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Research Paper

Differential Anti-tumor Activity of *Coriolus versicolor* (Yunzhi) Extract through p53- and/or Bcl-2-Dependent Apoptotic Pathway in Human Breast Cancer Cells

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KEY WORDS

apoptosis, anti-tumor, Bcl-2, breast cancer cells, *Coriolus versicolor*, p53, Yunzhi

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NOTES

*Any opinions, findings, conclusions or recommendations expressed in this material/event (or members of the project team) do not reflect the views of the Government of the Hong Kong Special Administrative Region or the Innovation and Technology Commission.

ABSTRACT

Coriolus versicolor (CV), also called Yunzhi, has been demonstrated to exert anti-tumor effects on various types of cancer cells, but the underlying mechanism has not been fully elucidated. The present study aimed to evaluate the in vitro anti-tumor activity of a standardized aqueous ethanol extract prepared from CV on four breast cancer cell lines using MTT assay, and test whether the mechanism involves apoptosis induction and modulation of p53 and Bcl-2 protein expressions using cell death detection ELISA, p53 and Bcl-2 ELISAs respectively. Our results demonstrated that the CV extract dose-dependently suppressed the proliferation of three breast tumor cell lines, with ascending order of IC_{50} values: T-47D, MCF-7, MDA-MB-231, while BT-20 cells were not significantly affected. Tumoricidal activity of the CV extract was found to be comparable to a chemotherapeutic anti-cancer drug, mitomycin C. Nucleosome productions in apoptotic MDA-MB-231, MCF-7 and T-47D cells were significantly augmented in a time-dependent manner and paralleled the anti-proliferative activity of CV extract. Expression of p53 protein was significantly upregulated only in T-47D cells treated with the CV extract in a dose- and time-dependent fashion, but not in MCF-7 (except at 400 µg/ml after 16 h) and MDA-MB-231 cells. The CV extract significantly induced a dose-dependent downregulation of Bcl-2 protein expression in MCF-7 and T-47D cells, but not in MDA-MB-231 cells. These results suggested that apoptosis induction, differentially dependent of p53 and Bcl-2 expressions, might be the possible mechanism of CV extract-mediated cytotoxicity in human breast cancer cells in vitro.

INTRODUCTION

Breast cancer is the most common cancer among women worldwide. The incidence and mortality rates of breast cancer in women still rank high in global epidemiologic studies over the past few years.[1] Improvements in hormonal and cytotoxic therapies have not led to sustained remission or cure in advanced breast cancer. Although anti-estrogens have provided the most effective endocrine therapy for breast cancers in advanced stages, therapeutic choices are limited for estrogen receptor (ER)-negative tumors, which are often more aggressive. In fact, only about two-thirds of ER-positive breast cancers respond to estrogen ablation.[2]

The limited therapeutic possibilities for treating breast cancer increase the necessity for exploring new approaches of therapy. Plant-derived extracts have been historically considered as important alternative remedies for enhancing immune status, and prevention and treatment of chronic diseases.[3] Recent studies have shown that mushroom extracts possess anti-bacterial, anti-viral, anti-tumor and immuno-potentiating activities.[4-8] *Coriolus versicolor* (CV), known as Yunzhi in China, is a mushroom belonging to species of the Basidiomycetes class (Polyporaceae family) of fungi. Its medicinal value was recorded in the *Compendium of Chinese Materia Medica* and *Shen Non-Compendium Medica* thousands of years ago in China; nowadays the therapeutic potentials have been gaining acceptance among patients worldwide.[5,8]

Polysaccharopeptide (PSP), one of the various bioactive components derived from CV, is found to possess anti-tumor and immunomodulatory activities. It is a protein-bound polysaccharide isolated from the deep-layer cultivated mycelia of *Coriolus versicolor* COV-1 strain.[9] The polysaccharide moiety of PSP is a β (1 $\rightarrow$ 3)-glucan branching at 4' and 6' positions, and consists of five different sugars including arabinose, glucose, galactose, mannose and xylose. The protein component is rich in aspartic and glutamic acids. The substance has a molecular weight of about 100,000 and is highly water-soluble.[6] Previous

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studies have reported that PSP and PSK (protein-bound polysaccharide Krestin)[10] could dose-dependently inhibit the proliferation of breast, lung and prostate tumor cell lines in vitro.[5,11-13] PSP was also found to induce the production of interferon-γ and interleukin-2, stimulate T-cell proliferation and counteract the immunosuppression induced by cytotoxic drugs such as cyclophosphamide.[14-16] In vivo administration of PSP or PSK to nude mice also effectively suppressed the growth of inoculated human hepatoma, lung and prostate adenocarcinoma, and extended the survival time.[5,6,16,17] In recent clinical trials, PSP administration to patients with oesophageal, gastric and lung cancer who are undergoing radiotherapy or chemotherapy, can help to alleviate symptoms and prevent the decline in immune status.[5,18]

Apoptosis has been well characterized by a variety of hallmark events including rapid reduction in cellular volume, chromatin condensation, and internucleosomal DNA cleavage.[19] Induction of apoptosis is suggested to be one of the major modes of action of chemotherapeutic anti-cancer drugs on malignant cells.[20-22] The extent of drug-induced apoptosis, in some cases, could act as a predictive marker of the tumor response in vivo.[23,24] The molecular mechanism implicated in apoptosis induced by extracts of CV has been partially delineated. Recent studies, including ours, have shown that extracts of CV and other mushrooms such as *Ganoderma lucidum* can induce apoptosis in breast and cervical tumor cell lines, as well as leukemia and lymphoma cell lines.[11,25-27] Tumor suppressor gene p53 is one of the pivotal molecules involved in apoptosis induction. Many studies raised the possibility that cells lacking p53 activity due to mutation might be more resistant to cancer chemotherapy.[28-30] In wild-type p53 ($p53^{wild}$)-positive cells, once p53 is activated, the outcome of cellular response is either cell cycle arrest or apoptosis. The apoptotic process is mediated by transcriptional transactivation of p53 on many apoptosis-related genes, including Fas and Bax.[31] Bcl-2 is one of the major members of the Bcl-2 family which works on mitochondria to prevent membrane permeabilization and hence the release of apoptogenic factors from the mitochondrial intermembrane space. It is frequently overexpressed in many types of tumors.[32] Although the mechanism of action is still unclear, Bcl-2 functions as a suppressor of apoptotic death and is negatively regulated by p53 for committing the cells to undergo apoptosis.[33]

The present study aimed to investigate whether the mechanism of in vitro anti-proliferative activity of a wild *Coriolus versicolor* ethanol-water standardized extract, supplied by Vita Green Health Products Company Ltd. and previously reported (see Refs. 27,34), on four breast cancer cell lines (BT-20, MDA-MB-231, MCF-7, and T-47D) involves induction of apoptosis, and modulation of p53 and Bcl-2 expressions. Cell death detection ELISA was used to quantify the nucleosome production resulting from nuclear DNA fragmentation during apoptosis, and p53 pan ELISA and Bcl-2 ELISA kits were employed to evaluate the protein expressions of p53 and Bcl-2 respectively.

MATERIALS AND METHODS

Preparation of CV extract. An ethanol-water extract from *Coriolus versicolor* L. was provided by Vita Green Health Products Company Limited, Hong Kong, in compliance with Good Manufacturing Practice (GMP) standard. The extract was obtained from the fruiting bodies of wildly grown *Coriolus versicolor* in the Guangxi province of mainland China. The *Coriolus versicolor* was authenticated by Prof Zhang Xiao-qing (Mycology expert, Institute of Microbiology Chinese Academy of Sciences, Beijing, China). Herbarium voucher specimen (no. 2003-2510) is deposited at the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong. The fruiting bodies were cleaned, crushed and extracted with ethanol-water at proprietary proportions and temperatures, followed by differential precipitation with ethanol. Further processing of specific ethanol fractions led to isolation of the extraction product in powder form (Batch no.: Yz03; shelf time: three years). The major components of the resulting CV extract were polysaccharides and triterpenoids, which were approximately 30% by weight. Plain RPMI medium 1640 (Invitrogen GIBCO, NY, U.S.A.) and Minimum Essential Medium (MEM) (Invitrogen GIBCO) were used to dissolve the CV extract as stock solutions of 3.0 mg/ml and 2.2 mg/ml respectively, for 48 h at room temperature with continuous agitation. Insoluble material was removed by centrifugation and the soluble supernatant was sterilized using a 0.22 μm filter, and further diluted with the plain culture medium to the defined concentrations as indicated.

Cell cultures. Human breast cancer cell lines (BT-20, MDA-MB-231, MCF-7, T-47D) were purchased from American Type Culture Collection (ATCC, MD, U.S.A.). BT-20 and MDA-MB-231, which do not express estrogen receptor (ER), originate from a primary breast carcinoma and a metastatic adenocarcinoma respectively. MCF-7 and T-47D are ER-positive metastatic adenocarcinoma and ductal carcinoma cells respectively. The cell lines were maintained in a humidified incubator at 37°C and in 5% CO_2 atmosphere. RPMI medium 1640, supplemented with 10% fetal bovine serum (FBS, Invitrogen GIBCO), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen GIBCO), was used for cell cultures of MDA-MB-231 and T-47D. MEM medium, supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate, was used as the culture medium of BT-20 and MCF-7 cells. After being trypsinized and harvested from culture flasks, the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. For BT-20, 10^4 cells from log phase cultures were seeded in 100 μl of MEM medium supplemented with 20% FBS per well of 96-well flat-bottom Costar culture plates (Corning Inc., MA, U.S.A.); while for the other three cell lines, 5 x 10^3 cells were seeded in 100 μl of culture medium supplemented with 20% FBS per well. One hundred microlitre of solutions containing 2X final concentrations of the CV extract in plain culture medium were added per well. Control wells were added with 100 μl of plain culture medium alone. A chemotherapeutic anti-tumor drug, mitomycin C (MMC, Sigma Chemical Co., MO, U.S.A.) at a final concentration of 20 μg/ml was added as the positive control. Cells were then incubated with the CV extract for a defined time (see below). Proliferative response and cell death of the CV extract-treated cells were determined using MTT cytotoxicity assay and cell death detection ELISA, respectively.

MTT cytotoxicity assay. The procedure of MTT assay had been described previously.[27] Briefly, after incubation of cells with the CV extract for 48 h (BT-20, MDA-MB-231 and MCF-7) or 72 h (T-47D) as mentioned before, 30 μl of 5 mg/ml MTT in phosphate buffered saline (PBS, Invitrogen GIBCO) was added to each well and the plate was incubated at 37°C for 2 h. The plate was then centrifuged followed by removal of supernatant. One hundred microliter of dimethylsulfoxide (DMSO, Sigma) was then added to each well. Absorbance of the dissolved solution was detected at 540 nm by a Benchmark microtiter plate reader (Bio-Rad Laboratories, CA, U.S.A.). The absorbance of untreated cells was considered as 100%.

Cell viability assay. To evaluate the effect of the CV extract on cell viability, cells were exposed to the CV extract at concentrations producing 50% growth inhibition (IC_{50}) for the tumor cell lines (as determined by MTT assay) for 16, 24 and 48 h. The viable cells were counted by trypan blue exclusion test. The percentage of dead cells was calculated from the ratio of dead cells to total number of cells x 100%.

Apoptosis assay. Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Basel, Switzerland) was used to quantify histone-complexed DNA fragments (nucleosomes) in cytoplasm of the apoptotic cells after induction of apoptosis. The assay is based on a one-step sandwich enzyme-linked immunoassay principle as described elsewhere.[22,27] Briefly, after incubation

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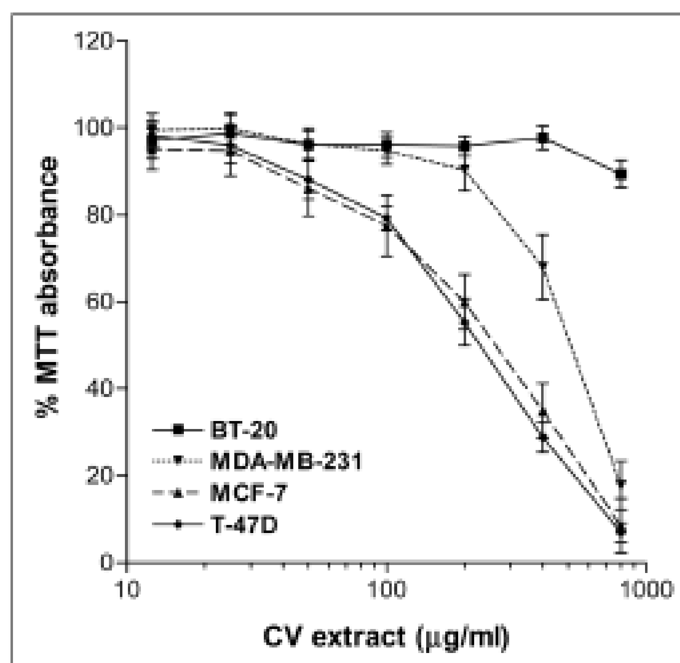

Figure 1. Anti-proliferative effects of CV extract on four human breast cancer cell lines. Cells were incubated with increasing concentrations (12.5–800 μg/ml with 2-fold increase) of the CV extract in culture medium for 48 h (except T-47D for 72 h) and the proliferative response was assessed by MTT assay. Results are expressed as the mean% MTT absorbance (ratio of absorbance in extract-treated well to that of control well x 100%) ± SD of three independent experiments with six wells each.

Table 1 **Concentrations producing 50% growth inhibition (IC_{50}) of CV extract and comparison of growth suppression between CV extract (800 μg/ml) and mitomycin C (MMC, 20 μg/ml) on four breast cancer cell lines**

Cell lines	IC_{50} (μg/ml)[a]	% Growth suppression	
		CV extract[b]	MMC
BT-20	>800	10.6 ± 3.2	92.8 ± 0.8
MDA-MB-231	514.0 ± 42.0	82.2 ± 5.6	85.1 ± 5.1
MCF-7	271.7 ± 20.5	91.5 ± 6.3	97.4 ± 0.7
T-47D	233.3 ± 21.9	93.0 ± 2.3	94.5 ± 1.3

Results are expressed as the mean ± SD of three independent experiments with six wells each. [a]IC_{50} values were determined from the results of MTT assay of three independent experiments. [b]Maximal % growth suppression (100%—% MTT absorbance from ratio of absorbance in extract-treated and control wells) on the cell lines by CV extract (800 μg/ml), as calculated from the results of MTT assay.

with the CV extract (at concentrations determined by MTT assay) for 16, 24 and 48 h, the cells were pelleted and lysed. The remaining steps were referred to the instructions supplied by the manufacturer. The resulting color development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured at 405 nm (with reference wavelength at 490 nm) using a Benchmark microtiter plate reader (Bio-Rad). Results were expressed as the apoptotic index which was calculated from the ratio of absorbance of treated (apoptotic) sample to that of the untreated (control) sample.

ELISA of p53 protein. For detecting the expression of p53 protein in the breast cancer cell lines, p53 pan ELISA kit (Roche Applied Science) was used. Briefly, cells were treated with or without the CV extract (at the same concentrations in apoptosis assay) for 16, 24 and 48 h, the samples to be assayed (the lysate collected from approximately 10^6 cells) were placed (triplicated) in a streptavidin-coated 96-well microtitre plate precoated with biotinylated mouse monoclonal antibody specific for both wild and mutant human p53 protein. After incubation with horseradish peroxidase-conjugated sheep polyclonal detector antibody for 2 h at room temperature, the unbound materials were removed by several washings. Finally, a chromogenic substrate (tetramethylbenzidine, TMB) was added and enzymatically converted to a colored solution with intensity proportional to the amount of p53 protein in the sample. The absorbance was measured at 450 nm (with reference wavelength at 690 nm), and p53 concentration was determined by interpolating from the standard curve obtained with known concentrations of p53 protein. Relative expression of p53 protein was calculated from the ratio of absorbance of test sample to that of control.

ELISA of Bcl-2 protein. Bcl-2 ELISA kit (Bender MedSystems GmbH, Vienna, Austria) was used to evaluate the expression of Bcl-2 protein in the breast cancer cell lines. Briefly, cells were treated with or without the CV extract (at the same concentrations in apoptosis assay) for 16, 24 and 48 h, the samples to be assayed (the lysate collected from approximately 5 x 10^5 cells) and the biotinylated specific detector antibody (mouse monoclonal) were placed (triplicated) in a 96-well microtitre plate coated with mouse monoclonal antibody specific for human Bcl-2, and were incubated for 2 h at room temperature. After removing the unbound materials by several washings, horseradish peroxidase-conjugated streptavidin was added to bind to the antibodies, which catalysed the conversion of a chromogenic substrate (TMB) to a colored solution with intensity proportional to the amount of Bcl-2 protein in the sample. The absorbance was measured at 450 nm (with reference wavelength at 620 nm), and Bcl-2 concentration was determined by interpolating from the standard curve obtained with known concentrations of Bcl-2 standards. Relative expression of Bcl-2 protein was calculated from the ratio of absorbance of test sample to that of control.

Statistical analysis. Results were expressed as the mean ± standard deviation (SD). Statistical differences were assessed by the Student's unpaired t-test, with $p<0.05$ as significant. All analyses were performed using the SigmaStat for Windows, version 3.0.1 (SPSS Inc., IL, USA).

RESULTS

Anti-tumor activities of CV extract on breast cancer cells. The CV extract could significantly inhibit the proliferation of ER-negative MDA-MB-231 (at 200 to 800 μg/ml) and, ER-positive MCF-7 and T-47D cells (at 50 to 800 μg/ml) in a dose-dependent manner, but no significant growth suppression on the ER-negative BT-20 cells (Fig. 1). Table 1 shows the IC_{50} values of the CV extract on the tested cell lines. Proliferation of ER-positive MCF-7 and T-47D cells were significantly inhibited by the CV extract from 50 μg/ml, with low IC_{50} values (271.7 ± 20.5 μg/ml and 233.3 ± 21.9 μg/ml respectively). The CV extract only at concentrations higher than 200 μg/ml could suppress the cell growth of ER-negative MDA-MB-231, resulting in higher IC_{50} value (514.0 ± 42.0 μg/ml). The CV extract exerted no significant growth inhibition on BT-20 cells with IC_{50} value greater than 800 μg/ml. MDA-MB-231, MCF-7 and T-47D cells were similarly susceptible to the cytotoxic effect of both CV extract and MMC with more than 80% growth suppression. However, much lower inhibition by the CV extract was observed in BT-20 cells when compared with MMC.

Apoptosis-inducing effects of CV extract on breast cancer cells. Based on the IC_{50} values ranging from 400 to 600 μg/ml as determined by MTT assay, 400 and 600 μg/ml of the CV extract were chosen and incubated with each of the three cell lines (MDA-MB-231, MCF-7, and T-47D). The cell viability was first evaluated (Fig. 2A). The proportion of dead MDA-MB-231 cells increased sharply (from 2.8 ± 2.2% to 9.5 ± 2.4%) upon 48 h incubation with the CV extract at 600 μg/ml when compared with 16 and 24 h. Meanwhile, the percentage of death of MCF-7 (13.5 ± 9.2%) and

T-47D cells (9.0 ± 1.0%) peaked at 24 and 48 h, respectively, when incubated with the CV extract of same concentration (600 μg/ml). These results implicated that the apoptotic induction of these cell lines should be evaluated at different time points. Figure 2B showed that using cell death detection ELISA, an increase in nucleosome production (apoptotic index, AI) greater than or equal to two-fold was considered significant when compared with the untreated control (AI = 1). There was no significant production of nucleosomes in all the three cell lines treated with the CV extract for 16 h. However, after 24 h, the CV extract at both 400 and 600 μg/ml significantly induced apoptosis of MDA-MB-231 cells in a dose-dependent manner (AI = 2.5 ± 0.2 and 4.5 ± 0.2 respectively). After 48 h, significant apoptosis could only be induced at 600 μg/ml of the CV extract (AI = 3.3 ± 0.7). The nucleosome production was found to slightly decline with increasing period of incubation with the CV extract. For the ER-positive breast tumor cell lines, MCF-7 underwent a sharp increase in apoptosis after 24 h incubation with the CV extract (AI = 4.0 ± 2.1 for 400 μg/ml and 7.3 ± 0.6 for 600 μg/ml), which was higher than those of 48 h incubation. Meanwhile, apoptosis of T-47D cells was significantly induced by the CV extract only at 600 μg/ml after 48 h (AI = 8.6 ± 3.9), with highest increase in nucleosome release when compared with the other two breast tumor cell lines.

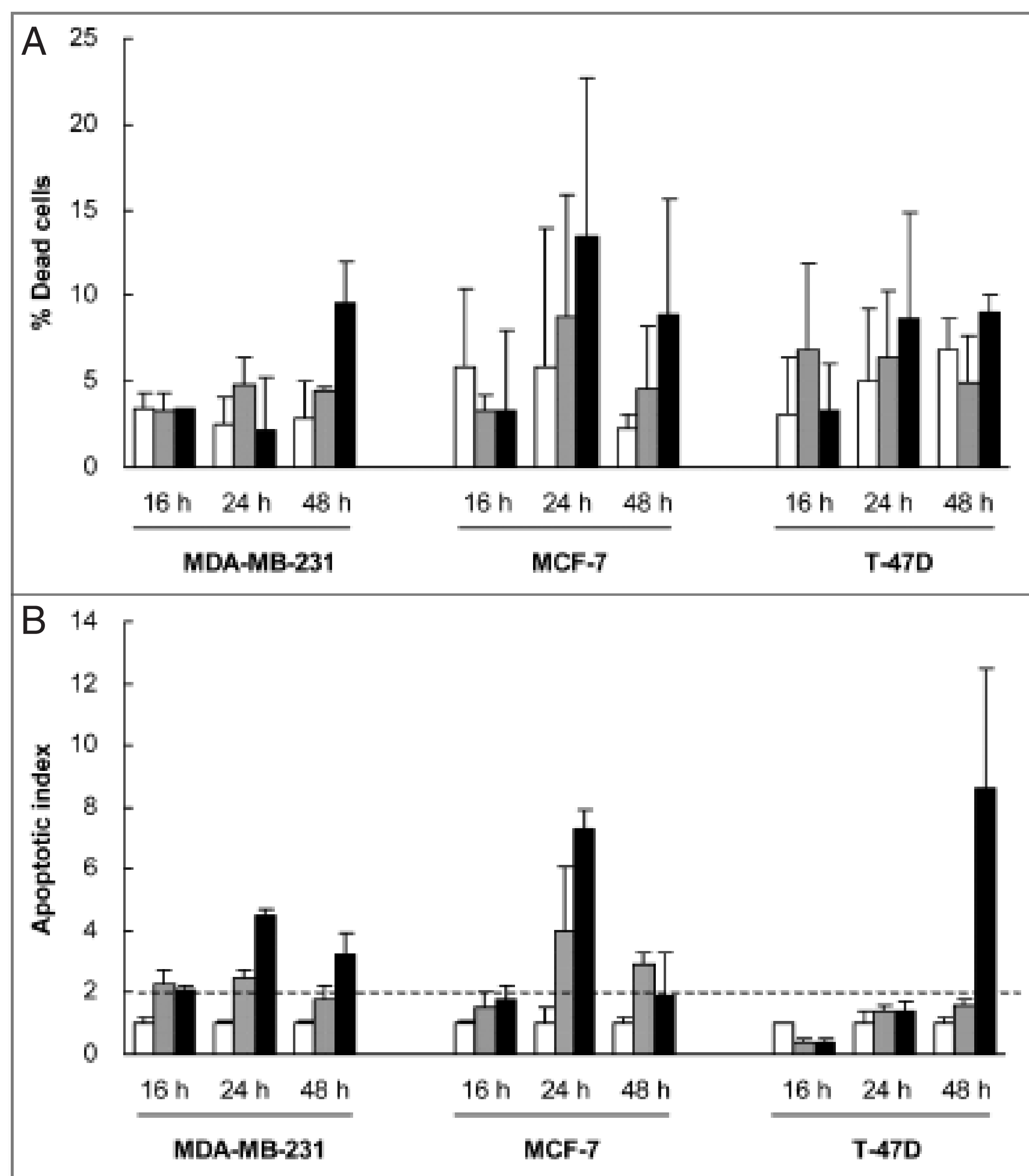

Figure 2. Induction of cell death by CV extract in three breast cancer cell lines. Cells were incubated with the CV extract in culture medium at concentrations derived from IC_{50}, 400 μg/ml (gray bar) and 600 μg/ml (black bar), or culture medium alone (white bar) for 16, 24 and 48 h. (A) Results are expressed as the mean percentage of dead cells ± SD in duplicate experiments with three wells each. Percentage of dead cells was calculated from the ratio of dead cells to total number of cells using trypan blue exclusion test. (B) The induced apoptosis (i.e., internucleosomal DNA fragmentation) was then assessed by cell death detection ELISA. Results are expressed as the apoptotic index (AI, mean ± SD), which was calculated from the ratio of absorbance reading in CV extract-treated well to that of control well in duplicate experiments with three wells each. AI greater than or equal to two (dashed line) was considered significant when compared with the untreated control.

Effects of CV extract on expression of p53 protein. In the study of p53 protein expression, $p53^{wild}$-negative MDA-MB-231 and $p53^{wild}$-positive MCF-7 and T-47D were treated with 400 or 600 μg/ml of the CV extract. Our results indicated that the relative expression of p53 (mostly mutant) in MDA-MB-231 cells was significantly downregulated only after 24 and 48 h of incubation with 600 μg/ml of the CV extract, but no obvious change was found in other conditions (Fig. 3A). For MCF-7 cells, the expression of p53 protein was markedly and transiently enhanced by 3.6-fold only after 16 h of incubation with 400 μg/ml, but not 600 μg/ml, of the CV extract. There was no prominent alteration in p53 expression for 24 h of incubation, but with a significant downregulation for both 400 and 600 μg/ml of the CV extract after 48 h of incubation. On the contrary, the p53 expression in T-47D was significantly increased by a factor of 2.3 with 600 μg/ml of the extract at 24 h, and a factor of 2.3 and 2.8 with 400 and 600 μg/ml at 48 h respectively, reflecting a dose- and time-dependent upregulation of p53 protein.

Effects of CV extract on expression of Bcl-2 protein. Regarding the expression of Bcl-2 protein, the present results demonstrated that there was no significant alteration in the relative Bcl-2 expression of MDA-MB-231 cells, except with a slight upregulation only after 48 h of incubation with 600 μg/ml of the CV extract (1.15-fold, Fig. 3B). For MCF-7 cells, the Bcl-2 protein expression was significantly lowered by 400 μg/ml of the CV extract after 16 h (0.76-fold). There were also 0.77- and 0.73-fold reductions in Bcl-2 level for 400 μg/ml of the extract after 24 h and 48 h respectively, despite the lack of statistical significance. However, the level of Bcl-2 protein was significantly downregulated by 0.42- and 0.46-fold for 600 μg/ml of the

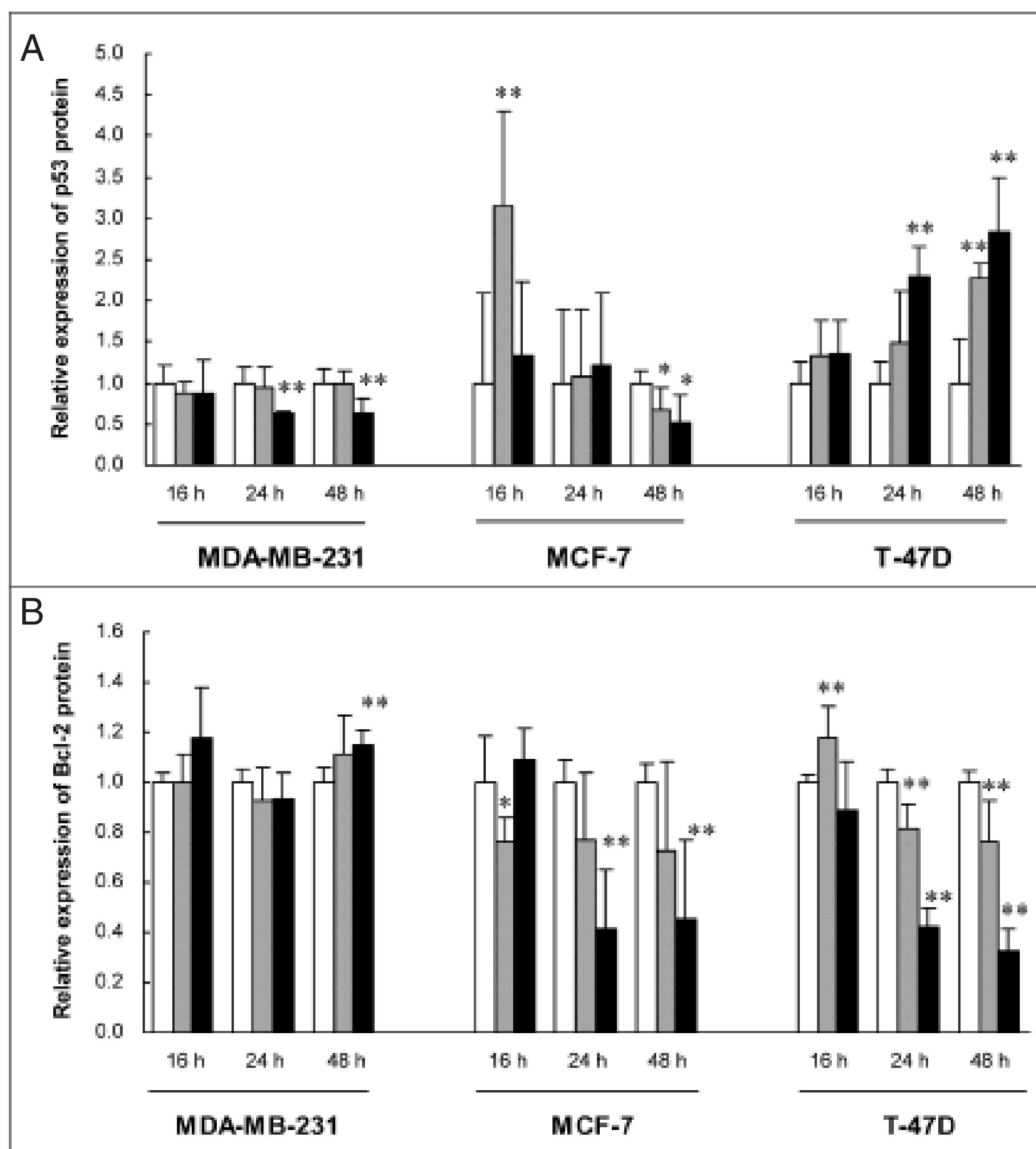

Figure 3. Effects of the CV extract on (A) p53 and (B) Bcl-2 expressions in three breast cancer cell lines. Cells were incubated with the CV extract in culture medium at concentrations derived from IC_{50}, 400 μg/ml (gray bar) and 600 μg/ml (black bar), or culture medium alone (white bar) for 16, 24 and 48 h. The levels of p53 and Bcl-2 protein were measured by p53 pan ELISA and Bcl-2 ELISA kits respectively. Results were expressed as the mean ± SD in duplicate experiments with three wells each, which was calculated from the ratio of absorbance reading in CV extract-treated well to that of control well. Differences between the treated and control wells were determined by Student's unpaired t-test. *$p<0.05$, **$p<0.01$.

CV extract after 24 and 48 h respectively, but not after 16 h of incubation. Expression of Bcl-2 protein in T-47D cells was significantly suppressed by the CV extract after 24 and 48 h (400 μg/ml at 24 h: 0.82-fold; 400 μg/ml at 48 h: 0.76-fold; 600 μg/ml at 24 h: 0.42-fold; 600 μg/ml at 48 h: 0.33-fold), in a dose- and time-dependent manner. Surprisingly, however, the Bcl-2 expression was prominently upregulated with 400 μg/ml at 16 h.

DISCUSSION

Our previous studies have reported that an ethanol-water extract of a widely used Chinese medicinal herb, *Coriolus versicolor* in its natural form possess cytotoxic effects on human leukemia and lymphoma cells, and immunomodulating activities on murine spleen lymphocytes.[27,34] The present findings revealed that the CV extract significantly suppressed the proliferation of human breast cancer cells (MDA-MB-231, MCF-7, T-47D) in a dose-dependent manner in vitro, except BT-20 cells (Fig. 1). Such antiproliferative activity of the CV extract was reflected by the relatively low IC_{50} value (Table 1). The absence of growth suppression in BT-20 cells treated with the CV extract reflected that ER-negative breast tumor cells might be resistant (e.g., BT-20) or less susceptible (e.g., MDA-MB-231, when compared with MCF-7 and T-47D) to the antitumor effect of CV extract. MDA-MB-231 is known as the most malignant among the four chosen cell lines in terms of autocrine growth factor production (e.g., fibroblast growth factor).[35,36] Therefore, discrepancy between the antiproliferative effects of the CV extract and the malignancy of BT-20 and MDA-MB-231 cells might be linked to the estrogen-independent signaling of proliferation (e.g., autocrine loop of growth factor stimulation). Comparing with the commonly used chemotherapeutic anti-tumor drug such as MMC, the CV extract (albeit at higher concentration) induced comparable anti-tumor activity on MDA-MB-231, MCF-7 and T-47D cells (Table 1).

Apoptosis is known as an important type of cell death in response to cytotoxic treatment. The administration of many natural compounds with anti-cancer effect has been shown to be capable of inducing the apoptotic death of cancer cells. Our results illustrated that the CV extract dose-dependently induced apoptosis in MDA-MB-231 cells, MCF-7 cells and T-47D cells in terms of nucleosome production (Fig. 2B). These suggested that the extract exerted its anti-tumor effect on the three breast tumor cells possibly

via an apoptotic mechanism. To our knowledge, there was no previous detailed mechanistic study on apoptosis induction of Yunzhi extract, PSP or PSK against breast cancer cells. The present findings showed that maximal increase in nucleosome production detected by cell death ELISA was observed earlier than MTT-based cytotoxicity in the three breast tumor cell lines. In addition, the results of induced apoptosis were found to be paralleled with those of MTT assays in a descending order (T-47D > MCF-7 > MDA-MB-231).

Genetic alterations resulting in loss of apoptosis or disturbance of apoptosis-signalling pathways are likely to be the crucial components of carcinogenesis. Apoptosis is well known to be modulated by anti-apoptotic and pro-apoptotic effectors, which involve a large number of proteins that act as a rheostat in regulating programmed cell death and as a target of anti-cancer therapy.[37,38] The pivotal role played by aberrant expression of p53 protein in human breast cancers is well studied.[31] About 30–50% of these tumors have a mutant p53 gene, and others have additional alterations in cellular localization of p53 protein.[2,31] MDA-MB-231 is a more malignant breast carcinoma cell line in terms of autocrine growth factor production and constitutive activation of signalling pathways for survival factors.[39] For MDA-MB-231 cells harboring a mutant, non-functional p53 protein, the CV extract suppressed the cell growth by inducing apoptosis, indicating that the growth inhibitory effect was not directly mediated by p53 (Fig. 3A). It is possible that the apoptosis of MDA-MB-231 cells induced by the CV extract was mediated by other pro-apoptotic molecules in a p53-independent manner. On the other hand, ER-positive MCF-7 cells are known to express functional p53 protein.[40] The present findings demonstrated that the CV extract augmented the p53 expression in MCF-7 cells at 16 h, but followed by a significant reduction at 48 h (Fig. 3A). Such transient increase in p53 expression at 16 h might precedently initiate the downstream apoptotic events including DNA fragmentation at 24 and 48 h (Fig. 2B), suggesting that the apoptosis of MCF-7 cells induced by the CV extract might only be partly or transiently regulated by functional p53 protein. On the contrary, the CV extract was found to significantly upregulate the expression of p53 in T-47D cells in a dose- and time-dependent fashion (Fig. 3A), resulting in the maximal apoptosis at 48 h (Fig. 2B). Interestingly, it seems likely that the apoptosis of early-phase breast tumor cell harbouring wild-type p53 (MCF-7, T-47D) was, at least partly, p53-dependent; meanwhile, the apoptosis of late-phase breast tumor cell (MDA-MB-231) was independent of p53 expression. These findings implicated that, as induced by the CV extract, the p53 protein might differentially act as a major upstream transcriptional activator for promoting apoptosis in different types of breast cancer cells.[41]

Bcl-2 is overexpressed in a high percentage of human breast cancer cells.[33,42] A number of molecular studies have found no structural abnormalities of the Bcl-2 locus in breast cancers.[43,44] Therefore, the elevated expression of Bcl-2 protein in breast cancers is probably not the result of a chromosomal translocation or amplification of the gene. Noninvasive or early-stage (mostly ER-positive) breast cancer cells usually have high Bcl-2 expression, while invasive or metastatic (mostly ER-negative) breast cancers often have lower expression of Bcl-2 protein.[45,46] In the present study, the lack of Bcl-2 suppression implicated that the apoptosis of MDA-MB-231 cells induced by the CV extract was probably mediated via a Bcl-2-independent pathway (Fig. 3B). On the other hand, the expressions of Bcl-2 protein in both ER-positive MCF-7 and T-47D cells were significantly suppressed by the CV extract after 24 and 48 h of incubation, which preceded the induction of DNA fragmentation, suggesting that the downregulation of Bcl-2 in turn facilitated the apoptosis induced by the CV extract in vitro. It is well known that p53 acts as a negative transcriptional regulator of Bcl-2 gene in vitro and in vivo.[31,41] Taken together, our results demonstrated that the CV extract might inhibit the growth of ER-negative MDA-MB-231 cells via induction of apoptosis, which was independent of p53 or Bcl-2 status. On the other hand, the CV extract triggered apoptosis of ER-positive MCF-7 and T-47D cells in a p53-dependent manner (partly for MCF-7), followed by a significant downregulation of Bcl-2 protein leading to final cell death.

In conclusion, the present results have suggested that the in vitro anti-proliferative effects of the CV extract on MDA-MB-231, MCF-7 and T-47D cells are mediated through apoptosis induction, which in turn is differentially regulated depending on p53 and Bcl-2 expression. Our findings have implied that CV extract has potential therapeutic value for treatment of breast cancer. In the future, it is worth evaluating the relationships between hormone receptor expression and apoptotic pathways of the breast cancer cells. Since p53 is known to regulate cell cycle arrest, mechanistic studies on cell cycle phase distribution and expressions of cyclins and cyclin-dependent kinases (CDK) and CDK inhibitors (e.g., p21/WAF1) will be conducted to delineate the anti-tumor mechanisms of the CV extract.

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References

1. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun M. Cancer statistics. CA Cancer J Clin 2003; 53:5-26.
2. Elstner E, Williamson EA, Zang C, Fritz J, Heber D, Fenner M, Possinger K, Koeffler HP. Novel therapeutic approach: Ligands for PPARgamma and retinoid receptors induce apoptosis in bcl-2-positive human breast cancer cells. Breast Cancer Res Treat 2002; 74:155-65.
3. Spencer JW. Essential issues in complementary/alternative medicine. In: Spencer JW, Jacobs JJ, eds. Complementary/Alternative Medicine, An Evidence-based Approach. St Louis: Mosby, 1999:3-36.
4. Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. Mushrooms, tumors, and immunity. Proc Soc Exp Biol Med 1999; 221:281-93.
5. Kidd PM. The use of mushroom glucans and proteoglycans in cancer treatment. Altern Med Rev 2000; 5:4-27.
6. Ng TB. A review of research on the protein-bound polysaccharide (polysaccharopeptide, PSP) from the Mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae). Gen Pharmacol 1998; 30:1-4.
7. Ooi VE, Liu F. A review of pharmacological activities of mushroom polysaccharides. Int J Med Mushrooms 1999; 1:195-206.
8. Wasser SP, Weis AL. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: A modern perspective. Crit Rev Immunol 1999; 19:65-96.
9. Yang MM, Chen Z, Kwok JS. The anti-tumor effect of a small polypeptide from *Coriolus versicolor* (SPCV). Am J Chin Med 1992; 20:221-32.
10. Sakagami H, Aoki T, Simpson A, Tanuma S. Induction of immunopotentiation activity by a protein-bound polysaccharide, PSK (review). Anticancer Res 1991; 11:993-9.
11. Chow LW, Lo CS, Loo WT, Hu XC, Sham JS. Polysaccharide peptide mediates apoptosis by upregulating p21 gene and downregulating cyclin *D1* gene. Am J Chin Med 2003; 31:1-9.
12. Hsieh TC, Wu JM. Cell growth and gene modulatory activities of Yunzhi (Windsor Wunxi) from mushroom *Trametes versicolor* in androgen-dependent and androgen-insensitive human prostate cancer cells. Int J Oncol 2001; 18:81-8.
13. Wang HX, Liu WK, Ng TB, Ooi VE, Chang ST. Immunomodulatory and anti-tumor activities of a polysaccharide-peptide complex from a mycelial culture of *Tricholoma sp.*, a local edible mushroom. Life Sci 1995; 57:269-81.
14. Qian ZM, Xu MF, Tang PL. Polysaccharide peptide (PSP) restores immunosuppression induced by cyclophosphamide in rats. Am J Chin Med 1997; 25:27-35.
15. Wang HX, Ng TB, Liu WK, Ooi VE, Chang ST. Polysaccharide-peptide complexes from the cultured mycelia of the mushroom *Coriolus versicolor* and their culture medium activate mouse lymphocytes and macrophages. Int J Biochem Cell Biol 1996; 28:601-7.
16. Dong Y, Kwan CY, Chen ZN, Yang MP. Anti-tumor effects of a refined polysaccharide peptide fraction isolated from *Coriolus versicolor* in vitro and in vivo studies. Res Commun Mol Pathol Pharmacol 1996; 92:140-8.
17. Mickey DD, Carvalho L, Foulkes K. Combined therapeutic effects of conventional agents and an immunomodulator, PSK, on rat prostatic adenocancer. J Urol 1989; 142:1594-8.
18. Tsang KW, Lam CL, Yan C, Mak JC, Ooi GC, Ho JC, Lam B, Man R, Sham JS, Lam WK. *Coriolus versicolor* polysaccharide peptide slows progression of advanced nonsmall cell lung cancer. Respir Med 2003; 97:618-24.
19. Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239-57.

20. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001; 411:342-8.
21. Makin G, Hickman JA. Apoptosis and cancer therapy. Cell Tissue Res 2000; 301:143-52.
22. Sellers WR, Fisher DE. Apoptosis and cancer drug targeting. J Clin Invest 1999; 104:1655-61.
23. Frankfurt OS, Krishan A. Apoptosis enzyme-linked immunosorbent assay distinguishes anticancer drugs from toxic chemicals and predicts drug synergism. Chem Biol Interact 2003; 145:89-99.
24. Johnson JI, Decker S, Zaharevitz D, Rubinstein LV, Venditti JM, Schepartz S, Kalyandrug S, Christian M, Arbuck S, Hollingshead M, Sausville EA. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. Br J Cancer 2001; 84:1424-31.
25. Han B, Toyomasu T, Shinozawa T. Induction of apoptosis by *Coprinus disseminatus* mycelial culture broth extract in human cervical cancer cells. Cell Struct Funct 1999; 24:209-15.
26. 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; 102:250-3.
27. Lau CB, Ho CY, Kim CF, Leung KN, Fung KP, Tse TF, Chan HH, Chow MS. Cytotoxic activities of *Coriolus versicolor* (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis. Life Sci 2004; 75:797-808.
28. Fisher DE. Apoptosis in cancer therapy: Crossing the threshold. Cell 1994; 78:539-42.
29. Falette N, Paperin MP, Treilleux I, Gratadour AC, Peloux N, Mignotte H, Tooke N, Lofman E, Inganas M, Bremond A, Ozturk M, Puisieux A. Prognostic value of *p53* gene mutations in a large series of node-negative breast cancer patients. Cancer Res 1998; 58:1451-5.
30. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res 1999; 59:1391-9.
31. May P, May E. Twenty years of p53 research: Structural and functional aspects of the p53 protein. Oncogene 1999; 18:7621-36.
32. Chan SL, Yu VC. Proteins of the bcl-2 family in apoptosis signalling: From mechanistic insights to therapeutic opportunities. Clin Exp Pharmacol Physiol 2004; 31:119-28.
33. Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol 1999; 17:2941-53.
34. Ho CY, Lau CB, Kim CF, Leung KN, Fung KP, Tse TF, Chan HH, Chow MS. Differential effect of *Coriolus versicolor* (Yunzhi) extract on cytokine production by murine lymphocytes in vitro. Int Immunopharmacol 2004; 4:1549-57.
35. Angus WG, Larsen MC, Jefcoate CR. Expression of CYP1A1 and CYP1B1 depends on cell-specific factors in human breast cancer cell lines: Role of estrogen receptor status. Carcinogenesis 1999; 20:947-55.
36. El Yazidi I, Renaud F, Laurent M, Courtois Y, Boilly-Marer Y. Production and oestrogen regulation of FGF1 in normal and cancer breast cells. Biochim Biophys Acta 1998; 1403:127-40.
37. Cuello MA, Nau M, Lipkowitz S. Apoptosis and the treatment of breast cancer. Breast Dis 2002; 15:71-82.
38. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. Ann Rev Biochem 2000; 69:217-45.
39. Toillon RA, Chopin V, Jouy N, Fauquette W, Boilly B, Le Bourhis X. Normal breast epithelial cells induce p53-dependent apoptosis and p53-independent cell cycle arrest of breast cancer cells. Breast Cancer Res Treat 2002; 71:269-80.
40. Xu J, Loo G. Different effects of genistein on molecular markers related to apoptosis in two phenotypically dissimilar breast cancer cell lines. J Cell Biochem 2001; 82:78-88.
41. Zhivotovsky B, Kroemer G. Apoptosis and genomic instability. Nat Rev Mol Cell Biol 2004; 5:752-62.
42. Duenas-Gonzalez A, Abad-Hernandez MM, Cruz-Hernandez JJ, Gonzalez-Sarmiento R. Analysis of bcl-2 in sporadic breast carcinoma. Cancer 1997; 80:2100-8.
43. Siziopikou KP, Prioleau JE, Harris JR, Schnitt SJ. Bcl-2 expression in the spectrum of preinvasive breast lesions. Cancer 1996; 77:499-506.
44. Leek RD, Kaklamanis L, Pezzella F, Gatter KC, Harris AL. Bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and in situ cancer. Br J Cancer 1994; 69:135-9.
45. Kobayashi S, Iwase H, Ito Y, Yamashita H, Iwata H, Yamashita T, Ito K, Toyama T, Nakamura T, Masaoka A. Clinical significance of *bcl-2* gene expression in human breast cancer tissues. Breast Cancer Res Treat 1997; 42:173-81.
46. van Slooten HJ, van de Vijve MJ, van de Velde CJ, van Dierendonck JH. Loss of Bcl-2 in invasive breast cancer is associated with high rates of cell death, but also with increased proliferative activity. Br J Cancer 1998; 77:789-96.

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