RESEARCH ARTICLE

Anti-metastasis Activity of Black Rice Anthocyanins Against Breast Cancer: Analyses Using an ErbB2 Positive Breast Cancer Cell Line and Tumoral Xenograft Model

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Abstract

Background: Increasing evidence from animal, epidemiological and clinical investigations suggest that dietary anthocyanins have potential to prevent chronic diseases, including cancers. It is also noteworthy that human epidermal growth factor receptor 2 (ErbB2) protein overexpression or *ErbB2* gene amplification has been included as an indicator for metastasis and higher risk of recurrence for breast cancer. Materials and Methods: The present experiments investigated the anti-metastasis effects of black rice anthocyanins (BRACs) on ErbB2 positive breast cancer cells *in vivo* and *in vitro*. Results: Oral administration of BRACs (150 mg/kg/day) reduced transplanted tumor growth, inhibited pulmonary metastasis, and decreased lung tumor nodules in BALB/c nude mice bearing ErbB2 positive breast cancer cell MDA-MB-453 xenografts. The capacity for migration, adhesion, motility and invasion was also inhibited by BRACs in MDA-MB-453 cells in a concentration dependent manner, accompanied by decreased activity of a transfer promoting factor, urokinase-type plasminogen activator (u-PA). Conclusions: Together, our results indicated that BRACs possess anti-metastasis potential against ErbB2 positive human breast cancer cells *in vivo* and *in vitro* through inhibition of metastasis promoting molecules.

Keywords: Breast cancer - anthocyanins - metastasis - tumor models - black rice

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Introduction

Breast cancer is now the most frequently diagnosed malignancy and the leading global cause of cancer motility in women, accounting for 23% of cancer diagnoses (1.38 million) and 14% of cancer deaths (458 000) each year (DeSantis et al., 2011; Jemal et al., 2011). The decrease in breast cancer-related deaths has been observed in the developed world since the early 1990s, partly due to improved screening and adjuvant treatment (Berry et al., 2005). Despite the advances that have been made in the treatment of breast cancer, metastasis remains the leading cause of breast cancer mortality (Siegel et al., 2012; Gretel et al., 2013).

Human epidermal growth factor receptor-2 (HER2/neu, ErbB2) is a family member of epidermal growth factor receptor (EGFR) membrane tyrosine kinase and plays an important role in cell proliferation, differentiation and migration (Gutierrez et al., 2011). Prior researches have revealed that breast cancer progression and poor prognosis are always positively correlated with ErbB2 amplification and resultant ErbB2 protein overexpression (Nahta et al., 2009; Bernasconi et al., 2012; Olson et al., 2013). While ErbB2 lacks an identified ligand, ErbB2 kinase activity can be activated in the absence of a ligand when overexpressed and/or through heteromeric associations with other ErbB family members (EGFR, ErbB3 and ErbB4) (Zhang et al., 2007; Aertgeerts et al., 2011). Previous studies reported that 47% (344 of 733) amplification of the *ErbB2* gene and 35% (279 of 798) overexpression of its product are detected in human breast cancers by FISH (fluorescence chromosomal in situ hybridization) and IHC (immunohistochemistry), respectively (Press et al., 2008).

Anthocyanins are found in foods of vegetable, fruit and cereal origin. Flavonoids, including anthocyanins, have attracted much attention for the potential health benefits in obesity control (Prior et al., 2008), diabetes control (Wedick et al., 2012), cardiovascular disease (CVD) prevention (Van et al., 2013), visual (Shim et al., 2012) and brain function (Gutierres et al., 2012) improvement, mainly due to its anti-inflammatory, antioxidant and chemoprotective properties (Wang et al., 2007; Stoner et al., 2011; Tsuda, 2012). In recent years, scientists newly found that anthocyanins can prevent carcinogenesis, and inhibit cancer progress and metastasis through cell signal

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transduction (Muserref et al., 2014), including breast cancer (Chang et al., 2010; Faria et al., 2010; Adams et al., 2011), lung cancer (Ho et al., 2010), colon cancer (Ratasark et al., 2014), prostate cancer (Bilal et al., 2008; Hafeez et al., 2008) and esophageal cancer (Stoner et al., 2010).

Black rice anthocyanins (BRACs) are a kind of anthocyanins that extracted from the aleurone layer of black rice, which is a major cereal crop existing since ancient times in China and other Eastern Asia countries (Ling et al., 2002). Our previous studies have shown that BRACs induced apoptosis in MDA-MB-453 cells via an intrinsic pathway (Chang et al., 2010). In BALB/c nude mice bearing MDA-MB-453 cell xenografts, oral administration of BRACs (100 mg/kg/day) suppressed tumor growth and angiogenesis (Chang et al., 2010).

The anthocyanin components in BRACs are about 26.3%, and cyaniding-3-O-glucoside and peonidin-3-O-glucoside are the main effective constituents, accounting for about 90%. Considering the lower abundance of effective anthocyanins in our BRACs (26.3%) than previous laboratory extract (AEBR) (43.2%) (Chang et al., 2010), as well as based on the preliminary experiment, we applied 150 mg/kg/day BRACs in our animal experiment.

It is proven that anthocyanins can inhibit the growth of breast cancer by inducing cell apoptosis and scavenging reactive oxygen species (ROS) (Xu et al., 2010). However, limited references are available about the potential in anti-metastasis by anthocyanins (Adams et al., 2010; Tsuda, 2012). The purpose of this study was to evaluate the metastasis inhibitory effects of BRACs on ErbB2 positive human breast cancer. We established xenograft in female BALB/c athymic mice with ErbB2 positive MDA-MB-453 malignant breast cancer cell line, which tends to metastasis to lungs *in vivo* (Neve et al., 2006). And then we investigated the protective effect of dietary BRACs on pulmonary metastasis from subcutaneous MDA-MB-453 xenograft. Furthermore, we studied the metastatic ability and metastatic promoting molecules variation after BRACs treatment *in vitro*. Our studies proved that bioactive anthocyanins isolated from black rice may act as potential candidate for cancer metastasis inhibition.

Materials and Methods

Chemicals and Reagents

BRA-90 anthocyanins (BRACs) were purchased from New Star natural plant development company (Jilin, China) and the dried powder was stored at 4°C protected from light. For animal experiment, the powder was dissolved with distilled water; for *in vitro* test, the powder was dissolved with RPMI/1640 medium and then filtrated with 0.2 μm membrane. Growth medium RPMI 1640, DMEM/high glucose, DMEM/F12 (1:1) medium and trypsin 0.25% solution were purchased from Hyclone (Beijing, China). Fetal bovine serum (FBS) and horse serum were purchased from Gibco (NY, USA). Polyethylene terephthalate (PET) membrane (8 μm pore size) Millicell® hanging cell culture inserts, Amicon Ultra-4 Centrifugal Filter Devices (10 000 NMWL), cell invasion assay kit (Cat. ECM550 and u-PA (urokinase-type plasminogen activator) activity assay kit (Cat. ECM600) were purchased from Millipore (Chemicon International, USA). SABC (StreptAvidin-Biotin-enzyme Complex) immunohistochemisty kit (SA2002) and DAB (Diaminobenzidine) kit (AR1022) were purchased from Boster (Wuhan, China). Rabbit anti-human monoclonal antibody of Ki-67 (D2H10, Cat. 9027) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Propidium iodide (PI) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

Human breast cancer MCF-7 (ErbB2 negative) and MDA-MB-453 (ErbB2 positive) (Neve et al., 2006; Holliday et al., 2011) and normal MCF-10A cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). MCF-7 and MDA-MB-453 cells were separately cultured in DMEM/high glucose or RPMI-1640 containing 10% FBS. MCF-10A cells were cultured in DMEM/F12 medium containing 5% horse serum in the presence of 10 μg/mL insulin (Wako), 20 ng/mL epidermal growth factor (PeproTech), 100 ng/mL cholera toxin and 0.5 μg/mL hydrocortisone (Herbert et al., 1990). All cells were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide.

Animal Experiments

The animal experimental procedures were approved by the Animal Care and Use Committee of the Chengdu Medical College. A total of 16 female nude mice (nu/nu mutant on a BALB/c back ground, n=16) (Dashuo experiment animal center, Chengdu) about four weeks old were housed with a regular 12 hours light and 12 hours dark cycle and ad libitum access to standard rodent chow diet AIN-93 formulation (Reeves et al., 1993). After 3 days adaptive feeding, mice were randomly divided into two groups (n=8 for each group). Mice were fed orally (intragastrically) with either control diet (distilled water) or BRACs supplemented diet (150 mg/kg/day) for three weeks, then the MDA-MB-453 tumor cells (8×10^5) were injected subcutaneously into the right dorsal. The control diet and BRACs supplemented diet were retained in each group during the course of the experiment.

Primary tumor growth was measured once every three days with calipers. Tumors were measured in two perpendicular dimensions, and the volumes were estimated by using the formula [Volume=0.52×(length)×(width)^2] for approximating the volume (mm^3) of an ellipsoid (Vantyghem et al., 2005). At the end point (six weeks), mice were sacrificed by decapitation under pentobarbital sodium anesthesia between 10 a.m. and noon, the xenograft tumors and vacuum aspirated lungs were fixed in 4% paraformaldehyde. Metastatic nodules on the surface of lungs were counted under the dissecting microscope.

Immunohistochemisty

The formalin fixed lungs were embedded in paraffin and thin sectioned (4 μm). Tissue sections were placed

onto APES (3-Aminopropyl-Triethoxysilane) pretreated slides, and then heat fixed, deparaffinized, rehydrated, 3% hydrogen peroxide treated, and followed by microwave antigen retrieval to block endogenous peroxidase activities. The slides were incubated with rabbit anti-human Ki-67 antibody after blocked by goat serum, and then incubated at 4°C overnight. Secondary goat anti-rabbit biotinylated antibody and signal development were then applied by using SABC and DAB kit according to the instructions, separately. Counterstaining was then performed with hematoxylin. Gray-staining cells were counted as Ki-67 positive cells. The percentage of Ki-67 positive nuclei was calculated with the aid of an image computer analyzer (IPP, image-proplus 6.0).

Cell Survival Assay

MCF-10A, MCF-7, MDA-MB-453 cells were plated in medium containing 10% FBS at a density of 1×10^6 cells/mL in 6-well plates, and allowed adhering for 24 hours. The media were then changed to fresh medium containing 0, 25, 50, 100, 200, 400 μg/mL BRACs, and the cells were further incubated for an additional 24 hours. Afterward, cells were harvested, resuspended with serum free medium (SFM), stained with PI, and viable cells were counted by flow cytometry.

Wound Healing (Scratch) Assay

MDA-MB-453 cells were plated in a 6-well plate at a concentration of 1×10^6 cells/well and allowed to form a confluent monolayer for 24 hours. Cells were then treated for 24 hours with fresh medium added 0, 100, 200, 400 μg/mL BRACs. Then the monolayer was scratched with 20 μL pipette tips, washed with SFM to remove floating cells and photographed (time 0). Cells were further incubated with SFM for 24 hours and photographed again (time 24). The numbers of cells migrated to time 0 wound area were counted.

Cell Adhesion Ability Assay

After 24 hours treatment with 0, 100, 200, and 400 μg/mL BRACs, MDA-MB-453 cells (5×10^4/well) were plated on 96-well plate and cultured for 3 hours. The 96-well cell culture plates were pre-coated with 20% FBS culture medium for 24 hours at 37°C. Nonadherent cells were removed by PBS washes; adherent cells were fixed in 4% paraformaldehyde for 10 min thereafter. Nonspecific binding was blocked by 3% BSA for 2 hours at room temperature. Then cells were stained with crystal violet for 10 min and rinsed with distilled water and laminar flow dried. Crystal violet was finally eluted with 33% acetic acid, and the absorbance (equivalently attached cells) was measured at 570 nm.

Cell Invasion and Motility Assays

MDA-MB-453 cells (0.5×10^6 cells/mL) pretreated with 0, 100, 200, and 400 μg/mL BRACs were plated into the upper chamber of the Millicell® hanging cell culture inserts containing SFM, and the inserts were placed into a 24-well plate containing complete medium in the lower chamber. An invasion assay was performed according to the manufacturer's instructions of Chemicon® cell invasion assay kit (ECM550). After 24 hours, the top surface of the insert was scraped using a cotton swab and the cells adhere to the bottom surface of the membrane were fixed with methanol and then stained with crystal violet. Cells which had migrated to the bottom of the membrane were visualized and counted using an inverted microscope. Cells in 5 randomly selected fields were counted in each well and averaged.

U-PA Activity Assay

After treated with 0, 100, 200, 400 μg/mL BRACs for 24 hours, the supernatants were collected and centrifuged to remove any cellular component. Then protein concentrations were determined following ultrafiltration by utilizing 10 000 NMWL Centrifugal Filter Devices. The u-PA activity assay was performed according to the manufacturer's protocol (ECM600).

Statistical Analysis

Values were expressed as means±SD. All *in vitro* experiments were repeated more than three times. Statistical analysis was performed using Student's t-test. $p<0.05$ was considered to be statistically significant. All analyses were performed by using GraphPad Prism 5 software (GraphPad Software, Inc.).

Results

Anti-cancer Effect of BRACs in Vivo

To evaluate the carcinogenesis chemopreventive and tumor growth inhibition potential of BRACs *in vivo*, the MDA-MB-453 xenograft model was were established. The control diet and BRACs supplemented diet were initiated 3 weeks prior to and continued 6 weeks after tumor cells injection. No apparent signs of toxicity as evidenced by body weight (Figure 1B), food and water intake (data not show) were monitored throughout the experiment. The xenograft tumors first became palpable 5 days after injection, and tumor take were 100% in both

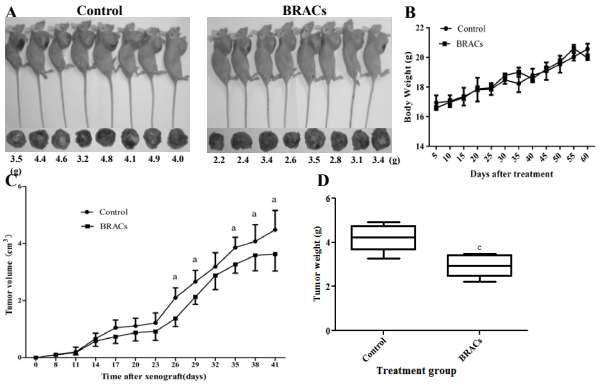

Figure 1. BRACs Reduced Primary Tumor Growth in MDA-MB-453 Xenograft. The MDA-MB-453 xenografts were established on BALB/c nude mice. (A) The anesthetic mice of control (left panel) and BRACs (right panel) treatment groups and the isolated primary tumors by day 42. (B) Body weights of mice were measured every five days from the day BRACs treatment started. (C) Tumor volumes were measured and calculated every three days. D: All mice were euthanized at the end point of the experiment, and tumors were removed and weighed. $^a p<0.05$; $^c p<0.001$ compared with control

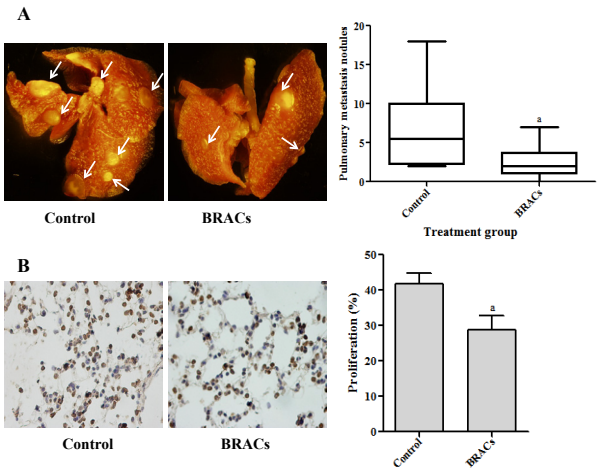

Figure 2. Effects of BRACs on Pulmonary Metastasis and Ki-67 Expression *in vivo*. (A) The pulmonary metastases on the surface of lungs (×70), arrowhead indicates metastases. (B) The expression of Ki-67. Brown or gray-staining cells were counted as Ki-67 positive (proliferating) cells (×400). The percentage of Ki-67 positive nuclei was calculated with the aid of an image computer analyzer. $p<0.05$ compared with control

diet groups, all mice were sacrificed by day 42 (Figure 1A). Tumor volumes were slightly but distinguishable lower in BRACs diet group after they reach a certain size about 0.6 cm^3 (day 14) (Figure 1C). The primary tumor weights were considerably decreased in BRACs treatment group versus control group ($p<0.01$) (Figure 1D). These data indicated that BRACs didn't prevent carcinogenesis, but do inhibit tumor growth *in vivo*.

BRACs Inhibit Pulmonary Metastasis and Ki-67 Expression

To assess the lungs metastatic potential of MDA-MB-453 cells, we checked the tumor nodules in the surface of lungs. After six weeks of primary tumor growth, metastases were histological evident in some mice in both groups. All eight mice bore pulmonary metastases in control diet group (eight of eight, 100%), but only five mice had surface metastases in BRACs group (five of eight, 62.5%). In addition, the superficial metastases in the lungs (tumor nodules) were counted under microscope, the BRACs-fed mice had fewer metastatic burden compared with the control-fed mice ($p<0.05$) (Figure 2A).

Antigen Ki-67 is a nuclear protein as a cellular marker for proliferation, and presents during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0) (Gerdes et al., 1984; Pathmanathan et al., 2013). We investigated the Ki-67 expression of the metastatic tumor tissues by immunohistochemical (IHC) method. The IHC result showed that proliferating (Ki-67 positive) tumor cells decreased by almost 15% in BRACs diet group compared with the control group ($P<0.05$) (Figure 2B).

Cytotoxic Effect of BRACs on Human Breast Cancer Cells

Previous study demonstrated that many berry anthocyanins have no cytotoxic effect on normal cells (Li et al., 2009; Adams et al., 2010), we verified it on BRACs. Cell survival assay indicated that BRACs reduced

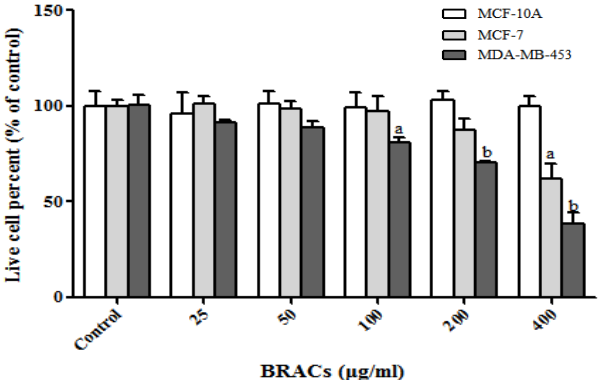

Figure 3. Cell Survival Inhibitory Effect of BRACs on Breast Cancer Cells. Breast cancer cells MCF-10A, MCF-7 and MDA-MB-453 were treated for 24 hours with 25, 50, 100, 200, and 400 μg/mL BRACs or not (control). Cell survival rate assay was performed by flow cytometry. $^ap<0.05$; $^bp<0.01$ compared with control

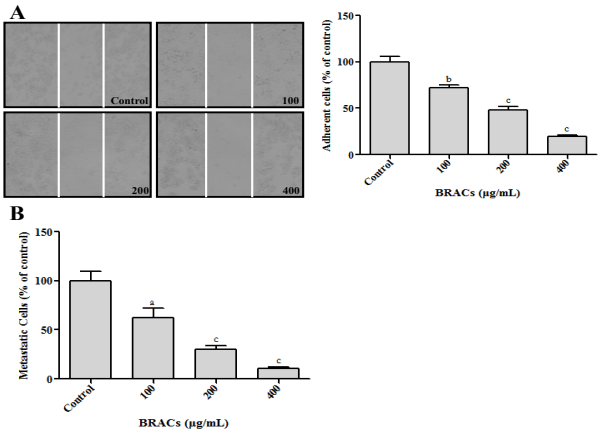

Figure 4. Effects of BRACs on Migration and Adhesion of MDA-MB-453 Cells. (A) MDA-MB-453 cells were treated with BRACs and then subjected to analyze for migration. The time 24 photographs were presented in the left panel (×40), and cells migrated to time 0 wound area were counted, data were presented in the right panel. (B) The BRACs treated MDA-MB-453 cells in indicated concentrations were performed adhesion assay following the procedures mentioned in Materials and Methods. $^ap<0.05$; $^bp<0.01$; $^cp<0.001$ compared with control

the survival rate of human breast cancer cell lines MCF-7 and MDA-MB-453 in a dose-dependent manner, but barely affected the untransformed MCF-10A cells (Figure 3). BRACs showed differential cytotoxic effects on the two kinds of breast cancer cell lines; the IC_{50} values over 24 hours were 354.95 μg and 652.57 μg of BRACs/mL for MDA-MB-453 and MCF-7 cells, respectively. This indicated that ErbB2 positive MDA-MB-453 cells were more sensitive to BRACs than ErbB2 negative MCF-7 cells. Based on this, we chose the MDA-MB-453 cell line and BRACs concentrations at 100, 200, 400 μg/mL for further metastatic inhibitory studies.

The Migration and Adhesion Inhibitory Effects of BRACs in MDA-MB-453 Cells

Migration and adhesion are critical procedures in cancer cell escaping from the primary tumor site. The migration inhibitory effect of BRACs on MDA-MB-453 cells was examined by wound healing (scratch) assay.

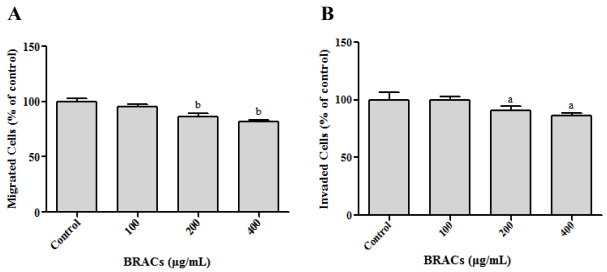

Figure 5. Effects of BRACs on Motility and Invasion of MDA-MB-453 Cells. MDA-MB-453 cells were treated with indicated concentrations of BRACs for 24 hours and then subjected to analyze for motility (A) and invasion (B) as described in Materials and Methods. $^{a}p<0.05$; $^{b}p<0.01$ compared with control

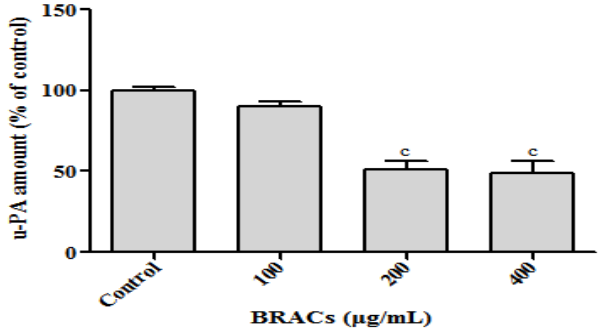

Figure 6. Effects of BRACs on u-PA activity of MDA-MB-453 Cells. Conditioned medium from cells was evaluated for regulation of u-PA activity was analyzed by using the u-PA activity assay kit. $^{c}p<0.001$ compared with control

Compared to control, cells migrated to the wound area were reduced about 35% after treated with 100 μg/mL BRACs (Figure 4A). The adhesion assay was then performed to study the inhibitory of cell adhering to culture plate after BRACs treatment. It showed that 100 μg/mL BRACs treatment reduced the adhesion cells to 70% (Fig.4B). The inhibitory effects were augmented as the concentration of BRACs increased in both scratch and adhesion assays.

The Motility and Invasion Effects of BRACs on MDA-MB-453 Cells

The motility and invasion abilities determine the deformation and ECM degradation capability, which are key movements in metastasis. The Millicell® hanging cell culture inserts were used to test the motility of MDA-MB-453 cells. In contrast to control group, the migration cells decreased approximately 15% in the 200 μg/mL BRACs treatment group (Figure 5A). Then the cell invasion assay (Cat. ECM550) was performed to observe the alteration of ECM invasion ability after BRACs treatment. The result showed that 200 μg/mL BRACs reduced the ECM invasion ability by about 10% ($p<0.05$) in MDA-MB-453 cells (Figure 5B). Both motility and invasion assays exhibited a concentration-dependent manner.

BRACs Decreased the Activities of U-PA

The secreted u-PA (urokinase-type plasminogen activator) is an important molecule in ECM degradation. The conditioned media were used for u-PA activity assay, a trend for decreased u-PA amount was observed in BRACs treated groups. In 200 μg/mL BRACs treatment group, the u-PA activity units had a 50% reduction in contrast with control group (Figure 6A).

Discussion

Our study demonstrated that nutritional ingredient BRACs have anti-cancer potential. Long-term BRACs supplemented diet can slightly reduce tumor growth, but significantly inhibit cancer cells pulmonary metastasis from subcutaneous ErbB2 positive breast tumor xenograft. Further, BRACs treatment decreased cancer cell metastatic abilities, including migration, adhesion, motility and invasion. These cancer metastasis inhibitory effects may partly due to the enzymatic activity suppression of transfer promoting factor u-PA by BRACs.

In recent years, natural plant products have gained increased attention in carcinogenesis occurrence and neoplastic progression prevention and intervention (Stoner et al., 2011; Tsuda, 2012; Muserref et al., 2014). Anthocyanins are abundant flavonoid constituents existing in colorful fruits and vegetables. It is reported that anthocyanins enriched foods can inhibit the development of cancer in carcinogen treated animals and in animals with a hereditary predisposition to cancer (Aiyer et al., 2010; Stoner et al., 2010).As shown in this study, the effective dose was much higher than normal chemical clinic medication mainly due to the low abundance of active ingredients in BRACs, poor absorption and rapid degradation of anthocyanins in gastrointestinal tract (Dreiseitel et al., 2009; Forester et al., 2010).

About 90% deaths from cancer are due to metastasis, a complex process involving cancer cells escaping the primary tumor, invading through surrounding tissues to spread and colonize distant organs (Wang, 2010). Our *in vivo* experiments indicated that oral intake BRACs can decrease primary tumor growth, as well as lower the pulmonary metastasis risk and the proliferation capability in metastatic tumors. Our study is in accordance with Adams's (2011) report, in which 5% blueberry powder-fed mice developed 70% fewer liver metastases and 25% fewer lymph node metastases compared to control mice in MDA-MB-231 breast tumor xenograft mode. These results suggest that anthocyanins could be a long-term strategy for breast cancer metastasis prevention and treatment.

Existing investigations indicated that anthocyanins selectively inhibit the growth of cancer cells without evident side effects to normal cells (Li LY et al., 2009; Faria et al., 2010). Our study is in line with that fact, even in the highest testing concentration (400 μg/mL), BRACs barely affected MCF-10A growth. Furthermore, different phenotypic breast cancer cell lines exhibited different reactions to BRACs, while ErbB2 positive MDA-MB-453 cells were more sensitive than ErbB2 negative MCF-7 breast cancer cells. But the exactly molecular mechanisms have not been clarified mainly due to the complicated pathways involved in anti-cancer effects.

The ability to invade the surrounding tissues and

then metastasize is the most important characteristic of malignant tumors. The complicated metastatic process can be subdivided into a number of procedures involving cell motility, cell invasion, surface adhesion and degradation of extracellular matrix (ECM) (Sahai, 2005). U-PA initiates the activation of an enzymatic cascade to activate plasmin and MMPs. These enzymes degrade the most components of ECM to allow tumor cells get access to the vascular and then invade into the target organ (Schmalfeldt et al., 2001). Our data demonstrated that BRACs can suppress the activity of u-PA, which maybe the causality of metastasis inhibition. Our previous animal experiments also proved that BRACs can decrease the expressing of u-PA in tumor tissue (Chang et al., 2010). Similar results were shown in many other anthocyanins research (Xu et al., 2010; Adams et al., 2010; Ho et al., 2010).

Previous studies revealed that anthocyanins can increase apoptosis (Bilal et al., 2008; Luigia et al., 2008), inhibit angiogenesis (Li et al., 2002) and induce G2-M cell cycle arrest (Chen et al., 2005; Bilal et al., 2008) to inhibit cancer. Our experiments further demonstrated that BRACs have metastatic inhibitory effect in ErbB2 positive breast cancer cells. It has been proved that blueberry phytochemicals can inhibit the metastatic potential in triple negative breast cancer cells by regulating the PI3 Kinase pathway (Adams et al., 2010). Cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) are the two main and most completely studied components of BRACs (Chang et al., 2010). Xu's (2010) report indicated that C3G can inhibit ethanol-induced invasion by blocking the ErbB2/cSrc/FAk pathway in ErbB2 overexpressing breast cancer cells. But P3G was identified possessing metastasis inhibitory effect by downregulation of proteinases activities and MAPK pathway in lung cancer cells (Ho et al., 2010). Another research showed that dietary delphinidin inhibited NF-κB signaling pathway in human prostate PC3 cells (Hafeez et al., 2008). EGFR family mediated four metastasis promoting signaling pathways, PLC-γ–PKC, Ras-Raf-MEK, PI3K-Akt-mTOR, JAK2-STAT3 (Han et al., 2012). As ErbB2 positive breast cancer cells are more sensitive to BRACs, we wondering which one or ones of these pathways are responsible for this metastasis inhibition.

As vegetable and fruit consumption may be associated with decreased cancer risk (Joanna et al., 2014), functional foods (like colored rice, fruit, vegetable and mushrooms) are best choice in cancer prevention for most people (Zeng et al., 2013). Although most fruits are seasonal available, advanced technologies such as drying process made it possible to extend the moisture content, and extraction and refining technics concentrat effective constituent (Kundu, et al., 2014). Even there are many delightful research in cancer prevention in rodent models (Marczylo et al., 2009 ; Thomasset et al., 2009), more clinic or human trials are needed (Gu et al., 2014; Muserref et al., 2014).

In conclusion, we manifested that BRACs inhibited pulmonary metastasis and lung nodules formation in ErbB2 positive MDA-MB-453 subcutaneous xenograft. Furthermore, the down regulation activities of invasion related factors were detected after BRACs treated in MDA-MB-453 cell line. Our work proved that as naturally nutritional ingredient, black rice anthocyanins may have promising roles in the fight against cancer metastasis.

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