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Inhibition of human breast carcinoma by BLC (*Sargassum fulvellum*) and BLC/HEN Egg *in vitro* and *in vivo*

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Abstract : Much of the interest on the chemopreventive properties of herbs and plants has been raised, whereas little is regarding to anti-tumor effect of farming and aquatic products. In the present study, the anti-tumor effect of hot-water extract of a seaweed, BLC (*Sargassum fulvellum*) and BLC/HEN egg was investigated using MCF-7 cells *in vitro* and *in vivo* systems. We found that the BLC extract and BLC/HEN egg inhibited cell proliferation in a dose-dependent manner, which might be mediated through up-regulation of p53. Furthermore, this test compound can directly induce apoptosis in MCF-7 cells, which might be mediated through up-regulation of a pro-apoptotic Bax protein and down-regulation of a anti-apoptotic Bcl-2 protein, not by immune system. Nude mice bearing established breast tumors (with exogenous estradiol) were treated with BLC extract and BLC/HEN egg. Treatment BLC extract and BLC/HEN egg caused a 42% and 71% inhibition of tumor growth, respectively. Both agents caused a significant inhibition of volume and weight growth of estrogen independent human breast tumors established from MCF-7 cells. Our results suggested that BLC extract and BLC/HEN egg have the efficacious effect of human breast cancer not only *in vitro* but also *in vivo*.

Key words : *Sargassum fulvellum*, MCF-7 cells, apoptosis, Bcl-2 family, xenograft

Introduction

Cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer [19]. Breast cancer is the most common form of cancer affecting women with cases in North America and Northern Europe being considerably higher than in Asia, the Far East, Africa and South America. The Risk factors of breast cancer include older age, personal medical history, reproductive history, family history and genetic risk factors, and lifestyle [4, 6, 13]. Lifestyle is a potential target for chemoprevention of breast cancer. The trial of breast cancer chemoprevention has shown that tamoxifen prevents the development of the second primary tumors and de novo breast cancer in high-risk patients [19]. Other chemopreventive agents are raloxifene (Razor), tibolone (Tizer), and the extract of herbs or plants including soy, grape and licorice root. Recently, chemoprevention with extract of herbs or plants has been got more attention for the treatment of cancer mortality [7, 8, 14].

The BLC (*Sargassum fulvellum* Sam-Hwa, Korea), a brown algae has been widely consumed product in Korean and other Asian sea. The fractional precipitation with ethanol from hot-water extract of BLC have been demonstrated to possess anti-tumor activities of sarcoma-180 [20] and antioxidant activities [21]. However, little is known regarding its anti-tumor effect of breast cancer. The BLC/HEN (Sam-Hwa, Korea) egg is the egg produced by HEN feeding with BLC. This study was designed to investigate the anti-tumor effects of hot-

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water extracts of BLC and BLC/HEN egg, and elucidate the potential mechanisms using an *in vitro* and *in vivo* system. We found that the hot-water extract of BLC and BLC/HEN egg were able to induce apoptosis in MCF-7 cells in time dependent manner, which were possibly mediated through up-regulation expression of p53 tumor suppress protein, down-regulation a anti-apoptotic Bcl-2 protein and up-regulation a pro-apoptotic Bax protein pathway. In addition, BLC and BLC/HEN egg inhibited the growth of MCF-7 xenografts in the range of 40-70%, when administered orally at a dose of 500 mg/kg/day (BLC) or orally at a dose of 1000 mg/kg/day (BLC/HEN egg). BLC and BLC/HEN egg decreased the number of breast metastasis found in mice inoculated s.c. with MCF-7 carcinoma, BLC/HEN egg 1000 mg/kg/day being the most effective.

Materials and Methods

Cell culture

MCF-7 cells (ATCC, USA) were cultured in D-media [12] supplemented with 5% fetal bovine serum (Gibco, USA).

Preparation of hot-water extract of BLC (*Sargassum fulvellum*) and BLC/HEN egg

The fresh fruiting bodies of BLC were washed, disintegrated, and extracted with hot-water for 1.5 hours. The crude extracts obtained were then subjected into silica gel chromatography, and the elutes were evaporated to dryness with a rotary evaporator. The BLC/HEN egg powder was made by the method of freeze drying.

Cell proliferation assay

The effects on cell proliferation were measured by MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded in 96-well microplates and incubated overnight. Then the cells were treated with different concentrations of the test compounds (12.5, 25, 50, 100, 200 and 400 μg/m*l*) or its vehicle, ethanol (0.1%) for 72 hours. At end of these periods, 20 ul of MTT stock solution (5 mg/m*l*, Sigma) were added to each well and the plates were further incubated for 4 h at 37°C. The supernatant was removed and 100 ul of DMSO were added to each well to solubilize the water insoluble purple formazan crystals. The absorbency at a wavelength of 570 nm was measured with Multiscan MCC 340 microplate reader (Titertek, USA). All the measurements were performed in triplicate. Results are expressed as the percentages proliferation with respect to vehicle-treated cells.

Apoptosis Assay

The apoptotic effect of BLC and BLC/HEN egg on MCF-7 cells was analyzed by nuclear DNA staining assay. The control and compound-treated cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed with PBS, stained with Hoechst 33258 at 1 μg/m*l* in PBS for 15 min. Stained cells were washed twice with PBS. The changes in nuclei were observed with a fluorescent microscope (Olympus, USA) through a UV filter.

Flow cytometric analysis of apoptosis

MCF-7 cells in the exponential phase of growth were treated with BLC and BLC/HEN egg (100 μg/m*l*) for the indicated times, then harvested by trypsinization, and washed twice with ice-cold PBS and fixed by 70% ethanol at -20°C for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 μg/m*l* of propidium iodide in the presence of 50 μg/m*l* RNase A for 30 min. Cell cycle distribution was analyzed using FACS Calibur (Becton & Dickinson, USA). Data from 10,000 cells per sample were collected and analyzed using the Cell Fit Cell analysis program.

Western blot analysis

MCF-7 cells were grown in a 10-cm dish, and when cell density reached 80-90% confluence, cells were treated with alcohol extract of BLC and BLC/HEN Egg (100 μg/m*l*) for the indicated times. The cells was then washed once with ice-cold PBS and lysed with lysis buffer (20% SDS containing 2 mM phenymethylsulgonyl fluoride, 10 mM iodoacetoamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride) for 10-20 min. The lysates were sonicated three times at 10-s intervals, aliquoted and stored at -20°C. The protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad, USA). Equal amounts of protein (20 μg/lane) were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently incubated with the corresponding primary antibodies, as indicated: a mouse

anti-p53 monoclonal antibody, a mouse anti-Bcl-2 monoclonal antibody (Zymed, USA); a rabbit anti-Bax antibody (Sigma, USA), a mouse anti-active PARP polyclonal antibody (Santa Cruz, USA). Antibody recognition was detected with the respective secondary antibody, ether anti-mouse IgG, or anti-rabbit IgG antibodies linked to horseradish peroxidase (Zymed, USA). Antibody-bound proteins were detected by the ECL western blotting analysis system (Amersham Pharmacia Biotech, UK).

MCF-7 tumor growth in vivo

Thirty 6-week-old female BALB/c nude mice purchased from Orient (Seoul, Korea) were used as host animals. During the experiment, the animals were housed five per cage in plastic cages with sterilized white pine chips as bedding. The animal room was kept specific pathogen-free, and was controlled for temperature (22±3°C), light (12 h light/dark cycle) and humidity (50±10%). Following one weeks acclimatization (at 7 weeks of age), the mice were randomized into three groups of seven mice each of which received AIN-76A diet and modified AIN-76A (500 mg/kg/day BLC or 1000 mg/kg/day BLC/HEN egg). Four weeks later, 3×10^7 MCF-7 cells/0.3 m*l* D-media supplemented with 10% FBS were injected with a 26-gauge needle. The mice were supplemented with 17β-estradiol (Sigma, USA) dissolved in 0.1% dimethylsulfoxide (Sigma, USA) [18]. During the experiment, the mice had free access to water. The mice were weighed, and locally growing tumors were checked once a week until the termination of the experiment. Tumor volume was calculated using the formula: Π×larger diameter×smaller diameter^2 [9]. The experiment was terminated 4 weeks after inoculation of MCF-7 cells. At the termination of the experiment, all mice were weighed and then sacrificed by cervical dislocation. At autopsy, the locally growing tumors were weighed. The growth inhibition was calculated as the formula described by Hawkin *et al.* [9].

Statistical analysis

The data were expressed as the mean plus or minus the standard error. Analyses were performed using SPSS statistical software. When ANOVA revealed $P<0.05$, the data were further analyzed by Sheffe's multiple range tests. Differences were considered statistically significant at $P<0.05$.

Results

Hot-water extracts of BLC and BLC/HEN egg inhibited proliferation of MCF-7 cells

To evaluate the effects of hot-water extract of BLC and BLC/HEN egg on the growth of MCF-7 cells, the cells were treated with series of concentrations from 12.5 to 400 μg/m*l* for 72 h. Cell growth was determined by MTT assay. As shown in Fig. 1, hot-water extract of BLC and BLC/HEN egg inhibited the proliferation of MCF-7 cells in a dose-dependent manner. After 72 h of treatment, at 400 μg/m*l*, this hot-water extract of BLC caused over the 60% inhibition of cell growth compared with control. And, same dose BLC/HEN egg caused the 30% inhibition of cell growth. As hot-water extract of BLC and BLC/HEN egg 100 μg/m*l* has no such effects on normal breast cells indicating that it is selectively cytotoxic for the tumor cells (data not shown), 100 μg/m*l* was used in all further experiments.

Hot-water extract of BLC and BLC/HEN egg induced apoptosis in MCF-7 cells

The ability of the hot-water extract of BLC and BLC/HEN egg to induce apoptosis in MCF-7 human breast cancer cells was assessed using Hoechst 33258 staining (Fig. 2). The cells shrank, turned around, and had a relatively smaller volume than control cells. It was clear that apoptosis was induced in MCF-7 cells within 48 hr.

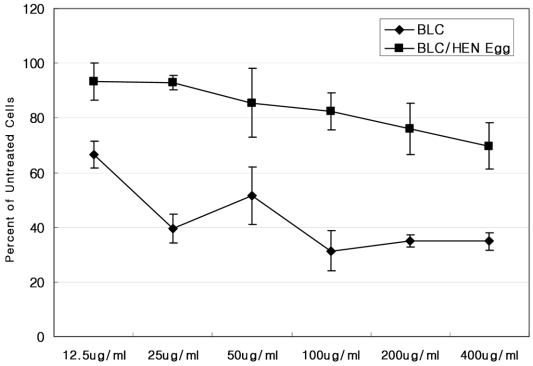

Fig. 1. Inhibition of proliferation by BLC extract and BLC/HEN egg. Cells were treated with various concentrations of test compound for 72 h, and cell viability was determined by the MTT assay. Results are expressed as percentages proliferation compared with untreated control (mean±SE., n=3).

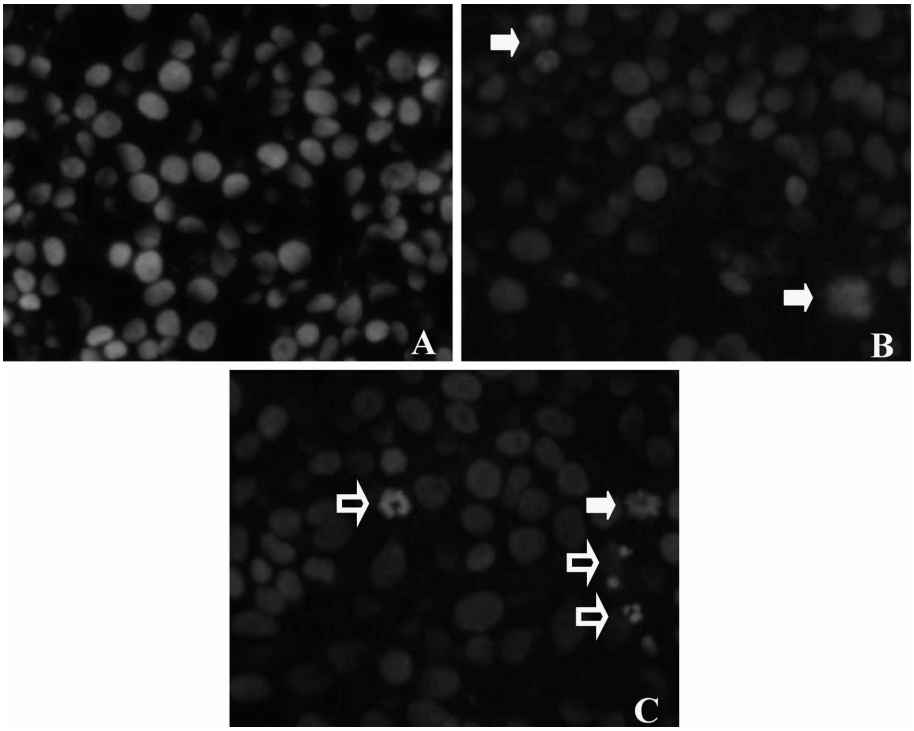

Fig. 2. Detection of apoptotic morphological changes in MCF-7 cells treated with alcohol extract of BLC extract and BLC/HEN egg. Nuclei were stained with Hoechst 33258 and examined by fluorescence microscopy. (A) Control; (B) treated with BLC extract; (C) treated with BLC/HEN egg.

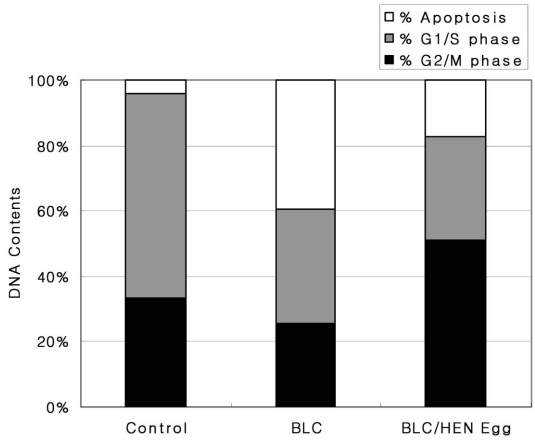

Fig. 3. Cell cycle distribution in MCF-7 cells treated with BLC extract and BLC/HEN egg. Results are expressed as percentages compared with untreated control (mean±SE., n=3). The number of cells at sub-G1 (apoptosis) phase increased gradually and the number of cells at G1/S phase decreased dramatically for 72 h after treatment.

Making sure of that BLC extract and BLC/HEN egg decreased cell proliferation and induced cell death, cell cycle analyses were performed with flow cytometry. As shown in Fig. 3, cells accumulated in the apoptosis (sub-G1) phase at 72 h after treatment with the test compound, whereas the number of cells in G1/S phase decreased in the same manner. BLC extract and BLC/HEN egg were found to be effective on the apoptosis of MCF-7 cells.

Effects of hot-water extract of BLC and BLC/HEN egg on the levels of p53, Bax and Bcl-2 in MCF-7 cells

To explore the potential signing pathways underlying BLC extract and BLC/HEN egg induced apoptosis, we evaluated transcription factor p53 and Bcl-2 family proteins by western blot. The Bax protein level was increased 48 h after treatment and remained elevated up to 72 h whereas, Bcl-2 protein level was decreased 48 h after treatment and remained elevated up to 72 h (Fig. 4). The results suggested that hot-water extract of BLC and BLC/HEN egg induced apoptosis in MCF-7 cells might be mediated through modulation of Bcl-2 family. And, tumor suppress protein, p53 was increased

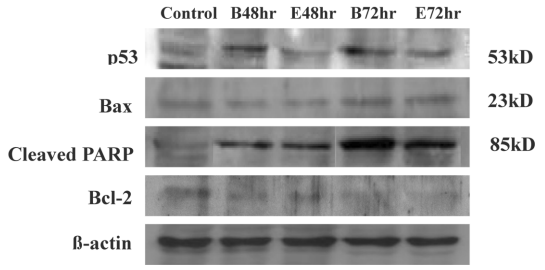

Fig. 4. Effects of BLC extract and BLC/HEN egg on expression of p53, Bax, Bcl-2 and cleaved PARP proteins. Cells were treated with hot-water extract of BLC and BLC/HEN egg from 48h to 72h. Total cellular proteins were prepared and western blot were performed with an antibody specific for corresponding proteins.

in time-dependent manner (Fig. 4).

Effects of hot-water extract of BLC and BLC/HEN egg on PARP expression and cleavage in MCF-7 cells

To assess the role of PARP in this apoptotic process, the expression of PARP was examined by western blot analysis. As shown in Fig. 4, the 116 Kd PARP was cleaved to its active 85 Kd in MCF-7 cells treated with BLC extract and BLC/HEN egg. This result showed that BLC extract and BLC/HEN egg induced apoptosis, since treatment resulted in cleavage of PARP, the substrate of several ICE-like proteases.

Effects of hot-water extract of BLC and BLC/HEN egg on tumor growth of MCF-7 cells *in vivo*

Direct injection of established tumors with BLC extract 500 mg/kg/day and BLC/HEN egg 1000 mg/kg/day administered for 8 weeks resulted in no significant body weight (Fig. 5). Tumor weights were 0.35±0.06, 0.20±0.11 and 0.10±0.02 g in the control group, BLC extract and BLC/HEN egg-administration groups respectively. Treatment with BLC suppressed the weight of breast tumors by 42% and BLC/HEN egg also decreased the weight of tumors by 71% (Table 1).

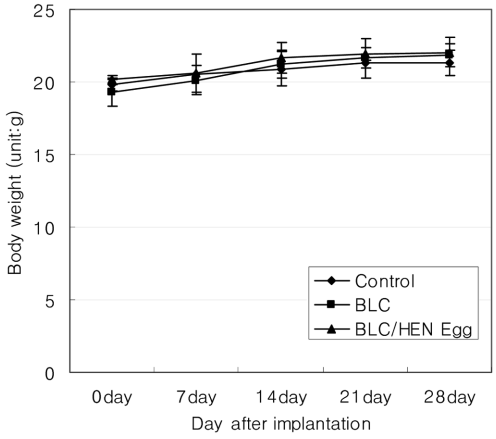

Fig. 5. Change of body weights for 4 weeks after implantation.

Table 1. Effects of BLC extract and BLC/HEN egg in xenograft nude mice

Group	Tumor weight (g)	Inhibited rate (%)
Control	0.35±0.06	
BLC	0.20±0.11	42.1
BLC/HEN Egg	0.10±0.02	71.4

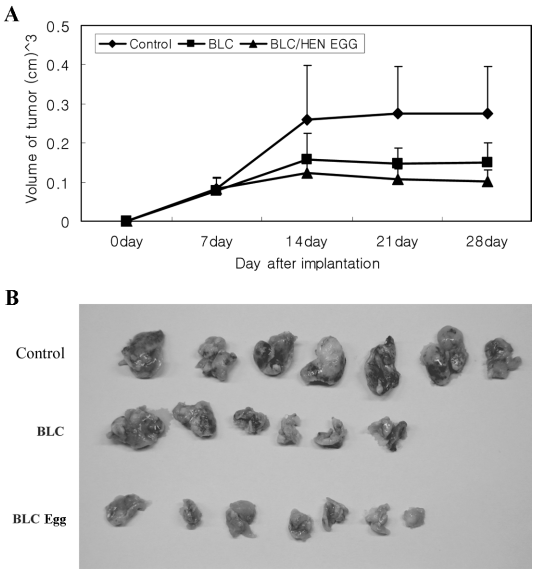

Fig. 6. Effects of BLC extract and BLC/HEN egg in MCF-7 xenograft nude mice. The treatments were administered as described in materials and methods. (A) Data represent the mean tumor volume size for each group; (B) The pictures were taken 4 weeks after implantation.

The volumes of tumour growth were rapidly reduced from 14 days after MCF-7 cells injection in BLC extract and BLC/HEN egg -administration groups (Fig. 6(A)). Fig. 6(B) shows that visually BLC extract and BLC/HEN egg are effective in suppressing *in vivo* growth of xenografted human MCF-7 breast tumors at the end of the experiment.

Discussion

Apoptosis, programmed cell death, plays a major role in the development, tissue homeostasis, and elimination of damaged cells [10]. Cancer prevention or therapy may be accomplished at different steps of this process by different mechanisms [11]. Apoptosis is the good target of chemoprevention. This study has shown that hot-water extract of BLC and BLC/HEN egg inhibited cell proliferation and induced apoptosis in human breast cancer cells. Furthermore, we found that both suppressed the tumor growth of xenografted human MCF-7 breast tumors.

Apoptosis is regulated by members of the Bcl-2 family members [16], which act upstream of a family of cysteine proteases known as the interleukin-1 converting enzyme (ICE) protease family, which are often called caspases [1]. The dysregulation of apoptosis contributes to the pathogenesis of breast cancer, at least in part, due to an imbalance between Bcl-2/Bcl-x and Bax [3]. BLC extract and BLC/HEN egg induced up-regulation of pro-apoptotic protein Bax and down-regulation of anti-apoptotic protein Bcl-2. These modulations of Bcl-2 family raised apoptosis of MCF-7 cells indicating nucleus fragment, shrinking and sub-G1 arrest in cell cycle analysis. The apoptosis-promoting Bax protein leads to mitochondrial dysfunction and the release of cytochrome c from the mitochondria and then cleaves several substrates including the PARP, a nuclear enzyme involved in DNA repair and maintenance of genome integrity and post-translational ribosylation of proteins, whereby apoptosis occurs [22]. In this study we found the cleavage of 116 Kd PARP to 85 Kd proteolytic fragments. The results are consistent with previous reports using MCF-7 cells [12]. The tumor suppressor protein p53 mutations are implicated in some 30±50% of all breast cancers. Therefore, designing alternative treatments strategies aimed specifically at either restoring p53 function to wild-type, or inducing optimal cellular outcome in response to damage, is a promising, rapidly-developing field in cancer research [23]. The hot-water extract of BLC and BLC/HEN egg induced p53 overexpression in time-dependent manner and then in human breast cancer cells.

The MCF-7 human breast cancer xenograft provides an excellent model of clinical breast cancer and many researchers used this method for experimenting the cancer prevention or therapy [2, 5, 9, 15, 17]. We tested BLC extract and BLC/HEN egg in MCF-7 xenograft tumor models to define that both have the anti-tumor effect on the human breast cancer. Our results show that BLC extract and BLC/HEN egg suppressed the weight of breast tumors by 42% and BLC/HEN egg decreased the weight of tumors by 71% tumor growth at the end of the experiment. The volumes of tumor growth were rapidly reduced from 14 days after implantation in BLC extract and BLC/HEN egg -administration groups.

In conclusion, this study shows that the hot-water extract of BLC and BLC/HEN egg induced apoptosis *in vitro* and reduced tumor growth *in vivo*. Additionally, we found that the modulation of Bcl-2 family and the stimulation of p53 pathway may be closely related to this cancer prevention and this may be an important mechanism whereby the BLC extract and BLC/HEN egg protects against tumor promotion. Therefore, the hot-water extract of BLC and BLC/HEN egg may give breast cancer preventive benefits to women.

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References

1. **Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J.** Human ICE/CED-3 protease nomenclature. Cell 1996, **87**, 171.
2. **Alpaugh ML, Tomlinson JS, Shao ZM, Barsky SH.** A novel human xenograft model of inflammatory breast cancer. Cancer Res 1999, **59**, 5079-5084.
3. **Bargou RC, Daniel PT, Mapara MY, Bommert K, Wagener C, Kallinich B, Royer HD, Dorken B.** Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-alpha expression in tumor cells correlates with resistance towards apoptosis. Int J Cancer 1995, **60**, 854-859.
4. **Becher H, Schmidt S, Chang-Claude J.** Reproductive factors and familial predisposition for breast cancer by age 50 years. A case-control-family study for assessing main effects and possible gene-environment interaction. Int J Epidemiol 2003, **32**, 38-48.
5. **Brady H, Desai S, Gayo-Fung LM, Khammungkhune S, McKie JA, O'Leary E, Pascasio L, Sutherland MK, Anderson DW, Bhagwat SS.** Effects of SP500263,

a novel, potent antiestrogen, on breast cancer cells and in xenograft models. Cancer Res 2002, **62**, 1439-1442.

6. **Brekelmans CT.** Risk factors and risk reduction of breast and ovarian cancer. Curr Opin Obstet Gynecol 2003 **15**, 63-68.
7. **Cho JH, Cho SD, Hu H, Kim SH, Lee SK, Lee YS, Kang KS.** The roles of ERK1/2 and p38 MAP kinases in the preventive mechanisms of mushroom Phellinus linteus against the inhibition of gap junctional intercellular communication by hydrogen peroxide. Carcinogenesis 2002, **23**, 1163-1169.
8. **Grube BJ, Eng ET, Kao YC, Kwon A, Chen S.** White button mushroom phytochemicals inhibit aromatase activity and breast cancer cell proliferation. J Nutr 2001, **131**, 3288-3293.
9. **Hawkin RA, Arends MJ, Ritchie AA, Langdon S, Miller WR.** Tamoxifen increases apoptosis but does not influence markers of proliferation in an MCF-7 xenograft model of breast cancer. Breast 2000, **9**, 96-106.
10. **Hayashi M, Nagata A, Endoh D, Arikawa J, Okui T.** High sensitivity of thymocytes of LEC strain rats to induction of apoptosis by X-irradiation. J Vet Med Sci 2002, **64**, 597-601.
11. **Hu H, Ahn NS, Yang X, Lee YS, Kang KS.** Ganoderma lucidum extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. Int J Cancer 2002, **102**, 250-253.
12. **Jo EH, Hong HD, Ahn NC, Jung JW, Yang SR, Park JS, Kim SH, Lee YS, Kang KS.** Modulations of the Bcl-2/Bax family were involved in the chemopreventive effects of licorice root (Glycyrrhiza uralensis Fisch) in MCF-7 human breast cancer cell. J Agric Food Chem 2004, **52**, 1715-1719.
13. **King MC, Marks JH, Mandell JB.** Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science 2003, **302**, 643-646.
14. **Kitts DD, Lim KT.** Antitumorigenic and cytotoxic properties of an ethanol extract derived from Rhus verniciflua Stokes (RVS). J Toxicol Environ Health A 2001, **64**, 357-371.
15. **Kobayashi H, Shirakawa K, Kawamoto S, Saga T, Sato N, Hiraga A, Watanabe I, Heike Y, Togashi K, Konishi J.** Rapid accumulation and internalization of radiolabeled herceptin in an inflammatory breast cancer xenograft with vasculogenic mimicry predicted by the contrast-enhanced dynamic MRI with the macromolecular contrast agent G6-(1B4M-Gd)(256). Cancer Res 2002, **62**, 860-866.
16. **Korsmeyer SJ.** Regulators of cell death. Trends Genet 1995, **11**, 101-105.
17. **Kumar S, Kishimoto H, Chua HL, Badve S, Miller KD, Bigsby RM, Nakshatri H.** Interleukin-1 alpha promotes tumor growth and cachexia in MCF-7 xenograft model of breast cancer. Am J Pathol 2003, **163**, 2531-2541.
18. **Leon-Blanco MM, Guerrero JM, Reiter RJ, Calvo JR, Pozo D.** Melatonin inhibits telomerase activity in the MCF-7 tumor cell line both in vivo and in vitro. J Pineal Res 2003, **35**, 204-211.
19. **Tsao AS, Kim ES, Hong WK.** Chemoprevention of cancer. CA Cancer J Clin 2004, **54**, 150-180.
20. **Yamamoto I, Nagumo T, Fujihara M, Takahashi M, Ando Y.** Antitumor effect of seaweeds. II. Fractionation and partial characterization of the polysaccharide with antitumor activity from Sargassum fulvellum. Jpn J Exp Med 1977, **47**, 133-140.
21. **Yan X, Chuda Y, Suzuki M, Nagata T.** Fucoxanthin as the major antioxidant in Hijikia fusiformis, a common edible seaweed. Biosci Biotechnol Biochem 1999, **63**, 605-607.
22. **Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR.** The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 1993, **75**, 641-652.
23. **Ziyaie D, Hupp TR, Thompson AM.** P53 and breast cancer. Breast 2000, **9**, 239-246.