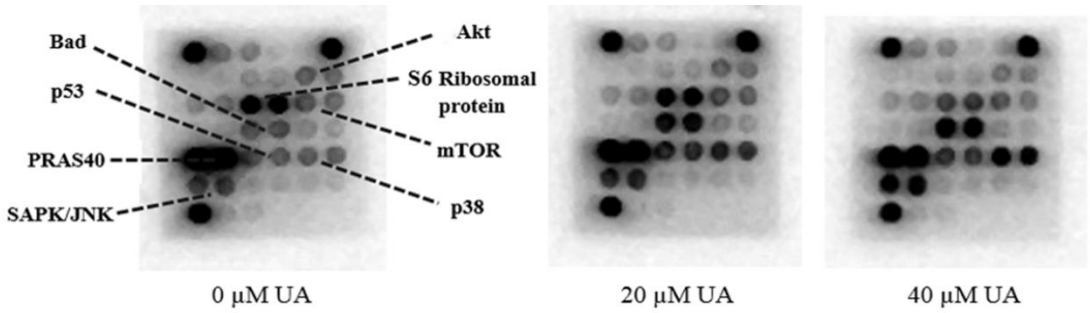


Figure 7. Key targeted protein of UA on cellular signal transduction in MDA-MB-231 human breast cancer cells. UA affected expression of 8 targeted protein in primary screening. All proteins were detected at their active forms.

According to the screening, UA activated tumor suppressor protein p53, pro-apoptotic protein Bad, and p38 and SAPK/JNK from MAPK pathways responsive to cellular stress. UA also downregulated survival proteins, such as: Akt, the central survival promoter regulating glucose metabolism, apoptosis, proliferation and migration; mTOR, affecter of Akt regulating cell proliferation, motility, protein synthesis and autophagy; S6 ribosomal protein, activator of mTOR pathway; PRAS40, a Akt substrate transducing Akt signal to mTOR. However, no obvious change was observed in ERK MAPK, and signal of other targets were not strong enough to be detected.

Table 1. Effects of UA on major molecular targets of cellular signal transduction pathways in MDA-MB-231 human breast cancer cells.

Target[a]	Phosphorylation site	Modification	UA's effects[b]
Positive Control	N/A	N/A	N/A
Negative Control	N/A	N/A	N/A
ERK1/2	Thr202/Tyr204	Phosphorylation	NC
Stat1	Tyr701	Phosphorylation	N/A
Stat3	Tyr705	Phosphorylation	N/A
Akt	Thr308	Phosphorylation	N/A
Akt	Ser473	Phosphorylation	Downregulation
AMPKα	Thr172	Phosphorylation	N/A
S6 Ribosomal Protein	Ser235/236	Phosphorylation	Downregulation
mTOR	Ser2448	Phosphorylation	Downregulation
HSP27	Ser78	Phosphorylation	N/A
Bad	Ser112	Phosphorylation	Upregulation
p70 S6 kinase	Thr389	Phosphorylation	N/A
PRAS40	Thr246	Phosphorylation	Downregulation
p53	Ser15	Phosphorylation	Upregulation
p38	Thr180/Tyr182	Phosphorylation	Upregulation
SAPK/JNK	Thr183/Tyr185	Phosphorylation	Upregulation
PARP	Asp214	Cleavage	N/A
Caspase-3	Asp175	Cleavage	N/A
GSK-3β	Ser9	Phosphorylation	N/A

[a] Targets are arranged in the order of the design of the kit.

[b] Upregulation represents the target was upregulated by UA; downregulation represents the target was downregulated by UA; NC represents no obvious change was observed; N/A represents the regulation result of UA on the current target was not applicable, including two situations：the signal for the current target was not strong enough for in the present detection condition, or the indicator was not applicable for positive and negative controls.

3.4.2 Effects of UA on membrane receptor EGFR

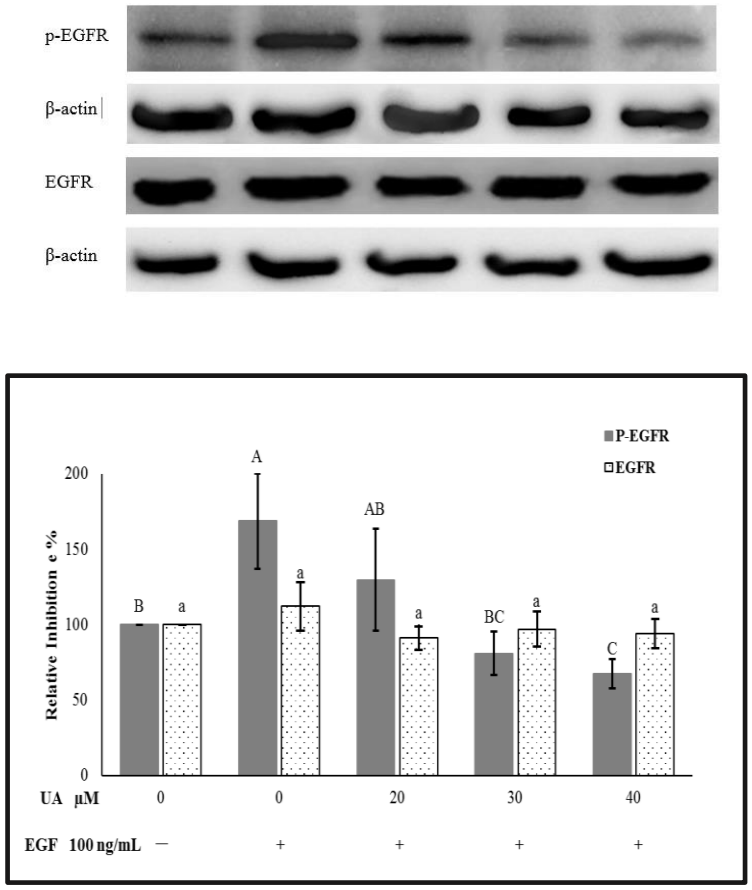


Figure 8 Effects of UA on EGFR expression. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

Effects of UA on EGFR expression in MDA-MB-231 human breast cancer cells is shown in Figure 8. EGF was used to stimulate EGF activation, which significantly upregulated phosphorylated EGFR, but did not affect total EGFR. UA downregulated EGFR phosphorylation while antagonizing EGF stimulation in a dose-response manner. UA at 40 μM significantly reduced p-EGFR (Tyr1068) expression to a level lower than blank control without EGF stimulation, which suggests UA not only

resisted EGF stimulation but also downregulated EGFR phosphorylation. Total EGFR expression was not affected by UA, indicating UA inhibited EGFR majorly via inhibiting its phosphorylation-induced activation.

3.4.3 Effects of UA on JAK2 expression

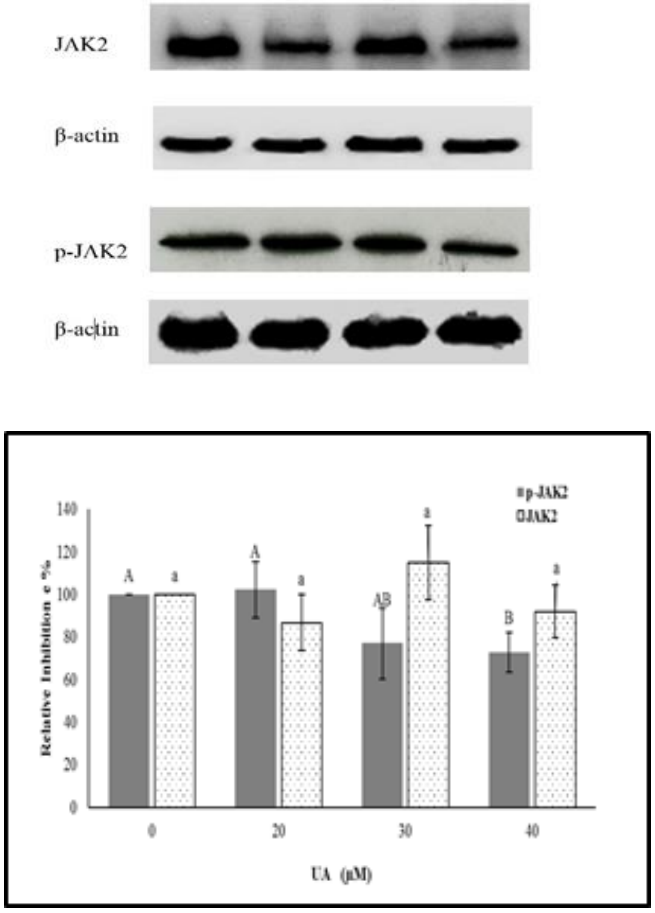


Figure 9. Effect of UA on JAK2 expression. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

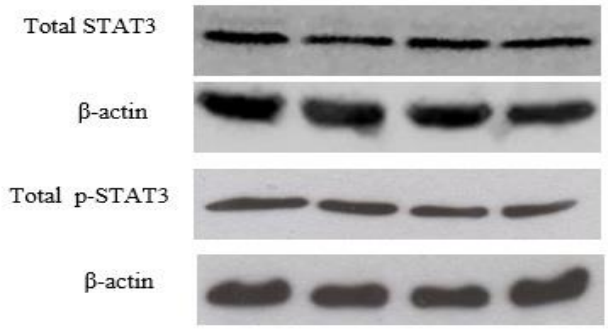
UA inhibited JAK2 activation by downregulating its active form of phosphorylation at Tyr1007/1008 residue in MDA-MB-231 human breast cancer cells, treatment at the

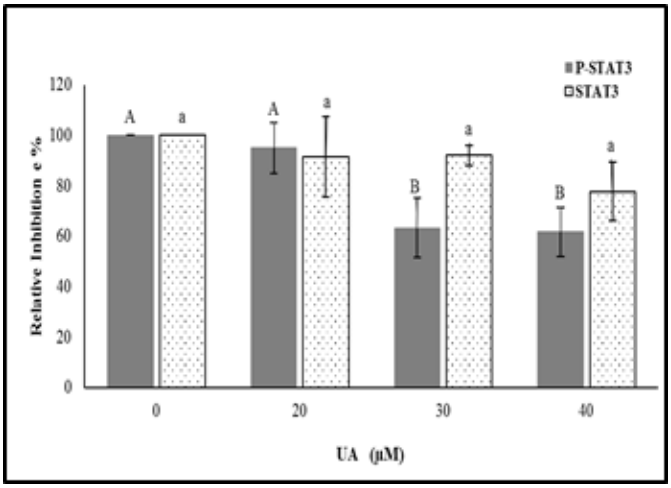
concentration of 40 μM showed statistical difference. The result is shown in Figure 9.

3.4.4 Effects of UA on STAT3 expression

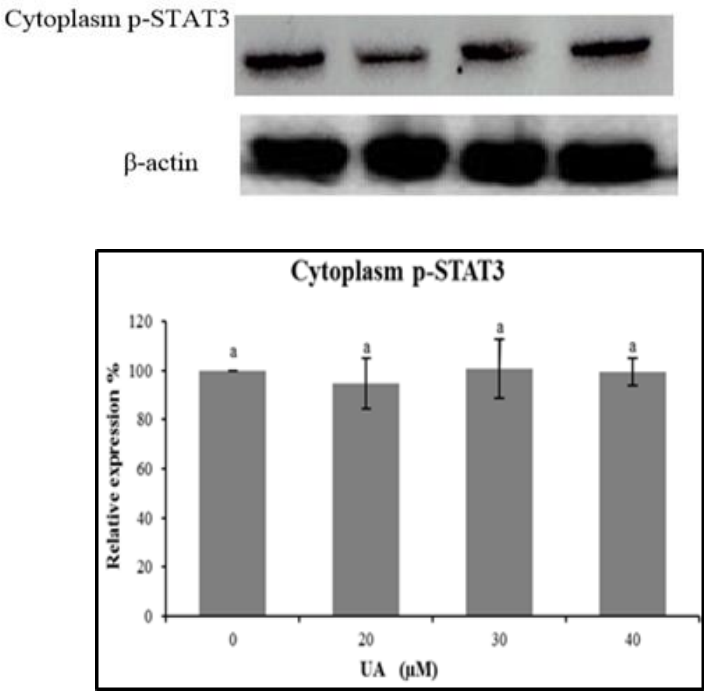
UA significantly inhibited activation of transcriptional factor STAT3 in MDA-MB-231 human breast cancer cells by downregulating its phosphorylated form (Tyr 705), while not affecting total STAT3 expression (Figure 10A). Further test suggested UA did not affect p-STAT3 in cytoplasm (Figure 10B), yet significantly inhibited p-STAT3 in nucleus, as its active form (Figure 10C). Taken together, UA significantly inhibited STAT3 activation by inhibiting its phosphorylation and reducing translocation of p-STAT3 to the nucleus.

A





B



C

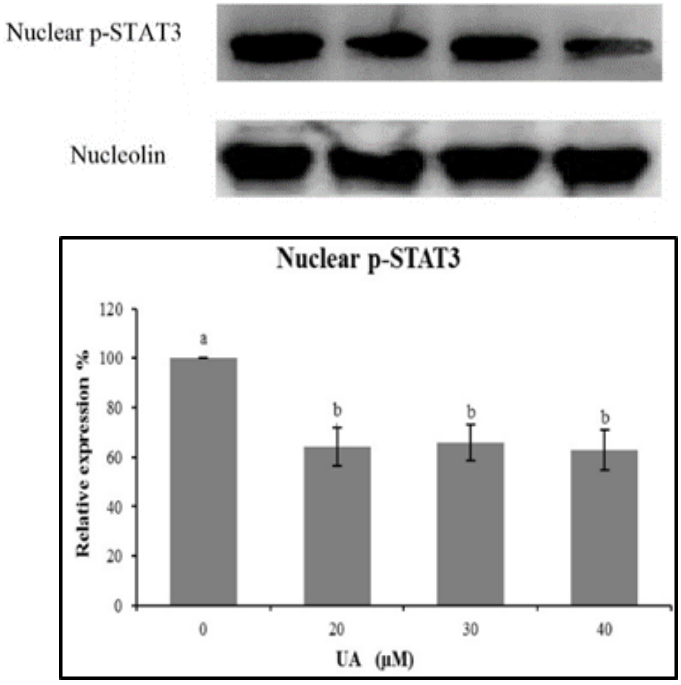


Figure 10. Effect of UA on STAT3 expression: (A) total STAT3; (B) cytoplasm STAT3; (C) nuclear STAT3**.** Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

3.4.5 Effects of UA on Akt expression

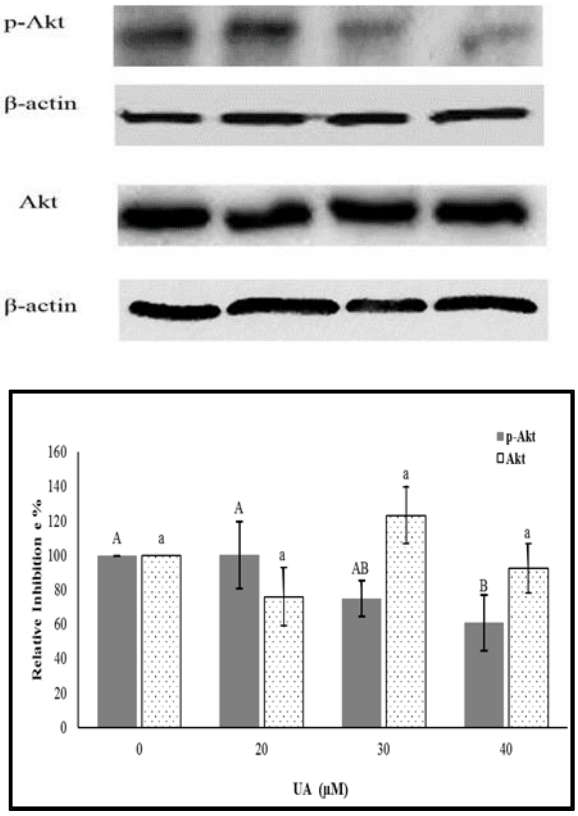


Figure 11. Effect of UA on Akt expression. Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

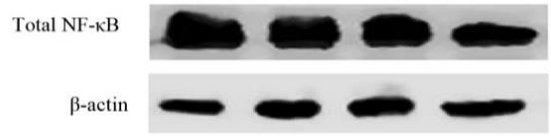
Akt activation was significantly inhibited by UA in MDA-MB-231 human breast cancer cells in a dose-dependent manner with decreased expression of its phosphorylated form (Ser 473), total expression of Akt was not affected. The result is shown in Figure 11.

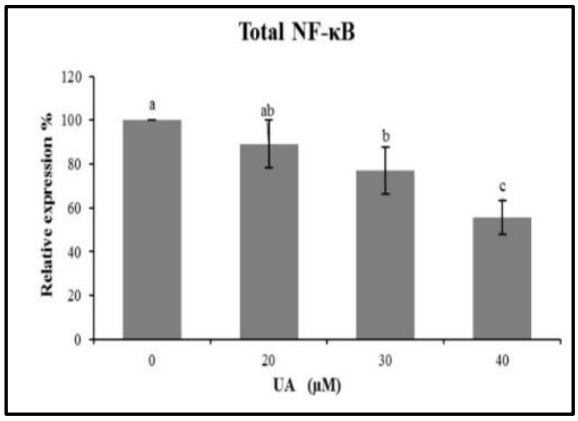
3.4.6 Effects of UA on NF-κB expression

UA significantly downregulated cellular expression of transcriptional factor NF-κB in

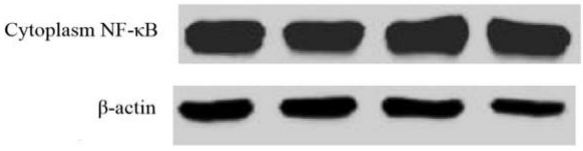
a dose-response manner in MDA-MB-231 human breast cancer cells (Figure 13 A). Additionally, UA significantly reduced nuclear distribution of NF-κB while cytoplasm NF-κB did not show significant change. The results indicate UA significantly reduced NF-κB expression and its active form in nucleus.

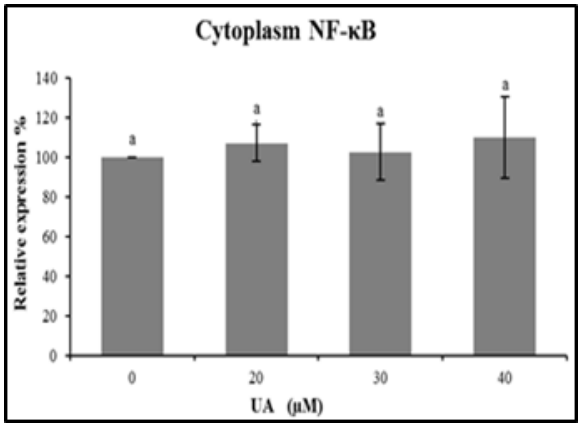
A





B





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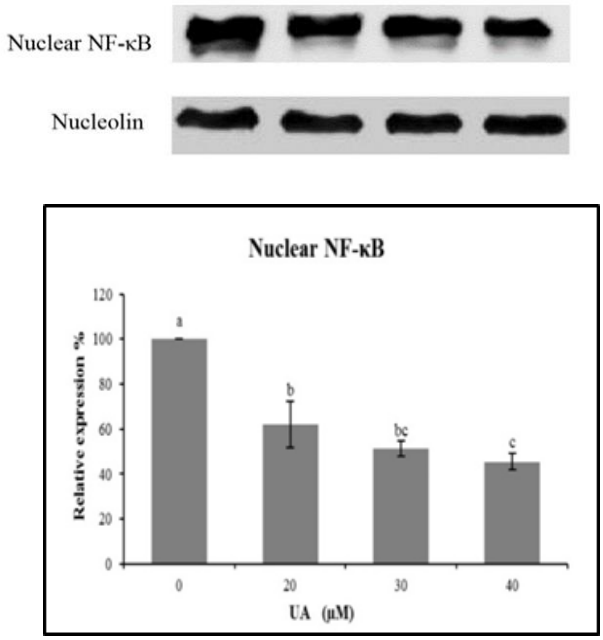

Figure 12. Effect of UA on NF-κB expression: (A) total NF-κB; (B) cytoplasm NF-κB; (C) nuclear NF-κB**.** Bars with no letters in common are significantly different ($p < 0.05$). Each value represents the mean ± SD of triplicates.

We reported anti-migration and anti-invasion effects of UA in the previous chapter. The current study found membrane receptor EGFR and molecular targets from JAK/STAT and Akt pathway were regulated by UA, which helps explain anti-proliferation and anti-invasion effects of UA in MDA-MB-231 human breast cancer cells (Figure 13).

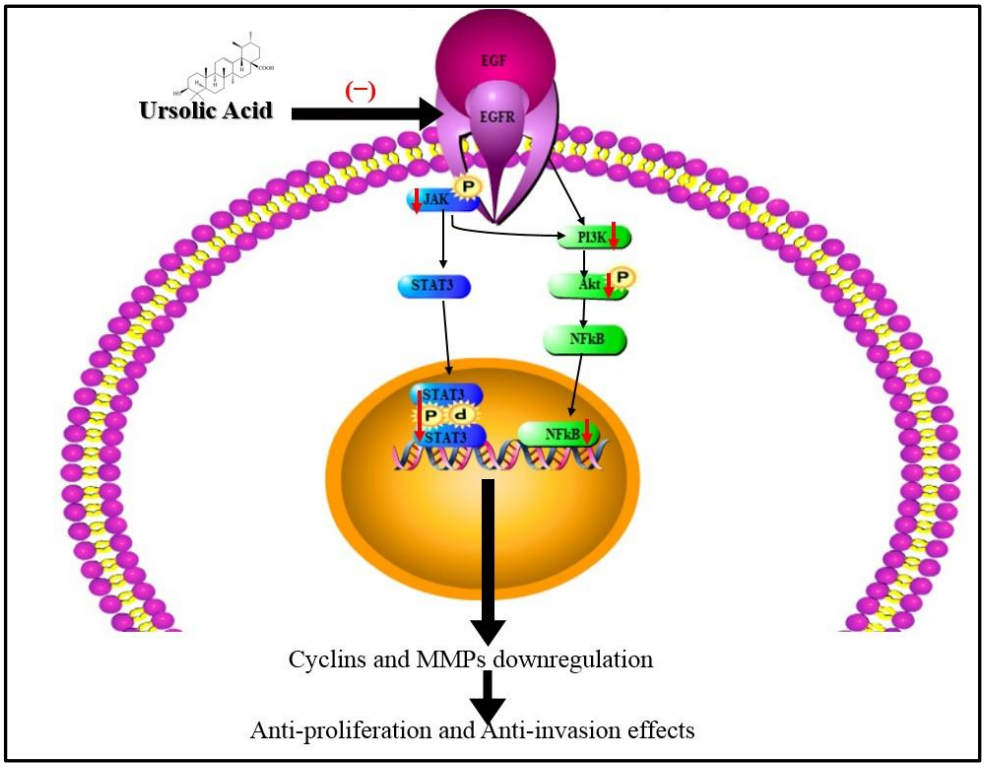

Figure 13. UA inhibited proliferation and invasion of MDA-MB-231 human breast cancer cells via the Akt and STAT3 signal transduction pathways.

3.5 Discussion

Hormone therapy and chemotherapy targeting ER, PR and HER receptors are not applicable in triple negative cancer treatment (Foulkes et al., 2010; Harbeck and Gnant, 2017). As cancer with more common distant recurrences in metastases (such as brain and visceral), triple negative cancer has poor more common distant recurrences in the brain and visceral metastases survival, it is necessary to find compounds that can effectively inhibit invasion of triple negative cancer through other molecular targets and with lower cytotoxicity (Chacon and Costanzo, 2010; Davies, 2016). Previously we reported ursolic acid significantly inhibited proliferation, migration and invasion in

at the concentrations without cytotoxicity and antagonized EGF stimulation in last chapter.

In the current study, we found the above anti-cancer effects were related with regulation on cellular signal transduction pathways. According to our primary screening, UA affected 8 targets with Akt, mTOR and MAPK pathways involved. UA upregulated tumor suppressor p53, which plays crucial role in anti-cancer defense especially via triggering apoptosis cascade and arresting the cell cycle. The tumor suppressor p53 is described as guardian of genome and prevents tumor formation from various approaches: it activates DNA repair proteins when sustained damage occurs on DNA; it arrests cell cycle at the G1/S regulation point hence stops growth and proliferation; it activates apoptosis in cancer cells; and it related to cell senescence (Muller and Vousden, 2013; Toshiyuki and Reed, 1995). The p53 protein majorly cooperates with p21 protein in signal transduction, it also has lots of crosstalk with pathways such as MAPK and Akt pathways (Rueda-Rincon et al., 2015; Wu, 2004). MAP kinases phosphorylate and activate p53 under upstream, such as stress stimuli, cytokines and growth factors (Toshiyuki and Reed, 1995; Wu, 2004). MDM2 protein is a key p53 function regulator, Akt activation induces phosphorylation in MDM2 and inactivates p53 (Gottlieb et al., 2002). Akt signaling was undermined by p53 due to membrane phospholipid composition modification (Rueda-Rincon et al., 2015).

Targets from PI3K/Akt/mTOR pathway were also affected by UA toward inhibitory signaling. The PI3K/AKT/mTOR pathway is activated in major cancers including

breast cancers, activation of PI3K is associated with increased activation of growth factor receptors (such as EGFR), loss of phosphatase and tensin homolog (PTEN), or Ras mutation (Fresno Vara et al., 2004; Karar and Maity, 2011). The pathway plays important role in regulating energy metabolism, ribosomal biosynthesis, cell cycle progression, autophagy and senescence (Elstrom et al., 2004; Karar and Maity, 2011; Populo et al., 2012; Sun et al., 2010). mTOR activation leads to increased synthesis of multiple proteins, including cyclin D1−the cell cycle and proliferation promoter, and HIF−the pro-angiogenic growth factor (such as VEGF trigger) (Karar and Maity, 2011; Populo et al., 2012; Porta et al., 2014).

UA activated p38 and SAPK/JNK MAPK in MDA-MB-231 breast cancer cells, but not ERK MAPK. The MAPK pathway can be activated by upstream signal from receptor and Ras, it consists of a three protein kinases central link: a MAPK kinase kinase (MAPKKK, e.g. Raf), a MAPK kinase (MAPKK, e.g. MEK) and a terminal MAPK, such as ERKs, JNKs (or SAPKs), and p38 kinases (Bonni et al., 1999; Dhillon et al., 2007). JNK MAPK and p38 MAPK can be both activated in response to cellular stresses including growth factors, inflammatory cytokines, lipopolysaccharides, radiation, and osmotic shock; they trigger a series of downstream signal toward promoting apoptosis and inhibiting proliferation and invasion (Burotto et al., 2014; Chen et al., 1996; Dhillon et al., 2007; Gallo and Johnson, 2002).

Our study found UA inhibited EGF-induced EGFR phosphorylation (Tyr 1068), which is in consistent with its inhibition toward EGF-induced migration and invasion. EGFR

is a promising therapeutic target in triple negative breast cancer, whose overexpression is found in approximately half of cases (Masuda et al., 2012). EGFR has been demonstrated to serve as upstream activator of multiple signaling nodes, such as JAK/STAT and PI3K/Akt, hence related with regulation of cell survival, growth, proliferation, migration and invasion (Amin et al., 2011; Nicholson et al., 2001). EGFR-targeting agents have been tested in advanced clinical development for the treatment of various human cancer types (such as metastatic non-small cell lung cancer, colorectal cancer squamous-cell carcinoma of the head and neck, bladder cancer and pancreatic cancer), the agents include anti-EGFR monoclonal antibodies, small molecules, and reversible EGFR tyrosine kinase inhibitors (Ciardiello and Tortora, 2008; Frattini et al., 2015; Gazdar, 2009; Mooso et al., 2015; Sartore-Bianchi et al., 2009). The current study suggested UA is an effective EGFR suppressor, which can be further investigated for anti-breast cancer applications.

Though during the primary screening, we did not collect signal of STAT3 that was strong enough for analysis due to methodology limitations. Lots of published paper suggested JAK/STAT3 pathway is a promising target for anti-cancer research and can be targeted by UA several cancers such as the prostate cancer (Shanmugam et al., 2012; Yu and Jove, 2004; Yu et al., 2014). The JAK/STAT pathway consist of cascade include: activation of JAK via phosphorylation following upstream stimuli of receptor tyrosine kinase; and activation of STAT via phosphorylation by JAK, forming homo- or heterodimers, and translocating to nucleus (Bromberg and Wang, 2009; Rawlings et al., 2004). STAT3 is a member from STAT3 family, it is typically phosphorylated at

Tyr 705 in response to JAK activation under stimuli from growth factors and cytokines, moreover STAT3 may alternatively be phosphorylated at Ser 727 by MAPK (Galdiero et al., 2006; Rawlings et al., 2004; Yu and Jove, 2004; Yu et al., 2014). Downstream effector genes of STAT3 include p53, cyclins, apoptotic proteins and MMPs (Yu and Jove, 2004; Yu et al., 2014). JAK/STATs pathway is also reported to regulate class IA PI3K on its p50α and p55α small regulatory subunits (Abell and Watson, 2005). Our study reported UA downregulated JAK2 phosphorylation at Tyr1007/1008 and inhibited STAT3 activation via downregulating its active form of phosphorylation at Tyr 705 and reducing nuclear distribution of p-STAT3. Hence UA might affect downstream effector genes of STAT3, which helps explain its anti-proliferation and anti-invasion effects.

NF-κB is a transcriptional factor activated by pro-inflammatory cytokines and growth factors, it is highly related with cancer progression and metastasis since it induces cell proliferation, anti-apoptotic, angiogenesis and invasive gene expression (Bours et al., 1994; Ghosh et al., 1998; Moretti et al., 2012). Aberrant NF-kB activity is a hallmark of cancer and chronic inflammatory diseases (Prasad et al., 2010). NF-kB is activated when it is released from its cytoplasm inhibitor IκBα and translocated to nucleus, degradation of IκBα requires phosphorylation by upstream kinase IKK, which is activated by upstream signal such as activated Akt (Baldwin, 1996). A FDA approved receptor activator of NF-kB ligand inhibitor, Denosumab, has been applied in patients with skeletal metastases from breast and other solid tumors, but not for patients with prostate tumor (Vassiliou, 2013). Our study reported UA as an effective inhibitor for

NF-κB expression. UA also downregulated active NF-κB in nucleus, with correlated downregulation of upstream activated Akt (phosphorylation at Ser 473).

3.6 Limitations and further directions

Cellular signaling pathways are highly interactive systems with lots of crosstalk. The current study only tested UA's effects on key targets and selected pathways. Future study should map more parts of the regulatory routes. Besides, to further confirm UA's regulation on downstream effectors is achieved or partly achieved from EGFR membrane receptor, additional inhibitor tests or knockdown by siRNA might be applied. Since both STAT3 and NF-κB are transcriptional factors, expression of downstream effector genes might be investigated to give more clues on UA's regulation on cell cycle progression and cell fate determination.

3.7 Conclusion

The objective of this chapter was to investigate effects of UA on anti-cancer cellular signal transduction in MDA-MB-231 human breast cancer cells and to determine specific molecular targets for UA. UA affected 8 targeted proteins in primary signaling screening, including activation of tumor suppressor and inhibition of survival proteins. Akt, mTOR and MAPK signaling pathways were involved in UA's effects against breast cancer cell migration and invasion. Western blots indicated UA significantly downregulated EGF-induced EGFR phosphorylation, which was correlated with reported inhibitory effects of UA on EGF-induced migration and invasion. UA inhibited JAK/STAT3 and Akt activation, as well as downregulated NF-κB expression

and activation. The results give clues in understanding the mechanisms behind anti-cancer activities of phytochemicals.

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Chapter 4 Anti-proliferation effects of combining ursolic acid with paclitaxel in inhibition of human breast cancer cells

4.1 Introduction

Breast cancer is the most common diagnosed cancer and the second leading cause of cancer death among American woman (Siegel et al., 2016). Treatment of breast cancer typically requires surgery, while most breast cancer patients are offered with adjuvant treatments such as radiotherapy, hormone therapy, chemotherapy and biological agents (Davies, 2016). Chemotherapy in breast cancer differs according to cancer development: monotherapy is usually applied in slow progression breast cancers, such as using anthracycline or taxane; combination chemotherapy is usually applied in rapid progression breast cancers, such as paclitaxel with gemcitabine, docetaxel with gemcitabine, docetaxel with capecitabine, anthracycline with taxane, and anthracycline with cyclophosphamide (Harbeck and Gnant, 2017; Hulka and Moorman, 2001; Mourouti et al., 2015).

Paclitaxel is a clinical anti-cancer drug that has been applied in breast cancer and other cancers (such as ovarian cancer and lung cancer), its antitumor activity is based on stabilization of the microtubule dynamics and thereby interfering cell cycle (de Weger et al., 2014; Liebmann et al., 1993). Paclitaxel binds to the microtubules and disrupts microtube assembly and disassembly in a GTP independent manner (de Weger et al., 2014; Weaver, 2014) Paclitaxel is typically combined with bevacizumab (a monoclonal antibody against VEGF) in initial treatment for metastatic breast cancer, this drug combination prolongs progression-free survival, but not overall survival, as

compared with paclitaxel alone in patients with metastatic breast cancer (Miller et al., 2007).

UA is a natural occurred triterpenoid, which can be isolated from peels of various fruits (such as apples and cranberries), herbs (such as peppermint) and spices (such as basil, rosemary and thyme) (Borovkov and Belova, 1967; Cargnin and Gnoatto, 2017; Hill and Connolly, 2012; Mezzetti et al., 1971; Xiang et al., 2015; Yeh et al., 2010). UA showed anticancer activities including induction of apoptotic cell death and cell cycle arrest, anti-proliferation and anti-invasion (Achiwa et al., 2013; Ikeda et al., 2008; Kassi et al., 2009; Kim et al., 2011; Leng et al., 2013; Shanmugam et al., 2013; Shanmugam et al., 2012; Wang et al., 2012; Wu et al., 2012; Yeh et al., 2010). EGF stimulation and EGFR overexpression has been associated with cancer development and invasion, and EGFR is served as crucial targets in treatment of invasive cancer and triple negative cancer (Ciardiello and Tortora, 2008; Masuda et al., 2012; Mooso et al., 2015; Nicholson et al., 2001). We previously found UA inhibited proliferation and invasion of MDA-MB-231 triple negative human breast cancer cells via downregulating EGF/EGFR downstream effectors in cell signaling transduction pathways.

A synergistic therapeutic effect is defined as the combination of two or more compounds displays a stronger effect than the additive effect of individual compounds when treated at equal concentrations (Chou, 2010). One major aim of drug combination is to acquire synergistic therapeutic benefits, which can be quantified

using indicators such as CI (combination index) and DRI (dose-reduction index) (Chou and Talalay, 1984; Chou, 2006). Previously, several combinations of bioactive compounds showed synergistic effects in various chemotherapeutic fields, including the inhibition of cancer cell proliferation (Chen et al., 2009; Yang and Liu, 2009). Baicalein, a flavonoid and silymarin, a flavonolignan showed additive effects at 24 h and synergistic effects at 48 h in inhibiting growth of HepG2 liver cancer cells; besides, combination of both compounds synergistically increased the percentages of cells in G0/G1 phase and reduced those in S-phase, which were associated with regulation on expression of Rb, p53, p21, p27, cyclin D1, cyclin E, and CDK4 (Chen et al., 2009). Previously, we reported of apple extracts and quercetin 3-β-D-glucoside (Q3G) exhibited synergistic effects toward MCF-7 human breast cancer cell proliferation using Chou-Talalay model. (Chou and Talalay, 1984; Yang and Liu, 2009). In the two-way combination, the EC_{50} values of apple extracts and Q3G were 2- and 4-fold lower, and CI values at 50 and 95% inhibition rates were 0.76 and 0.42 (Yang and Liu, 2009).

The objective of this research is to investigate the potential synergistic effects of combining ursolic acid and paclitaxel toward breast cancer proliferation.

4.2 Objectives

To investigate the potential synergistic effects of combining ursolic acid with paclitaxel toward breast cancer proliferation. The specific objects are designed as follow:

a) To determine anti-proliferation effects of combining ursolic acid with paclitaxel toward MDA-MB-231 human breast cancer cells

b) To determine anti-proliferation effects of combining ursolic acid with paclitaxel toward MCF7 human breast cancer cells

4.3 Materials and methods

4.3.1 Chemicals

Ursolic acid was isolated from apple peels in our lab as reported previously (He and Liu, 2007). UA was diluted in Dimethyl sulfoxide (DMSO) first and then dilute by 1: 100 in medium before use. Dimethyl sulfoxide (DMSO) and Epidermal Growth Factor (EGF) were purchased from VWR (Radnor, PA, USA). Phosphate-buffered saline (PBS), Anti-anti, insulin and α-minimum essential medium (α-MEM) were received from Gibco BRL Life Technologies (Grand Island, NY, USA). Fetal bovine serum was received from Atlanta Biologicals (Lawrenceville, GA, USA).

4.3.2 Cell culture

MDA-MB-231 human breast cancer cells and MCF7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231cells were maintained in α-MEM containing 10 mM Hepes, 1% antibiotic-antimycotic and 10% heat-inactivated fetal bovine serum as described previously (Jiang et al., 2016). MCF 7 cells were maintained in α-MEM containing 10 mM Hepes, 1% antibiotic-antimycotic, 5μg/mL insulin and 10% heat-inactivated fetal bovine serum as reported (Yoon and Liu, 2008). All the cells were cultured at 37 °C in a 5% CO_2 humidity

atmosphere.

4.3.3 Anti-proliferation assay

The anti-proliferative activities towards MDA-MB-231 and MCF7 human breast cancer cells were assessed by lab-developed methylene blue assay as reported previously (Felice et al., 2009; Jiang et al., 2016; Yoon and Liu, 2007). 100 µL fresh medium with cells was seeded in 96-well plate at the concentration of 2.5 x 10^4cells/well. After 8 hours incubation at 37 °C and 5% CO_2 to allow cells to get attached, growth medium was removed and cells was treated with 100 µL fresh medium with different concentrations of UA (0 ~ 100 µM), paclitaxel (0 ~ 40 µM), UA and paclitaxel combination (0.25 × ~ 1.25 × EC_{50}), or Dimethyl sulfoxide (DMSO) control. After 72 hours incubation, cells was washed with 100 µL phosphate-buffered saline (PBS). Then 50 µL methylene blue staining buffer (98% Hanks balanced salt solution, 0.67% glutaraldehyde, 0.6% methylene blue) was added to each well, and incubated for 1 hour. After the staining, the methylene blue staining solution was removed, and the plate was immersed and rinsed in deionized water for four times. After the wells were dried, a volume of 100 µl elution buffer containing (1% acetic acid, 49% PBS, and 50% ethanol) was added to each well and the plate was shaken on a bench shaker for 15 minutes. Finally, cell numbers were referring to the absorbance a read at 570 nm using the MRX Microplate Reader. The anti-proliferation activity was measured as percentage compared to the control. All measurements were conducted in triplicates.

4.3.4 Experimental Design of Combination Study

A two-way combination of ursolic acid plus paclitaxel toward cell proliferation in each cell line was designed based on the method reported previously (Chou and Talalay, 1984; Yang and Liu, 2009). The EC_{50} values of ursolic acid and paclitaxel were determined based on the dose-response curve respectively (Felice et al., 2009; Yang and Liu, 2009). The combination dosages were designed referring to EC_{50} of each compound in each cell line, for each compound, the combination dosage was applied as 0.25 × EC_{50}, 0.5 × EC_{50}, 0.75 × EC_{50}, 1 × EC_{50}, and 1.25 × EC_{50}. For MDA-MB-231 cells, the five were combination groups were 1 μM paclitaxel + 7.75 μM UA, 2 μM paclitaxel + 15.5 μM UA, 3 μM paclitaxel + 23.25 μM UA, 4 μM paclitaxel + 31 μM UA, 5 μM paclitaxel + 38.75 μM UA. For MCF7 cells, the five were combination groups were 3 μM paclitaxel + 7 μM UA, 6 μM paclitaxel + 14 μM UA, 9 μM paclitaxel + 21 μM UA, 12 μM paclitaxel + 28 μM UA, 15 μM paclitaxel + 35 μM UA. The combination effects were analyzed and combination index (CI) were calculated by Chou and Talalay's method invoking the Compusyn software (ComboSyn, Inc. Paramus, NJ, USA) (Chou and Talalay, 1984; Chou, 2010). Briefly, the CI in Chou-Talalay's method is described as follow (Chou and Talalay, 1984; Yang and Liu, 2009):

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$$

$(D)_1$ and $(D)_2$ respectively represent doses of ursolic acid and paclitaxel in the combination system; $(Dx)_1$ and $(Dx)_2$ respectively represent the doses of ursolic acid and paclitaxel alone. For data analysis of combinations, CI < 1, CI = 1, and CI > 1

indicate respectively synergistic, additive, or antagonistic effects (Chou and Talalay, 1984; Chou, 2010; Yang and Liu, 2009).

4.3.5 Data analysis.

Data were analyzed and presented using Sigmaplot software version 11.0 (Systat Software, Inc. Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD) for at least three replicates. Combination effects were analyzed adopting the Compusyn software (ComboSyn, Inc. Paramus, NJ, USA).

4.4 Results

Combination effects of paclitaxel and ursolic acid toward MDA-MB-231 human breast cancer cell proliferation is shown in Figure 14 and Table 2. Ursolic acid, paclitaxel and their combination inhibited cell proliferation in MDA-MB-231 breast cancer cells in a dose-dependent manner. Only additive effect (No synergism) was observed at 50% inhibition rate with CI_{50} of 0.98, synergistic effects were observed at 95% inhibition rate with CI_{95} of 0.68.

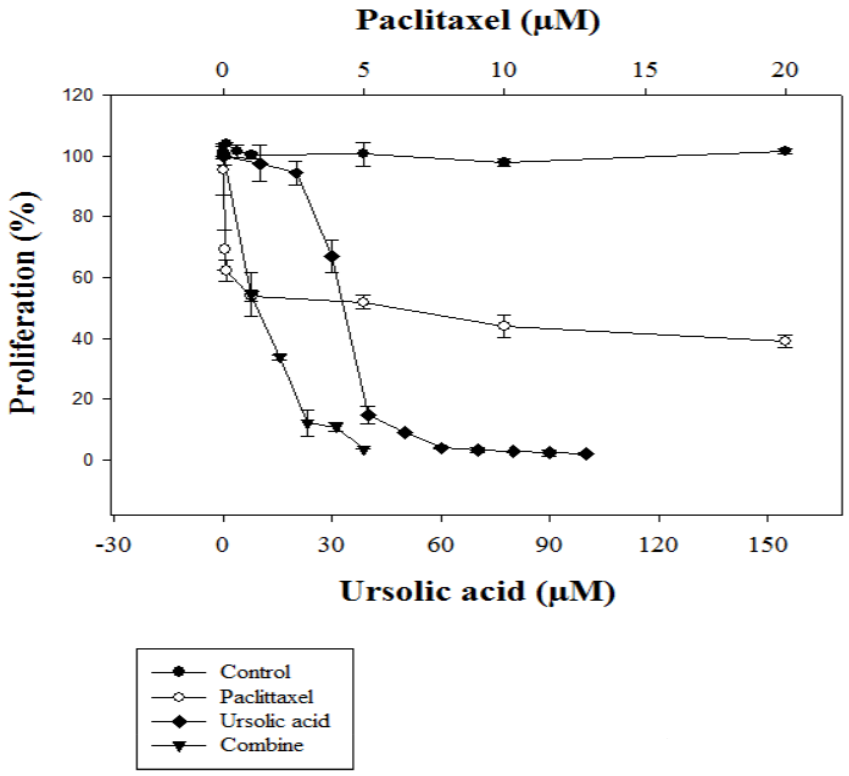

Figure 14. Inhibition effects of paclitaxel and ursolic acid combination in MDA-MB-231 human breast cancer cells.

Table 2. Combination effects of paclitaxel and ursolic acid toward MDA-MB-231 human breast cancer cell proliferation.

Component	EC_{50}		CI_{50}	CI_{95}
	Single	Combined		
Ursolic acid	30.67 ± 1.95	14.61 ± 3.67	0.98 ± 0.17	0.68 ± 0.18
Paclitaxel	3.73 ± 0.55	1.83 ± 0.51		

Proliferation inhibition effects of combination of paclitaxel and ursolic acid in MCF7 human breast cancer cells is shown in Figure 15 and Table 3. Ursolic acid, paclitaxel and their combination inhibited cell proliferation in MCF7 breast cancer cells in a dose-dependent manner. Modest synergistic effect was observed at 50% inhibition rate with CI_{50} of 0.87, additive effect (no synergism) was observed at 95% inhibition rate with CI_{95} of 1.07.

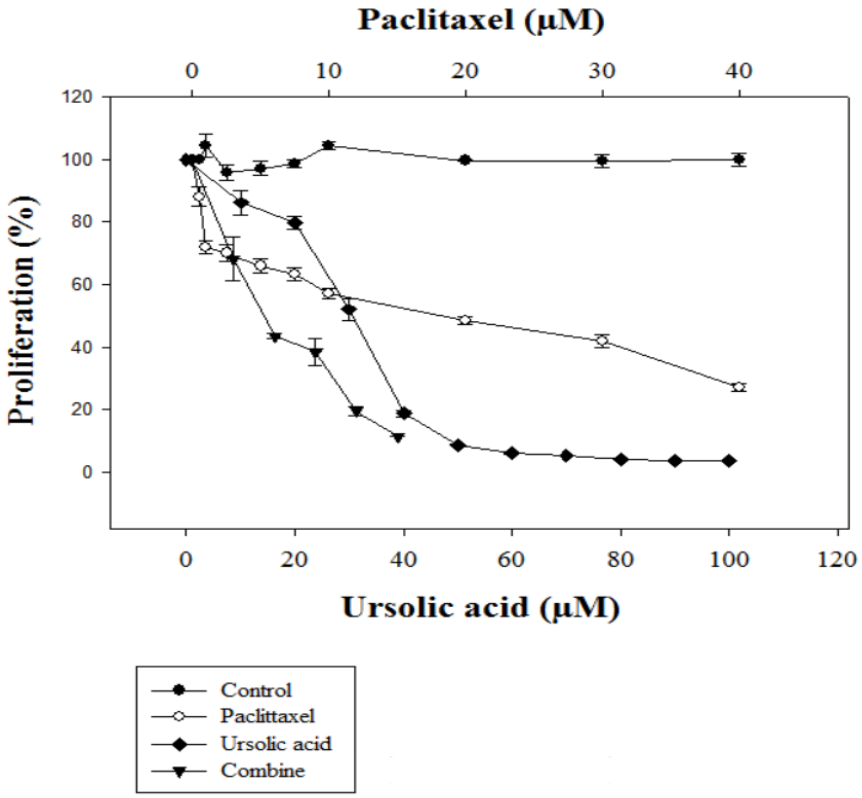

Figure 15. Inhibition effects of paclitaxel and ursolic acid combination in MCF7 human breast cancer cells.

Table 3. Combination effects of paclitaxel and ursolic acid toward MCF7 human breast cancer cell proliferation.

Component	EC_{50}		CI_{50}	CI_{95}
	Single	Combined		
Ursolic acid	29.47 ± 3.42	12.46 ± 2.11	0.87 ± 0.21	1.07 ± 0.22
Paclitaxel	12.41 ± 1.62	5.06 ± 1.55		

4.5 Discussion

Drug combination is widely applied in cancer treatment to achieve potential benefits, such as synergistic therapeutic effects, dose and toxicity reduction, and diminishing or postponing the induction of drug resistance (Chou, 2006). Previously Chou and Talalay developed the method to quantify synergistic effects of drug combination based on the median-effect equation, derived from the mass-action law principle (Chou and Talalay, 1984). The Compusyn software referring to Chou-Talalay method provides the offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations (Chou, 2010). Our results suggested ursolic acid and paclitaxel combination showed synergistic effects against MDA-MB-231 breast cancer cell proliferation at 95% inhibition rate, and modest synergistic effects against MCF7 breast cancer cell proliferation at 50% inhibition rate.

Paclitaxel is an important anticancer agent used in clinical practice in the treatment of various tumors including breast cancer, which is metabolized by CYP2C8 and CYP3A enzymes of the cytochrome P450 system and inhibits tumor development via

microtube dynamic stabilization and cell cycle disruption (de Weger et al., 2014). Paclitaxel induces mitotic arrest upon activation of the mitotic checkpoint (i.e. the spindle assembly checkpoint), the major cell cycle regulating action during mitosis to prevent chromosome missegregation (Weaver, 2014). Besides anti-proliferation effects induced by halting cell cycle arrest, paclitaxel also had an effect on tumor vasculature and microenvironment(Muta et al., 2009). Paclitaxel targeted FOXM1 to regulate KIF20A in mitotic catastrophe and breast cancer paclitaxel resistance while paclitaxel-resistant MCF-7 cells exhibited upregulated expression levels of FOXM1 and KIF20A (Khongkow et al., 2016). Combination of paclitaxel and a VEGF inhibitor (bevacizumab) prolonged progression-free survival in patients with metastatic breast cancer (Miller et al., 2007).

Ursolic acid is a natural phytochemical with pentacyclic triterpenoid structure, which was first identified in the epicuticular waxes of apples as early as 1920 and widely distributed in various fruit peels, herbs and spices such as rosemary and thyme (Borovkov and Belova, 1967; Cargnin and Gnoatto, 2017; Mezzetti et al., 1971; Yeh et al., 2010). Earlier we found UA inhibited proliferation and invasion in MDA-MB-231 human breast cancer cells via regulating key molecular targets such as EGFR, JAK/STAT3, Akt, p38 and JNK MAPK, and proteins from mTOR pathway. Ursolic acid is also associated with downregulation of apoptosis and induction of cell cycle arrest in cancer cells through EGFR and other receptors such as TNFα (Achiwa et al., 2013; Kassi et al., 2009; Kim et al., 2011; Shanmugam et al., 2013; Wang et al., 2012; Wu et al., 2012). Signaling pathways include MAPK, PI3K/Akt, JAK/STAT3 and key

cancer progression regulators p53 and NF-κB are involved in anti-cancer activities of UA (Achiwa et al., 2013; Ikeda et al., 2008; Kassi et al., 2009; Kim et al., 2011; Leng et al., 2013; Shanmugam et al., 2013; Shanmugam et al., 2012; Wang et al., 2012; Wu et al., 2012; Yeh et al., 2010). EGF stimulation and EGFR overexpression has been linked to development and invasion of cancer, especially invasive breast cancer and triple negative breast cancer (Ciardiello and Tortora, 2008; Masuda et al., 2012; Mooso et al., 2015; Nicholson et al., 2001). Previously we reported UA inhibited proliferation and EGF-induced invasion in MDA-MB-231 breast cancer cells and downregulated EGFR receptor and its downstream effector proteins.

In the current study, we adopted two human breast cancer cell lines to investigate the potential synergism in anti-proliferation effects of combining paclitaxel with ursolic acid, one is MDA-MB-231 breast cancer cells, which is triple negative and invasive; the other is MCF7 breast cancer cells, which is ER^{+}, $PR^{+/-}$, $HER2^{-}$ and noninvasive (Holliday and Speirs, 2011). Our results suggest the two types of cells were both responsive to treatments of paclitaxel, ursolic acid or their combination in dose-dependent manner, but with different sensitivity.

4.6 Limitations and future directions

The methodology to quantify synergism effects is always controversial (Chou, 2006) We used CI values as our indicators for synergism based on the Compusyn software and Chou-Talalay model (ComboSyn, Inc. Paramus, NJ, USA). Since sensitivity of cancer cells upon drug treatment differs according to cell type, cell stress status, and

drug preparing methods, it takes more effort to identify the dose-response curve precisely and find the most appropriate dosage that should be deliver. Besides, the authors suggested that, for in vivo studies, synergy conclusion may still be vague and not quantitative (Chou, 2010), hence it is necessary but takes long to attest and promote synergistic effects in animals considering the complicity of in vivo test environment and more interference factors. Finally, the mechanisms behind why synergy occurs remains unclear.

4.7 Conclusion

Ursolic acid, paclitaxel and their combination treatment significantly inhibited MDA-MB-231 breast cancer cell and MCF7 breast cancer cell proliferation in a dose-dependent manner. Synergistic effect was observed at 95% inhibition rate with CI_{95} of 0.68 in MDA-MB-231 breast cancer cells, yet no synergism effect was observed at 50% inhibition rate. Modest synergistic effect occurred at 50% inhibition rate with CI_{50} of 0.87 in MCF7 cells, while no synergism occurred at 95% inhibition rate. Our results gave information on the combination use of clinical anti-cancer drugs and bioactive compounds with potential synergistic effects.

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