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Anti-cancer Effects of *Anemarrhena asphodeloides Bunge* and *Coptis chinensis* in Conditionally Reprogrammed Breast Cancer Cells and Breast Cancer Cell Lines

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Abstract

Background: The author investigated the herbal regimens with enhanced anti-cancer effects and reduced side effects of chemotherapeutic agents. The synergistic effects of *Anemarrhena asphodeloides bunge* (AAB) and *Coptis chinensis* (CC) combined with tamoxifen (TMX) and paclitaxel (PTX) were evaluated in breast cancer cells.

Methods: Seven breast cancer cells were used for determination of anti-cancer effects of combinations of chemotherapeutic agents. A cell culture was obtained with four breast cancer cells derived from breast cancer patients and three cancer cell lines using conditionally reprogrammed cell (CRC) methodology. The cultured cells were verified as cancer cells with MAGE A1-6, hTERT and mammaglobin RT-PCR. The combinational chemo-sensitivity test was performed using the MTS assay. The synergistic anti-cancer effects were evaluated with the dosage reducing index (DRI) and combination index using CompuSyn program.

Result: All the four cancer tissues showed excellent growth with the CRC method. All cultured cells expressed hTERT and MAGE A1-6 mRNA. AAB showed synergistic effects with TMX and PTX in the three cells. Their DRI was higher with the PTX combination. CC showed synergistic effects in three cells with TMX and in all the seven cells with PTX. Their DRI was also higher with the PTX combination.

Conclusion: AAB showed synergistic effects in specific breast cancer cells with PTX or TMX. CC exhibited 100% synergy when used with PTX, suggesting a potential role as an effective anti-cancer adjuvant. As different synergistic effects were found among the breast cancer cells, individualized sensitivity tests were necessary for the clinical applications.

Keywords Synergy; Culture; *Anemarrhena*; *Coptis*; Tamoxifen; Paclitaxel

Introduction

Breast cancer is the most common malignancy in women worldwide, and one of the leading causes of cancer death [1]. Though the overall 5-year survival for all stages combined is 89%, the survival rates vary by stage [2]. *Anemarrhena asphodeloides Bunge* (AAB) is effective in treating diabetes and inhibiting platelet aggregation. Timosaponin A-III (TAIII), a notable saponin isolated from AAB induced autophagy in HeLa cells, followed by mitochondria-dependent apoptotic cell death [3]. *Coptis chinensis* (CC) contains berberine (BBR) and its structurally related compound coptisine [4]. BBR exhibited cytotoxic activities against several human cancer cells by inducing cell cycle arrest, apoptosis and/or inhibiting cell migration and invasion [5]. BBR has been shown to have anticancer effects without affecting normal cells [6], indicating that BBR is an attractive anti-cancer compound [7].

Tamoxifen (TMX) is a prodrug, and its anticancer activity is mediated via its active metabolite, 4-hydroxy TMX and its desmethyl analogue endoxifen [8]. TMX also induces cancer cell death by apoptosis but its antiproliferative potential was 30%-40% lower than that of 4-hydroxy-TMX [9]. Tamoxifen is inexpensive and readily available to healthcare systems explaining its worldwide popularity as a amazing drug for breast cancer [10]. Paclitaxel (PTX) is one of the most widely used taxanes in breast cancer treatment. The "taxanes" are a class of anticancer drugs that act by binding to tubulins/microtubules, which play a key role in cell division [11]. We supplemented conventional chemotherapeutic agents, TMX and PTX with herbal medicines. The combinational regimens are expected to reduce the side effects of TMX or PTX, or to enhance their cytotoxic activities against breast cancer cells.

Materials and Methods

Breast cancer cells and conditionally reprogrammed cell (CRC) culture

We used four breast cancer cells and three breast cancer cell lines. The four breast cancer cells were cultured from the biopsied tumor tissue obtained from patients diagnosed with breast cancer using conditionally reprogrammed cell (CRC) methodology. The Lombardi Cancer Center of Georgetown University (Washington, USA) kindly

donated the Swiss 3T3-J2 cell line. For the CRC culture, we used the same protocol as the Lombardi Cancer Center [12].

The patients were diagnosed and operated at the Daegu Catholic University Medical Center (Daegu, Republic of Korea). Human mammary tumor specimens were collected with the informed consent of the patients, according to Daegu Catholic University Medical Center Institutional Review Board (Daegu, Korea) protocols. The three cancer cell lines (MDA-MB-231, MDA-MB-361, MDA-MB-453), were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea).

Reverse transcription (RT) PCR and immunohistochemical analysis

After successful colony formation from CRC culture using primary tumor tissue, we extracted RNA with TRIzol using a few cultured cells. RT-PCR of Melanoma Associated Gene (MAGE) A1-6, human telomerase reverse transcriptase (hTERT), and mammaglobin was performed. These three genes were expressed in the breast cancer cell. The sequences of each gene and PCR conditions are presented in Table 1. Cells were confirmed as cancer cells if they expressed more than two types of cancer mRNA including MAGE, hTERT and mammaglobin. The amplification of all mRNAs was performed using the LightCycler 480 (Roche, Basel, Switzerland). We interpreted the PCR products as positive using fluorescent signals and melting temperature analysis.

The immuno-histochemical staining was also performed using anti-ER, anti-PR and anti-HER2 antibodies for the comparison of cultured cells and the original tumor tissue. The ER/PR PolyDetector HRP/DAB Detection system and HER-2 neu PolyDetector HRP/DAB Detection system (Santa Barbara, CA) were used for the staining.

Gene	Sequences	Products	Ta (°C)	Cycles
MAGE A1-6	5'-AAG GAG AAG ATC TGC CAG TG 5'-GAG GCT CCC TGA GGA CTC T	262 bp	62	25
hTERT	5-CGG GCT GCT CCT GCG TTT GGT G 5'-AGC CGC GGT TGA AGG TGA GAC TGG	311 bp	68	30
Mammaglobin	5'-TTT CCA AGA CAA TCA ATC CAC A 5'-GTA GTA AAA AGA CAT AAG AAA GAG AAG G	299 bp	58	33
GAPD	5'-TCG GAG TCA ACG GAT TTG GTC GTA 5'-CAA ATG AGC CCC AGC CTT CTC CA	320 bp	59	33
Abbreviation: MAGE-Melanoma Antigen-encoding Gene; hTERT-human TElomerase Reverse Transcriptase; GAPD-glyceraldehyde-3-phosphate dehydrogenase				

Table 1: Gene-specific primers used for polymerase chain reactions.

Combinational anti-cancer susceptibility test with MTS assay

We purchased PTX (T7402, Merck KGaA, Germany), TMX (T5648, Merck KGaA), AAB (ANAS2006, Semyung University, Jecheon, Korea) and CC (COTE2013, Semyung University, Jecheon, Korea). The components of AAB and CC were verified with thin layer chromatography-fingerprint analysis. Based on our previous study, the drug concentrations for the combinational anti-cancer susceptibility test were selected as shown in Table 2.

Drug	Conc1	Conc2	Conc3	Conc4	Conc5	Conc6	Conc7
AAB	No	2000	1000	500	250	125	62.5
PTX	No	250	125	62.5	31.3	15.6	7.8
AAB+PTX	No	2250	1125	562.5	281.3	140.6	70.3
TMX	No	40	20	10	5	2.5	1.3
AAB + TMX	No	2040	1020	510	255	127.5	63.8
CC	No	1000	500	250	125	62.5	31.3
PTX	No	250	125	62.5	31.25	15.6	7.8
CC + PTX	No	1250	625	312.5	156.3	78.1	39.1
TMX	No	40	20	10	5	2.5	1.3
CC + TMX	N0	1040	520	260	130	65	32.5

Abbreviation: AAB-*Anemarrhena asphodeloides* Bunge; CC-*Coptis chinensis*; TMX-Tamoxifen; PTX-Paclitaxel

Table 2: Anticancer drug concentrations in the cytotoxicity tests (μg/mL).

An equal number of cells (7,000/well) cultured from cancer cells and cell lines were inoculated into each well of 96-well culture plates and cultured at 37°C and 5% CO_2 for two days. Subsequently, the anticancer agent alone or in combination with herbal medicines was added and cultured at 37°C and 5% CO_2. Each 100-fold concentrated drug was dissolved in dimethyl sulfoxide (67-68-5, Merck KGaA, PTX and TMX) and distilled water (AAB and CC). After that, 2 drugs were mixed and 8 ml of mixed drug was added to 192 ml of medium to make a 2-fold concentrate, then the concentrated mixture was diluted 2-fold, and the sensitivity test was evaluated. For the single agent, a 100-fold concentrate was diluted 2-fold, then used in the same way as the mixed agent. These mixtures were reacted at 37°C and 5% CO_2 for two days, and the drugs were removed, supplemented with 200 μL of media, and incubated for a single day. After removal of media, 100 μL of new media and 20 μL of MTS (tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] were sequentially added to each well and incubated at 37°C for 4 h. Finally, 25 μL of SDS was added and the plates were read at 490 nm with FLUOstar OPTIMA microplate reader (BMG LABTECH, Cary, NC). The sensitivity tests were conducted in triplicate and the mean optical values were used.

Evaluation of anti-cancer synergistic effects of anti-cancer drugs (PTX, TMX) and herbs (AAB and CC)

To evaluate synergistic anti-cancer effects, we used CompuSyn software [13]. This program is based on the combination index (CI) for two drugs, and the CI value quantitatively defines synergism (CI<1), additive effect (CI=1) and antagonism (CI>1). The program also reported the dose reduction index (DRI) showing the extent of reduction in the dose of each drug in a synergistic combination, at a given effect level, compared with the doses of each drug alone.

Caspase activity assay for Caspase 3 and 9

To understand the cellular apoptotic pathway, the MDA-MB-453 cell line, which displayed anti-cancer synergy with both herbs, was selected. Using the same MTS assay protocol, the cells were cultured and treated with the combination of agents. However, simple combination regimens were used for treatment: AAB 500 μg/mL + TAM 10 μg/mL and PTX 62.5 μg/mL, CC 62.5 μg/mL + TAM 2.5 μg/mL and PTX 15.6 μg/mL. The caspase 3 and 9 activities of 20,000 cells were measured using Caspase-Glo 9 and 3/7 assay (Promega, Madison, WI) and FLUOstar OPTIMA microplate reader.

Results

Growth of breast cancer tissue

All the four cancer tissues grew profusely with the CRC method (Fig. 1). The CRC was very useful for the culture of breast cancer epithelial cells.

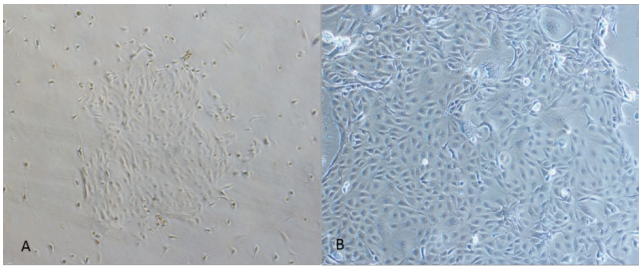

Figure 1: Growth of cancer cells from breast cancer tissue with conditionally reprogrammed cell (CRC) technique (CR1684). The CRC method successfully cultured cancer cells in the tissue within one month(A-40X, B-100X).

RT-PCR of cultured cells

All cultured cells showed the expression of hTERT and MAGE A1-6 mRNA, whereas two cells disclosed the expression of mammaglobin mRNA (Table 3). Thus, the cultured cells were verified as cancer cells.

Cell No	RT-PCR				ER		PR		HER2	
	hTERT	MAGE A1-6	Mammaglobin	GAPD	Culture	Tissue	Culture	Tissue	Culture	Tissue
1684	+	+	-	+	neg	neg	neg	neg	neg	weak
1693	+	+	+	+	mod	mod	mod	strong	mod	neg
16101	+	+	-	+	mod	mod	neg	mod	mod	mod
16138	+	+	+	+	mod	mod	mod	strong	mod	mod
Abbreviation: neg-negative; mod-moderate										

Table 3: Genetic analysis and immunohistochemical staining of cultured cells derived from cancer tissues.

IHC of cultured cells and the original cancer tissue

Comparison of IHC results between cultured cells and the original cancer tissue revealed that the two cells (1684, 16138) showed 100% concordance (3/3 markers), while another two cells (1693 and 16101) exhibited 67% concordance (2/3 markers) with the tissue specimen (Table 3) as shown in Fig. 2.

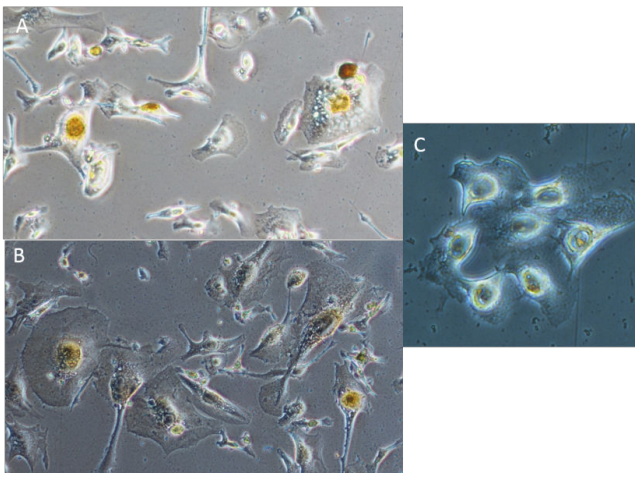

Figure 2: Immuno-histochemical staining of cultured cells. The yellow signal indicated positive reactions for estrogen receptor (A), progesterone receptor (B) and HER2 antigen (C)(A-100X, B-100X, C-200X)

Synergistic anti-cancer effects of herbs (AAB and CC) with anti-cancer drugs (TMX, PTX)

Anti-cancer effects of AAB with anti-cancer drugs (TMX, PTX)

Among the seven breast cancer cells, AAB showed synergistic effects with TMX and PTX in the same three cells (MDA-MB-453, 16101 and 16138, Table 4).

With the AAB+TMX combination, MDA-MB-453, 16101 and 16103 cells showed 31.5, 29.3 and 46.6 μg/mL of median effect doses, while their DRI of TMX was 1.67, 2.62 and 1.96 (bold letters in Table 4) at ED95% respectively.

With the AAB+PTX combination, the 16101, 16138 and MDA-MB-453 cells showed 6.7, 5.17 and 43.2 μg/mL of median effect doses, while their DRI of PTX was 8.55, 4.56 and 3.38 (bold letters in Table 4) at ED95%, respectively.

These results suggested that AAB could reduce the dosage of TMX by two times and the dosage of PTX by three times or more when used in combination.

Anti-cancer effects of CC with anti-cancer drugs (TMX, PTX)

In the same three cells, CC showed synergistic effects with TMX (MDA-MB-453, 16101 and 16138) and in all seven cells with PTX (Table 5).

With the CC+TMX combination, MDA-MB-453, 16101 and 16138 cells showed 19.8, 29.3 and 48.4 μg/mL of median effect doses, while the DRI of TMX was 2.89, 2.62 and 2.16 (bold letters in Table 5) at ED 95%, respectively.

With the CC+PTX combination, the median effect dose ranged from 23.9 μg/mL to 69.1 μg/mL whereas the DRI of PTX ranged from 2.48 to 7.95 at ED 95%.

These results suggested that CC could reduce the dosage of TMX by two times and the dosage of PTX by two times or more when used in combination.

Different synergistic effects

We found different synergistic effects among seven breast cancer cells, three cells showed synergistic effect while the other four cells showed no synergistic effect. These results meant that individualized sensitivity testing is required for clinical application.

Cell No.	Drug	Combination ratio	Dm	m	CI value			
					DRI value of AAB/TMX or AAB/PTX			
					ED50%	ED75%	ED90%	ED95%
MDA-MB-453	AAB+TMX	50	31.5	1.69	0.85 4.58/1.59	0.81 5.19/1.62	0.77 6.01/1.65	0.75 6.64/**1.67**
16101	AAB+TMX	50	29.3	2.44	0.81 1.93/3.44	0.88 1.81/3.11	0.95 1.69/2.81	0.99 1.62/**2.62**
16138	AAB+TMX	50	46.6	1.29	0.37 3.8/9.69	0.56 2.67/5.34	0.87 1.88/2.94	1.19 1348/**1.96**
1693	AAB+TMX	50	105.9	1.52	1.24 1.21/2.38	1.33 1.21/1.94	1.44 1.22/1.59	1.53 1.22/1.38
MDA-MB-231	AAB+TMX	50	179.1	3.22	1.72 0.79/2.0	1.71 0.8/1.86	1.69 0.81/1.73	1.68 0.82/1.65
1684	AAB+TMX	50	24.9	0.98	2.89	2.35	2.1	2.14

					0.36/7.41	0.47/3.73	0.63/1.88	0.76/1.18
MDA-MB-361	AAB+TMX	50	184.3	2.21	3.22 0.38/1.61	3.19 0.4/1.52	3.16 0.41/1.43	3.14 0.41/1.38
16101	AAB+PTX	8	6.7	1.46	0.37 3.34/13.3	0.4 3.22/11.3	0.43 3.10/9.55	0.45 3.02/**8.55**
16138	AAB+PTX	8	5.17	0.93	0.08 37.8/17.4	0.14 19.1/10.5	0.26 9.63/6.4	0.38 6.04/**4.56**
MDA-MB-453	AAB+PTX	8	43.2	1.09	0.93 3.53/1.55	0.83 2.87/2.07	0.79 2.32/2.77	0.79 2.02/**3.38**
1693	AAB+PTX	8	101.3	1.33	1.21 1.4/1.99	1.46 1.27/1.46	1.79 1.15/1.08	2.06 1.08/0.87
MDA-MB-231	AAB+PTX	8	183.7	2.66	1.2 1.34/2.2	1.19 1.21/2.72	1.2 1.1/3.35	1.23 1.03/3.87
MDA-MB-361	AAB+PTX	8	62	1.91	1.06 1.86/1.89	1.12 1.63/1.96	1.19 1.43/2.02	1.24 1.31/2.07
1684	AAB+PTX	8	25.7	1.1	1.08 1.57/2.24	1.53 1.08/1.65	2.17 0.74/1.21	2.75 0.57/0.98

Abbreviation: Dm-Median effect dose (μg/mL); m-shape of dose effect curve; ED-Expected death; CI- Combination index; 1<, =1 and >1 indicates synergism, additive effect, and antagonism; DRI-Dosage reducing index

Table 4: Cytotoxic effects of *Anemarrhena asphodeloides* combined with anti-cancer drugs in breast cancer cells.

Cell No.	Drug	Combination ratio	Dm	m	CI value			
					DRI value of AAB/TMX or AAB/PTX			
					ED50%	ED75%	ED90%	ED95%
MDA-MB-453	CC+TMX	25	19.8	1.68	1.08 1.22/3.78	0.91 1.6/3.42	0.79 2.12/3.09	0.74 2.56/**2.89**
16101	CC+TMX	25	29.3	2.44	0.81 1.93/3.44	0.88 1.81/3.11	0.95 1.69/2.81	0.99 1.62/**2.62**
16138	CC+TMX	25	48.4	2.15	0.72 2.16/3.83	0.81 2.04/3.1	0.92 1.92/2.49	1 1.85/**2.16**
1684	CC+TMX	25	68.4	2.22	1.04 1.91/1.9	1.05 1.84/1.96	1.05 1.77/2.02	1.05 1.73/2.07
MDA-MB-231	CC+TMX	25	70.4	3.14	1.3 1.32/1.85	1.28 1.34/1.87	1.26 1.37/1.89	1.24 1.39/1.9
1693	CC+TMX	25	100.7	2.91	1.53 0.93/2.17	1.44 1.02/2.13	1.35 1.13/2.09	1.31 1.21/2.07
MDA-MB-361	CC+TMX	25	67.9	2.15	1.51 1.36/1.25	1.53 1.42/1.21	1.55 1.45/1.16	1.56 1.47/1.14
1684	CC+PTX	4	30.6	1.63	0.72 5.13/1.86	0.6 4.14/2.77	0.54 3.34/4.1	0.53 2.89/5.36
MDA-MB-361	CC+PTX	4	23.9	1.57	0.37 4.5/7.1	0.43 3.56/6.64	0.52 2.82/6.2	0.59 2.4/5.93

16101	CC+PTX	4	46.6	1.84	0.8 1.89/3.61	0.75 2.21/3.34	0.71 2.59/3.08	0.69 2.88/2.91
MDA-MB-231	CC+PTX	4	40.4	1.6	0.4 3.06/13.9	0.5 2.45/11.3	0.62 1.96/9.17	0.72 1.68/7.95
1693	CC+PTX	4	30.6	1.63	0.48 3.66/4.73	0.59 3.01/3.76	0.73 2.48/2.98	0.85 2.17/2.55
MDA-MB-453	CC+PTX	4	29.2	1.6	1.41 0.99/2.43	1.18 1.28/2.49	1 1.63/2.55	0.91 1.93/2.59
16138	CC+PTX	4	69.1	2.21	0.86 1.82/3.18	0.92 1.74/2.9	0.98 1.67/2.64	1.02 1.62/2.48
Abbreviation: Dm-Median effect dose (μg/mL); m-shape of dose effect curve; ED-Expected death; CI- Combination index; 1<, =1 and >1 indicates synergism, additive effect, and antagonism; DRI-Dosage reducing index								

Table 5: Cytotoxic effects of *Coptis chinensis* combined with anti-cancer drugs in breast cancer cells.

Activity of caspases 3 and 9

The caspase activities are summarized in the Fig. 3. The caspase 3 and 9 activities were increased with the AAB and CC combination with anti-cancer drug compared with single drug treatment.

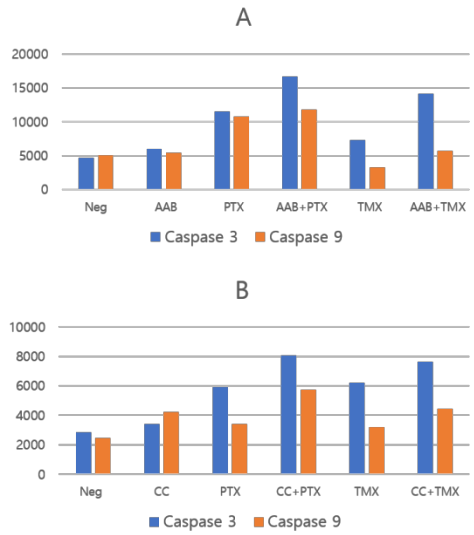

Figure 3: Caspase activity under combinational treatments of MDA453. When used in combination with PTX or TMX, AAB and CC showed increased caspase 3 and 9 activity of MDA453 than their single treatments. **A:** Anemarrhena asphodeloides bunge (AAB) combination with Paclitaxel (PTX) or Tamoxifen (TMX); **B:** Coptis chinensis (CC) combination with PTX or TMX; No drug (Neg).

Discussion

The CRC method using feeders and Y-27632 facilitated the culture of breast cancer epithelial cells. With the biopsy specimen, all four breast cancer cells proliferated and resulted in colonies within 1 week.

To verify the cultured cells, hTERT, MAGE A1-6 and mammaglobin genes were used. The hTERT gene was utilized as a pan-tumor marker [14, 15], however, its expression was increased in the CRC culture. Thus, additional markers were needed for cancer cell verification. MAGE A1-6 primer was used to detect MAGE A1 to A6 mRNA simultaneously. In the 12 breast cancer tissues, 11 tissues expressed more than one MAGE A1 to A6 genes [16]. Mammaglobin is a specific marker for breast cancer [17]. Watson MA reported 91% positive rates in the immunohistochemical analysis of breast cancer tissue [18]. Since all culture cells expressed more than two cancer-related genes, we verified the cultured cells as cancer cells.

We compared the IHC results between cultured cells and the original cancer tissue. Studies comparing IHC results between the CRC and the original tissue were rarely reported. The CRC showed stem cell phenotypes, however, maintaining tissue-specific differentiation potential [19]. In our IHC results, 1684 and 16138 cell lines showed concordance with the tissue, while the 1693 and 16101 cells showed discordance only with the Her2 and PR stain respectively. In the genetic marker study using 16 short tandem repeat alleles, the CRCs cultured from colorectal and gastric cancer tissue showed 100% concordance with their original tissue, but not with the IHC results (Data not shown). We concluded that the cultured cells originated from the cancer tissue.

In the combination treatments with TMX and PTX, AAB showed synergistic effects with the same three cells. In another four cells with CI value >1, we found dose-reducing effects of TMX because the DRI of TMX was >1. Compared with AAB, the CC showed higher synergistic effects with TMA and PTX. The same three cells in the AAB combination revealed synergy with CC and TMX combination, however with PTX, the CC showed synergy with all breast cancer cells. As anticancer effects of the CC have been already evaluated [5-7], it represents an attractive combination regimen.

In the breast cancer cells, we found different synergistic effects, warranting individualized sensitivity tests for clinical applications. Though, the Swiss 3T3 fibroblasts were necessary, the CRC culture method was very useful for individual cancer cell culture.

In the combination containing AAB and CC with anti-cancer drug, the caspase 3 and 9 activities were increased compared with single

drug treatment. In the cancer cell lines, AAB [3] and CC [20] increased caspase 3 and caspase 9 activities. The PTX induced reactive oxygen species leading to the activation of caspase [21]. The TMX also promoted caspase 3 signaling pathway [22].

As limitations of this study, the in vitro chemosensitivity tests could not reflect in vivo situation, especially the effect of combination therapy is difficult to understand by in vitro tests [23]. Correlations of in vitro results with clinical outcome have indicated predictive accuracies of 57–83 % for drug sensitivity and >90 % for drug resistance [23,24]. Although this study did not perform, it is necessary to carry out genetic studies to understand their resistance mechanism.

Conclusion

The seven breast cancer cells were used for determination of anti-cancer effects of combinations of chemotherapeutic agents. AAB showed synergistic effects in a few breast cancer cells when used with PTX and TMX. CC displayed 100% synergy when used with PTX, suggesting its potential role as an effective anti-cancer adjuvant. As different synergistic effects were found among the breast cancer cells, individualized sensitivity tests were necessary for the clinical applications. The CRC culture method was very useful for individual cancer cell culture.

Conflict of interest

The authors have declared that there is no conflict of interest.

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