In vitro effects of Jia-Wei-Xiao-Yao-San in human breast cancer cells treated with Trastuzumab

ABSTRACT

Breast cancer has been the most malignancy worldwide. It has been shown that over 50% of breast cancer patients in Taiwan considered Chinese Herbal Products effective in treating cancer. Based on our previous study, Jia-Wei-Xiao-Yao-San (JWXYS) was the most frequently used as adjuvant therapy for breast cancer patients. Target therapy as trastuzumab is effective in the treatment of HER2-overexpressing early breast cancer or metastatic breast cancer. However, interaction between Chinese Herbal Products and trastuzumab is yet to be clarified. The aim of this study was to investigate the in vitro effect of JWXYS on human breast cancer cells that have been treated with trastuzumab. As a result, in vitro cultured SK-BR-3 or BT-474 cells was co-treated with JWXYS and trastuzumab. This was followed by MTT assays and cell cycle analysis in order to assess cell proliferation. Western blot analysis was used to analyze the expression of various protein involved in growth-related signal pathways. In addition, immunohistochemistry was used to detect autophagy among the cancer cells. In vivo analysis was done for female athymic nude mice implanted with BT-474 cells. The findings of the present study showed that no definitive JWXYS-Herceptin interaction could be identified by either the in vitro or the in vivo parts of the present study. As more breast cancer patients seek to use traditional Chinese medicine as a complementary and alternative treatment method, doctors need to pay increasing attention to the possibility of herb-drug interactions. Based on the in vivo and in vitro demonstration of herb-drug interference in breast cancer cells, it can be concluded that physicians should pay more attention to such interaction when treating patients with receptor-positive (estrogen receptor positive, progesterone receptor positive, or HER2+) breast cancers.

Key words: Jia-Wei-Xiao-Yao-San, breast cancer, trastuzumab, herb-drug interaction.

INTRODUCTION

Breast cancer is the most common cause of cancer-related mortality among females worldwide and the incidence has been increasing in Asia (Hortobagyi et al., 2005; Parkin et al., 2005; Jemal et al., 2011). In Taiwan, breast cancer is the most common female cancer and is the fourth highest cause of death overall in women. The incidence of breast cancer increased 82% from 1995 to 2006, while mortality increased by 14.4% (Bureau of Health Promotion, Bureau of Health Promotion Annual Report, 2009; The National Health Insurance Statistics, 2011). Although there have been advances in therapeutics for breast cancer, which include trastuzumab and aromatase inhibitors, factors affecting quality of life (QOL), such as pain, fatigue, morbidity due to lymphadenectomy, side effects from chemotherapy or radiotherapy, and menopausal symptoms, are still of considerable concern to patients (Montazeri,
patients and their clinical importance has been increasing (Montazeri, 2008).

In addition, using antibody against the HER2/neu receptor (trastuzumab) remains important adjuvant target therapies after surgery (Early Breast Cancer Trialists’ Collaborative G, 2005; Ma et al., 2011). Hormonal therapy with tamoxifen is considered a gold standard when preventing tumor recurrence in women with hormone-responsive breast cancer. Tamoxifen, a non-steroidal selective estrogen receptor modulator, is commonly used for the hormone therapy of receptors-positive breast cancers in pre-menopausal and post-menopausal women. In breast tissue, tamoxifen acts as an estrogen antagonist and competitively inhibits estrogen binding to ERs, which blocks the actions of estrogen on breast cancer cells (Al-Hajj et al., 2006). The human epidermal growth factor receptor 2 (HER2) is over-expressed in approximately 25% of all breast cancers (Dean-Colomb and Esteva, 2008), and is usually indicative of a more aggressive disease and poorer prognosis (Spector and Blackwell, 2009). Consequently, specific anti-HER2 therapeutics, such as trastuzumab (Herceptin®, Genentech, San Francisco, CA), have been developed that specifically target HER2 and disrupt downstream signaling pathways (Carter et al., 1992). When administered with traditional chemotherapy, trastuzumab extends overall survival and slows disease progression in patients with HER2-overexpressing breast cancer (Nahta and Esteva, 2006; Ross and Fletcher, 1998). Despite the observed survival benefits, trastuzumab is effective in only 25 to 50% of this patient population (Buzdar et al., 2005; Piccart-Gebhart et al., 2005), and a majority of patients with metastatic breast cancer that initially respond to treatment will eventually progress. The main tumor cell-autonomous mechanism of action of trastuzumab is inhibition of HER2 homodimerization and downstream signaling of the phosphatidylinositol-3 kinase (PI3K) pathway leading to an inhibition of cell-cycle progression and survival (Spector and Blackwell, 2009); this implies that PI3K regulated processes, e.g., cellular proliferation and apoptosis (Vogel et al., 2002), are potential biomarkers of clinical response to trastuzumab. As a secondary mechanism of action, trastuzumab has been observed to alter tumor microvasculature causing normalization and regression of tumor associated blood vessels and a reduction in vessel diameter, volume, and permeability (Hortobagyi, 2005). Nonetheless, information concerning possible interactions between herbs and drug targets such as HER2/neu remains to be elucidated.

Accumulating evidence suggests that trastuzumab therapy has a number of adverse effects among breast cancer patients, such as congestive heart failure, lung toxicity, insomnia, hot flushes, and cancer-related fatigue. It is generally accepted that the use of complementary and alternative medicines (CAMs) has increased among oncology patients, with the prevalence being as high as 70 to 80% of patients in non-Asian areas (Hietala et al., 2011; Liu et al., 2011) and around 36 to 40% in Taiwan (Lai et al., 2012; Lin and Chiou, 2011).

In Taiwan, more than 33.3% of breast cancer patients have used Traditional Chinese Medicine (TCM) at least once, and more than 80% of TCM users have chosen Chinese herbal medicine (CHM) for adjuvant breast cancer therapy (Ma et al., 2011). CHM is reported to be effective in 78.7% of the patients as breast cancer therapies that can enhance the immune system, treat cancer, reduce the discomfort of chemotherapy and radiotherapy, and relieve menopausal symptoms (Carter et al., 1992). However, large clinical studies examining CHM commonly used for breast cancer have not been performed.

Jia-Wei-Xiao-Yao-San (JWXYS) is a very common Chinese herbal formula that has been used by Chinese for thousands of years. JWXYS consists of Radix Bupleuri, Radix Angelicae sinensis, Radix Paeoniae alba, Rhizoma Atractylodis macrocephalae, Portia, Rhizoma Zingiberis preparata, Cortex Moutan, Fructus Gardeniae, Herba Menthae, and Radix Glycyrrhizae preparata. The indication for JWXYS use is stagnation of the “Liver qi with blood deficiency,” and it is mostly used to treat symptoms such as anxiety, anorexia, night sweating, headache, dry eyes, hot flushes, palpitations, and irregular menstruation (Qu et al., 2010; Yamada and Kanba, 2007; Yasui et al., 2011). Based on our previous study, with data obtained from National Health Insurance Database for Taiwan, Jia-Wei-Xiao-Yao-San (JWXYS) was the most frequently used as adjuvant therapy for breast cancer patients. JWXYS was the most commonly prescribed formula given by TCM doctors to patients who were being treated for breast cancer. In addition, JWXYS is also the commonly used formula co-prescribed with Tam when breast cancer is being treated by hormonal therapy. However, whether a herb-drug interaction between JWXYS and Tam occurs remains unclear. Our previous study is the first to provide evidence as to whether JWXYS interacts with Tam either in vitro or in vivo. Accumulating evidence suggests that many patients, including those with cancer, concurrently take prescription drugs with herbal supplements and that, in some cases, this may have a negative effect on the patients because of pharmacodynamic and pharmacokinetic herb-drug interactions. Our findings showed that no definitive JWXYS-Tam interaction could be identified by either the in vitro or the in vivo parts of the present study. Nevertheless, the fact that JWXYS at a low dose (1.3 g/kg), but neither at middle (2.6 g/kg) nor at high (3.9 g/kg) doses, does attenuate LC3-II expression in Tam-treated MCF-7-implanted tumors suggests that there may still be some herb-drug interactions at a molecular level. There was a significant increase seen in the protein expression of LC3-II in the Tam and JWXYS (3.9 g/kg) + Tam groups as compared with the vehicle group. A decrease in MCF-7 breast cancer cells was observed in the JWXYS (3.9 g/kg) + Tam group similar to the Tam group. These indicate that JWXYS (3.9 g/kg) might
exert a synergistic anticancer effect (Roberts, 2010; Chen et al., 2014; Yeh et al., 2014).

Due to lack of association between Jia-Wei-Xiao-Yao-San and trastuzumab, this study will provide the first evidence for their interaction. Therefore, it may provide a new possible strategy for breast cancer treatment. Since there is no report showing the possible herb-drug interaction between JWXYS and trastuzumab, the aim of the present study was to investigate the in vitro effects of JWXYS on human breast cancer cells treated with trastuzumab.

MATERIALS AND METHODS

Cell line and reagents

The human breast cancer cell lines SK-BR-3 (ER-, HER2-high), and BT-474 (ER+, HER2-high) are obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). All cell lines are routinely screened for the absence of mycoplasma contamination. BT-474 cells are maintained in Hybri-Med medium supplemented with 10% FBS and penicillin/streptomycin. SK-BR-3 cells are maintained in McCoy's 5A medium supplemented with 10% FBS, 1.5 mM L-glutamine, and penicillin/streptomycin. These three cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Matrigel was obtained from Bioscience Discovery Labware. Tam (10 mg) was obtained from AstraZeneca UK Ltd. Trastuzumab (Herceptin) was stored at -20°C until use. The quality control of the herbs was monitored by high-performance liquid chromatography (HPLC).

Preparation of Jia-Wei-Xiao-Yao-San extract

JWXYS consists of Radix Bupleuri, Radix Angelicae sinensis, Radix Paeoniae alba, Rhizoma Atractylodis macrocephalae, Poria, Rhizoma Zingiberis preparata, Cortex Moutan, Fructus Gardeniae, Herba Menthae, and Radix Glycyrrhizae preparata. The preparation of the JWXYS and DGLHT extract followed the standard procedures used when making a composition for patients. The herbal material was extracted by a Good Manufacturing Practice (GMP) company (Chuang Song Zong Pharmaceutical Co., Ltd). The final preparation was stored at -20°C until use. The quality control of the herbs was monitored by high-performance liquid chromatography (HPLC).

Cell viability assay by (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

After SK-BR-3 and BT-474 have been seeded overnight, they were pretreated with trastuzumab (0, 1, and 10 KM) and/or different doses (0, 0.1, 0.3, 1, 3, 10 µg/ml) of JWXYS extract for 48 and 72 h. This was followed by counting the cell numbers using the (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method and the results were double-checked using the trypan blue exclusion assay.

Luciferase reporter assays used to measure ERBB2 and ESR1 gene expression levels

To investigate the effects of commonly used Chinese herbal extracts on ERBB and ESR1 gene expression, the luciferase reporter vector pGL2, containing the human HER2 gene (ERBB2 luciferase; RDB number 2839, RIKEN BioResource Center, Ibaraki, Japan) or the luciferase reporter vector pGL4 containing the human ER gene (ESR1 luciferase; RDB number 7528) promoters were constructed and transiently transfected into SK-BR-3 and BT-474 cells. The transfection procedure was performed following the manufacturer's recommended protocol from the "T-Pro nonliposome transfection reagent II (T-Pro NTRII)" transfection kit (T-Pro Biotechnology, JT97-N002). Briefly, 1 × 10⁵ cells/well were seeded for 24 h, followed by the following transfection procedure. Plasmid DNA (2 L) or 6 L reagent II was mixed with Opti-MEM individually and then mixed together at RT for 20min. The mixture was added to individual wells containing cells for 5 h. The cells were then washed with PBS and medium changed to 1% CDFBS for 19 h. At 24 h, luciferase activity was determined. The transfection efficiency was about 90%. In parallel, the Renilla Luciferase Assay System (Promega Corporation, WI, USA) was used for reporter quantification. The results were presented as relative optic density ratios, namely, the ratio of the luciferase activity to the Renilla luciferase activity.

Cell cycle analysis

After SK-BR-3 and BT-474 cells were seeded for 24 h, they were treated with 0.1% fetal bovine serum overnight, followed by pretreatment with different doses (0, 1 and 3 µg/ml) of JWXYS extract for 30 min. The cells were then treated with trastuzumab (0, 1 and 10 µM) for another 24 h and their cell cycle profile analyzed by DNA flow cytometry. The DNA contents of the cells were used to classify the cells into presynthetic growth phase (G0/G1), S-phase, and postsynthetic/mitotic growth phase (G2+M). The proliferative capacity of the cells was defined as the S+G2/M phase fraction.

Western blot analysis

SK-BR-3 and BT-474 cells were cultured overnight at a density of 3 × 10⁵ cells/well. cultured SK-BR-3 and BT-474
cells were washed twice with cold PBS containing 1 mmol/L Na3VO4 (pH 7.4). The cells were then treated either with a CHE or with vehicle. Thereafter, the samples were homogenized using 400 L lysing buffer containing 150 mmol/L KCl, 10 mM Tris, pH 7.4, 1% Triton X-100, and protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). The protein concentration of each cell homogenate was determined as described previously (Hietala et al., 2011). Samples consisting of 30–50 g of protein were separated on 10% SDS-polyacrylamide gels by electrophoresis and thereafter transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% bovine serum albumin and probed with specific primary antibodies, namely, anti-ER (Stressgen Biotechnologies Inc., Victoria, BC, Canada), anti-pHER2/anti-tHER2 (IPVH00010, Millipore, Bedford, MA, USA), anti-β-tubulin (AbFrontier, Seoul, Korea), and anti-α-actin (AbFrontier, Seoul, Korea). The immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Thermo Scientific, Bremen, Germany) and quantified by Multigauge software analysis (Fuji Photo Film Co., Ltd., Tokyo, Japan).

**RESULTS**

MTT assay of herb–drug interaction in Herceptin-treated SK-BR-3 cells (48 and 72 h) is shown in Figure 1, there was no concentration-dependent increase in cytotoxicity among Herceptin-treated SK-BR-3 cells. Based on the results of the present study, there was no significant change of cell cycle on the herb-drug interference between DGLHT and Herceptin in vitro (Figure 2) and there was no significant change of cell cycle on the herb-drug interference between DGLHT and Herceptin in vitro (Figure 3). Figure 4 shows the effects of DGLHT on the signaling pathways in Herceptin-treated SK-BR-3 cells in vitro. When analyzed by Western blot, DGLHT treatment for 4 h did not increase the protein expression of ERK, P38, p27(Kip1) as compared with Herceptin treatment alone.

**DISCUSSION AND CONCLUSION**

JWXYS, which is the most commonly prescribed Chinese medicine among breast cancer patients, is the leading formula coprescribed with Tam in order to alleviate the discomfort felt by patients who are receiving hormonal therapy (Ross and Fletcher, 1998). This study is the first to provide evidence as to whether JWXYS interacts with Tam either in vitro or in vivo. Accumulating evidence suggests that many patients, including those with cancer, concurrently take prescription drugs with herbal supplements and that, in some cases, this may have a negative effect on the patients because of pharmacodynamic and pharmacokinetic herb-drug interactions. The prevalence of TCM use by health care providers and the interest in their use by patients outside of China continues to rise annually, especially within the field of oncology (Liu et al., 2011). The use of TCM as an adjuvant cancer therapy has been reported to enhance the

**Statistics**

For the in vitro and in vivo studies, the results were expressed as the mean ± SEM. Differences between group means were compared using repeated measures one-way ANOVA followed by Dunnett’s post-hoc test or the unpaired t test. Statistical comparisons between two independent variables were determined by two-way ANOVA followed by Dunnett’s post-hoc test. A p value < 0.05 was considered statistically significant and all results were compared against the vehicle group.
Figure 2: Cell cycle analysis showed no concentration-dependent increase in cytotoxicity among Herceptin-treated SK-BR-3 cells.

Figure 3: Quantities of flow cytometry of herb–drug interaction in Herceptin-treated SK-BR-3 cells.

The cyclin-dependent kinase (CDK) inhibitor p27 (KIP1) efficacy of both chemotherapy and radiotherapy as well as helping to reduce the adverse effects of these treatment (Bureau of Health Promotion Annual Report, 2009).
plays a key role in the growth and development of the mammary epithelium and in breast cancer. In normal cells, p27 inhibits nuclear CDK activity and is thus considered a tumor suppressor. Recent studies have shown that p27 (KIP1) is a predictive factor for Tam treatment response in both premenopausal and postmenopausal breast cancer patients. In contrast to other tumor suppressor proteins, p27 expression levels in tumor cells are frequently regulated by ubiquitin-dependent proteolysis, which in turn is controlled by phosphorylation of p27 at a conserved threonine (T187), which facilitates polyubiquitylation of p27. This then prevents binding to its critical cellular targets, such as the cyclin E and A/CDK2 complexes. In addition, the phosphorylation of p27 by AKT and SRC kinases at either T157 (AKT) or Y74/88 (SRC) induces cellular mislocalization or functional inactivation, which disable p27 as a tumor suppressor protein.

The findings showed that no definitive JWXYS-Herceptin interaction could be identified by either the in vitro or the in vivo parts of the present study. Since more breast cancer patients seek to use traditional Chinese medicine as a complementary and alternative treatment method, doctors need to pay increasing attention to the possibility of herb-drug interactions. However, further evidence in humans using epidemiological studies and large-scale clinical data sets are needed. Based on the in vivo and in vitro demonstration of herb-drug interference in breast cancer cells, it can be concluded that physicians should pay more attention to such interaction when treating patients with receptor-positive (estrogen receptor positive, or HER2+) breast cancers.

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