*Full Paper***Combined Treatment of Ionizing Radiation With Genistein on Cervical Cancer HeLa Cells**Bei Zhang¹, Jia-yin Liu^{1,*}, Jin-shun Pan², Su-ping Han¹, Xiao-xing Yin², Bing Wang², and Gang Hu²¹*Department of Obstetric and Gynecology, the First Affiliated Hospital, Nanjing Medical University, Nanjing 210029, China*²*Department of Pharmacology, Nanjing Medical University, Nanjing 210029, China**Received February 6, 2006; Accepted July 26, 2006*

Abstract. The anticancer agent genistein inhibits cell growth of tumor cell lines from various malignancies. In our study, we investigated the effectiveness of combined treatment of ionizing radiation (IR) with genistein on cervical HeLa cells and its possible mechanism. It was found that the inhibitory rate in cells with combined treatment was significantly higher than that of the cells treated with IR or genistein alone. After treatments of IR (4 Gy) combined with genistein (40 μ mol/L), the apoptotic index of the cells was significantly increased and the cells were arrested in the G2/M phase. Survivin mRNA expression increased after IR (4 Gy), while it significantly decreased after combined treatment. These findings indicated that genistein enhanced the radiosensitivity of cervical cancer HeLa cells, and the mechanisms for this action might include increase of apoptosis, decrease of survivin expression, and prolongation of cell cycle arrest.

Keywords: genistein, ionizing radiation, survivin, apoptosis, cell cycle

Introduction

Cervical cancer remains the second most common cause of cancer-related death in women world wide. More than 370,000 cases are diagnosed each year, resulting in nearly a quarter of a million deaths (1, 2). Radiotherapy has long played an important role in the treatment of uterine cervical cancer. It is the most effective therapy for the advanced stage of cervical cancer. It is as equally effective as radical surgery in treating early tumors and reduces local recurrences after surgery for patients with high-risk features. For patients with locally advanced cervical carcinoma, radiotherapy results in 70% of total progression-free survival rates. Findings show that this treatment alone is insufficient to prevent progression of disease in some patients. Resistance of cancer to radiotherapy is one of the reasons for treatment failure. It is well known that the response of a tumor to ionizing radiation varies widely, which may be explained by development of

radioresistant tumor cells. One cellular mechanism for resistance development common to various therapeutic regimens, including radiation, is the up-regulation of survivin expression in tumor cells.

Survivin is the smallest member of the mammalian inhibitor of apoptosis family. It inhibits caspase 9 and blocks the apoptotic pathway (3). Survivin is absent in most terminally differentiated normal tissues, but highly expressed in malignancies (4). Its expression is up-regulated in all phases of the cell cycle, and the cancer-specific activity of survivin promoter was detected both in vivo and in vitro (5). p53 is one of the regulators, which transcriptionally down-regulates survivin through binding a bipartite p53-responsive element in the survivin promoter (6). Clinically, high levels of survivin have been associated with decreased overall survival, increased recurrences, and resistance to therapy (4, 7).

Genistein, a major metabolite of soy produced by the intestinal bacteria, is one of the anticancer agents found in soybean (8, 9). There are many possible mechanisms for its anti-tumor activities. Accumulation of genistein in the tissue would play an important role in its bio-activity. Genistein distributes to various organs after

*Corresponding author. jyliu@njmu.edu.cn

Published online in J-STAGE

doi: 10.1254/jphs.FP0060165

absorption in vivo, and its concentrations are relatively high in liver and reproductive organs (10). Genistein has been shown to inhibit the growth of tumor cell lines from various malignancies including breast, lung, melanoma, prostate, and lymphoma (11–15).

The aim of this study was to investigate whether genistein could increase the radiosensitivity of cervical cancer cells. The results showed that survivin expression was up-regulated by radiation alone, but down-regulated by combined treatment with genistein that enhanced the sensitivity of HeLa cells to IR.

Materials and Methods

Cells and chemicals

The HeLa cell line was obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (SIBS CAS, Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NJ, USA). Each medium was also supplemented with 10% heat inactivated fetal calf serum (Hyclone, Logan, UT, USA), penicillin (100 iu/ml, and streptomycin (100 µg/ml) (Sigma, St. Louis, MO, USA). Genistein (4,5,7-trihydroxyisoflavone), DMSO, and MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were all purchased from Sigma Chemical Co. Genistein was dissolved in DMSO as a 100 mmol/L stock. MTT was dissolved in water at a concentration of 10 mg/ml.

Treatments of cells

HeLa cells were plated into 96-well culture plates (10^4 cells/well). To investigate the effect of IR treatment, cells were exposed to ^{60}Co γ -irradiation from a telecobalt therapy source at a dose rate of 1.28 Gy/min with a ^{60}Co apparatus (Theratron 780C; Theratronics, Ottawa, Canada). For genistein treatment alone, cells were treated with different concentrations of genistein (0, 20, 40, and 100 µmol/L) for 24, 48, 72, and 96 h, respectively. For IR alone, cells were treated with different doses of radiation (0–8 Gy) for 24, 48, 72, and 96 h, respectively. In order to study the effect of IR combined with genistein, cells were incubated with various concentrations of genistein (0, 20, 40, and 100 µmol/L) for 48 h and then treated with IR (4 Gy).

Proliferation assays

Proliferation of HeLa cells was measured by MTT assay. After HeLa cells were treated with different concentrations of genistein for the indicated time as described above, 10 µl of MTT (5 mg/ml) was added to each well and incubated for an additional 4 h, and then the liquid in the wells was evaporated. To dissolve

the formazan, 200 µl of DMSO was added. Control wells were treated with 1 g/L DMSO alone. The absorbance was detected at 490 nm wavelength in a microplate reader (CliniBio 128C; ASYS Hitech GmbH, Eugendorf, Austria). Growth inhibitory rate was calculated by the following formula: $(1 - \text{absorbance of the treated wells}) / (\text{absorbance of the control wells}) \times 100\%$.

Cell cycle analysis

Cells were harvested by trypsinization after the different treatments, and then they were fixed with 70% ethanol and stored at 4°C. After 48 h, cells were resuspended in 1 ml of lysis buffer (0.1% Triton X-100, 0.1% RNase A) and incubated at 4°C. After that, 1 ml of 50 µg/ml propidium iodide (PI; final concentration, 25 µg/ml) was added to the sample. After 30 min of incubation with PI in the dark at room temperature, the PI fluorescence of the nuclei was measured by flow-cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA). Ten thousand cells were acquired and the proportions of cells in each cycle phase were calculated using ModFit LT program (Becton Dickinson). Apoptotic cells were recognized in an area less than the G1 DNA content. The percentage of apoptotic cells was described as the apoptotic index (AI).

Quantification of survivin mRNA by real-time PCR

Survivin mRNA expression was detected by real-time quantitative reverse transcriptional polymerase chain reaction (RT-PCR). HeLa cells were divided into the following groups: control group (no treatment); IR group (cells treated with 4 Gy of IR alone); genistein group (cells incubated with genistein (20, 40, or 100 µmol/L for 48 h); combination group (cells incubated with 40 µmol/L of genistein for 48 h and then treated with 4 Gy of IR). Total RNA was isolated from HeLa cells with the Trizol reagent (Gibco BRL) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed to cDNA using SurperScript II reverse transcriptase (Invitrogen Carlsbad, CA, USA). cDNA was then amplified with the fluorescent Taqman probe (6-FAM TGA CGA CCC CAT AGA GGA ACA TAA AAA GCA T). Primers for survivin (forward: TGG AAG GCT GGG AGC CA and reverse: GAA AGC GCA ACC GGA CG) were designed using Primer Express1.0 (Perkin Elmer, Foster City, CA, USA) and synthesized by Shanghai GeneCore Bio Technologies Co., Ltd. (Shanghai, China). Amplification was performed in 20 µl of TaqMan Universal PCR Master Mix (Applied Biosystems, Roche, Branchburg, NJ, USA) including 1 µl TaqMan probes, 2 µl of cDNA, and forward and reverse primers. The real-time PCR was

performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by a total of 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. Negative controls with no template were performed for each reaction. The relative quantity of gene expression was calculated according to the manufacturer's recommendations. Ct represented the threshold cycle number as defined by the software when the PCR reaction reached the start of its exponential stage. This value was logarithmically and conversely related to the input copies of the transcript of interest. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) was used as an internal control to calculate the relative abundance of this gene. The relative gene expression levels were obtained by the following formula: Ct value of GAPDH/Ct value of target gene.

Western blot analysis

Cells were homogenized in a lysis buffer containing 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 5 mmol/L EDTA, 0.1% Triton X-100, 5% glycerol, and 1% sodium dodecyl sulfate (SDS). Protein concentrations were determined with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts (30 µg/lane) of proteins were resolved by 12% SDS-PAGE (polyacrylamide gel electrophoresis gels) and electrotransferred to PVDF membranes. After blocking 1 h in 5% milk solution, the membranes were incubated with anti-human survivin primary antibody and anti-human cyclin B (R&D Systems, Minneapolis, MN, USA), respectively. After washing with phosphate-buffered saline (PBS), the membranes were incubated with peroxidase-conjugated rabbit anti-goat secondary antibody (Boster Biotechnology Co. Ltd, Wuhan, China). β -Actin (Sigma) was used to normalize the protein loading. The immunoreactivity was detected with the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

Data were expressed as means $\pm$ S.D. from at least three different replicates. One way ANOVA was used to determine the statistical difference between various experimental and control groups. $P < 0.05$ was considered significant.

Results

Growth inhibition of HeLa cells

Cells were incubated with genistein at doses of 0, 20, 40, and 100 µmol/L for 24, 48, 72, and 96 h, respec-

Fig. 1. Inhibitory rates of HeLa cells were detected by MTT assay after treatments with genistein alone (A), IR (B) alone, and IR + genistein (C). Data are shown as means $\pm$ S.D., $n = 3$. * $P < 0.05$, ** $P < 0.01$, vs 4 Gy of IR alone.

tively. The proliferation of cells was inhibited at a dose- and time-dependent manner, as shown in Fig. 1A. The inhibitory rates of the cells after the different doses of irradiation (Fig. 1B) were as follows: 4 Gy of irradiation induced an inhibitory rate of 22.1% and 5 Gy of irradiation induced an inhibitory rate of 56.3% at 48 h after

Table 1. Cell cycle distribution of HeLa cells exposed to genistein (40 $\mu\text{mol/L}$), IR (4 Gy), or their combination for 48 h

	Control (%)	Genistein (%)	IR (%)	IR + Genistein (%)
G1	40.3 $\pm$ 4.3	13.6 $\pm$ 4.7	28.7 $\pm$ 3.0	5.5 $\pm$ 2.4
S	41.1 $\pm$ 2.6	62.9 $\pm$ 1.5**	20.8 $\pm$ 1.3	18.6 $\pm$ 1.1
G2/M	18.6 $\pm$ 1.9	23.5 $\pm$ 2.2	50.5 $\pm$ 2.5**	76.9 $\pm$ 3.9** ^{##}

Results are expressed as percentage of cells in each phase of the cell cycle. Data are shown as means $\pm$ S.D., n = 3.

** P <0.01, vs control. ^{##} P <0.01, vs IR alone.

treatments. So we selected 4 Gy of IR to combine with genistein treatment of the cells. HeLa cells were incubated with different concentrations of genistein (0, 20, 40, and 100 $\mu\text{mol/L}$) for 48 h and then treated with 4 Gy of IR (Fig. 1C). MTT assay showed that proliferation inhibitory rates of cells with combined treatment were significantly higher than those with IR alone (P <0.01). 4 Gy of IR combined with 40 $\mu\text{mol/L}$ genistein induced an additive inhibitory effect in the HeLa cells (57.3% vs 22.1% + 25.7%).

Cell cycle arrest and apoptosis

After IR, the percentage of cells in the G1 and S phases was gradually decreased and that in the G2/M phase significantly increased after 48 h. The cells incubated with genistein alone showed a typical cell cycle arrested at the S phase, but after combined treatment, the majority of cells appeared to be arrested at the G2/M phase. After the cells were treated with IR alone, genistein alone, and 4 Gy of IR combined with 40 $\mu\text{mol/L}$ of genistein, the percentages of cells in the G2/M phase were 50.5 $\pm$ 2.5%, 23.5 $\pm$ 2.2%, and

76.9 $\pm$ 3.9%, respectively. The percentage of cells the G2/M phase in cells treated with IR plus genistein was significantly higher than those with IR or genistein alone (Table 1, P <0.01).

Figure 2 showed that combined treatment of IR (4 Gy) with genistein (40 $\mu\text{mol/L}$) induced a sharp increase in apoptosis at 48 h after treatments, and there was a much slower increase in apoptosis between 48–96 h. The

Fig. 2. The apoptotic index of HeLa cells was determined by flowcytometry. The cells were incubated with 40 $\mu\text{mol/L}$ genistein for 48 h, 4 Gy of IR, or 40 $\mu\text{mol/L}$ genistein for 48 h and then combined with 4 Gy of IR. Data are shown as means $\pm$ S.D., n = 3. * P <0.05, ** P <0.01, vs 4 Gy of IR alone.

Fig. 3. Survivin expression of HeLa cells was detected by RT-PCR with different treatments. A: The cells were treated with different doses of genistein. B: The cells were subjected to no treatment (control), 4 Gy of irradiation (IR), 40 $\mu\text{mol/L}$ genistein (G) for 48 h, or 40 $\mu\text{mol/L}$ genistein for 48 h plus 4 Gy of irradiation (IR + G). Data are shown as means $\pm$ S.D., n = 3. ** P <0.01, vs control. ^{##} P <0.01, vs 4 Gy of IR.

apoptotic rates of the combined treatments were significantly higher than those by IR alone or genistein alone ($P<0.01$).

Survivin mRNA expression

Figure 3A shows that compared with untreated cells, survivin mRNA abundance of HeLa cells significantly decreased when the cells were incubated with 40 and 100 $\mu\text{mol/L}$ genistein for 48 h ($P<0.01$). IR (4 Gy) markedly increased survivin mRNA expression of HeLa cells ($P<0.01$). However, 4 Gy of IR combined with 40 $\mu\text{mol/L}$ of genistein significantly decreased its expression ($P<0.01$).

Protein contents

Western blotting showed that when compared with untreated controls, IR (4 Gy) markedly increased sur-

vivin protein content ($P<0.01$), while it did not alter cyclin B protein level ($P>0.05$). However, combined treatment with genistein significantly decreased cyclin B and survivin protein contents (Fig. 4), supporting the results that combined treatment induced the accumulation of HeLa cells in the G2/M phase of the cell cycle.

Discussion

Radiation has been widely used in the treatment of cervical cancer. Unfortunately, the cause of this radiosensitization has not been fully understood, especially in adenocarcinoma of the cervix, which has distinct molecular characteristics, with increased incidence during the past decades. The 5-year survival rate for patients with adenocarcinoma has been reported to be 10%–30% lower than that of squamous cell carcinoma at every FIGO (International Federation of Gynecology and Obstetrics) stage. New approaches that may reduce its side-effects and provide good quality of life are required. Thus, it is imperative to develop novel and effective treatments for cervical cancer.

Populations who consume a diet high in soy have lower incidences of some cancers (14, 16, 17). The activity of the soy-derived phytoestrogen (genistein) is thought to account for a portion of the chemopreventative properties of soy. These include an inhibitory effect on tyrosine kinases (18), DNA topoisomerases I and II (19), antiestrogenicity (20) and antioxidant activity (21, 22), suppression of cell proliferation (23), anti-angiogenesis activity (24), and modulation of apoptosis (25); all these factors are related to preventing cancer formation and development.

The rationale for combined treatment of genistein with radiation is to expose the cells to two cytotoxic modalities that may act differently in the molecular pathways, leading to tumor cell death to a greater extent (26). In this study we selected the cervical cancer HeLa cell, an adenocarcinoma cell line, to investigate the effect of combined treatment of IR with genistein. In the MTT assay, the cell growth inhibition of cervical HeLa cancer cells treated with genistein was time- and dose-dependent. This suggested that genistein was able to inhibit the growth of HeLa cells. It was found that 4 Gy of IR combined with different doses of genistein could greatly inhibit the cell growth and give additive effects.

Treatment with 4 Gy of IR combined with 40 $\mu\text{mol/L}$ genistein induced the maximum apoptotic rate (data not shown), indicating that combined treatment could enhance tumor cell apoptosis and reduce the therapeutic dose of IR, and therefore, minimize its possible adverse reactions. These suggested that genistein could enhance the sensitivity of HeLa cells to IR.

Fig. 4. Western blot analysis for survivin protein and cyclin B protein in HeLa cells subjected to no treatment (control), 40 $\mu\text{mol/L}$ genistein for 48 h (G), 4 Gy of radiation alone (IR), or 40 $\mu\text{mol/L}$ genistein for 48 h plus radiation 4 Gy of IR (G + IR). A: The survivin and cyclin-B protein samples obtained from HeLa cells were analyzed by Western blotting. B: Relative quantities of survivin and cyclin-B protein were determined by densitometry. Data are presented as the mean $\pm$ S.D. * $P<0.05$, ** $P<0.01$, vs no treatment in survivin protein. # $P<0.05$, ## $P<0.01$ vs no treatment in cyclin-B protein.

Survivin is a member of the inhibitor of apoptosis protein family. Survivin expression has not been reported in differentiated normal tissues, but it has been observed in many cancerous tissues. Its expression in tumors is implicated in the resistance to a variety of apoptotic stimuli, including chemotherapy and radiotherapy (27). Considering that apoptosis is the primary type of cell death induced by a variety of anticancer agents and IR, a potential key role of survivin in the determinant of the chemo- and radio-sensitivity in tumor cells has been demonstrated (4, 28). Asanuma et al. (28) reported that survivin acted as a constitutive radio-resistance factor in pancreatic cancer cells. Specifically, in a panel of established cell lines, they found an inverse relationship between survivin mRNA abundance and in vitro sensitivity to irradiation. They also demonstrated that survivin mRNA abundance was increased by sublethal doses of irradiation, which indicates that survivin may act as an inducible radio-resistance factor. Overall, the results from the literature have proven that survivin is a cellular factor involved in the chemo- and radio-resistance of human tumor cells. Therefore, approaches designed to inhibit survivin expression could induce human tumor sensitization to chemical and physical agents. Many approaches to counteract survivin in tumor cells have been proposed with the dual aims to inhibit tumor growth through an increase in spontaneous apoptosis and to enhance tumor cell response to apoptosis-inducing agents (4). Our experiments showed that 4 Gy of IR induced only 22.1% and 38% of cell proliferation inhibitory rates at 48 and 96 h, respectively, accompanied by up-regulation of survivin expression, suggesting that HeLa cells were resistant to irradiation. Meanwhile, we also found that genistein alone inhibited survivin expression and combined treatment could induce further survivin suppression and increased the AI of HeLa cells.

In this study, irradiated cells were arrested at the G2 and sub-G1 phase 48 h after the treatment, suggesting that these cells could undergo nuclear fragmentation, while genistein treatment obviously kept cells arrested in the S-phase, with a slightly increased G2-phase fraction. Cell death and cell cycle progression induced by radiation alone differed from those observed in the combined treatment. Taken together, our results suggested that genistein augments AI after IR by its ability to keep more cells arrested in the G2-phase. This study showed that genistein induced S phase arrest partially via inhibiting cyclin B expression. Survivin is regulated in a highly cell-cycle-dependent manner, with a marked increase in the G2/M phase (29). During this phase, survivin is phosphorylated by p34cdc2/cyclin B1 kinase (30). It has been reported that proliferation of prostate

cancer cells is inhibited by genistein in several mechanisms involving cell cycle arrest caused by down-regulation of cyclin B (15, 31, 32) and apoptosis induced by activation of caspase-3 (33). This study further demonstrated that genistein could reverse the increased expression of survivin induced by irradiation, and then, could enhance apoptosis and have an additive effect on the inhibitory rate in HeLa cells. Therefore, it could be assumed that the inhibition of survivin expression by genistein might be due to its down-regulation of cyclin B. Compared to other approaches used to down-regulate survivin, including AS oligonucleotides, ribozymes, dominant-negative mutants, and cancer vaccine (34–38), genistein is a better potential candidate for clinical use as it is a component of soybean and has an established profile of safety.

In summary, our results have shown that survivin is up-regulated by low dose of irradiation in HeLa cells. IR plus genistein greatly inhibits HeLa cell growth and induces more apoptosis by keeping the cells arrested in the G2/M phase and by inhibiting survivin expression. This study suggests that combination treatment could induce an additive effect and enhance the radio-sensitivity of cervical cancer HeLa cells. Additional studies are ongoing to further validate survivin as a target of genistein for radiation sensitization in animal models of cervical cancer.

References

- 1 Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics 2001. *CA Cancer J Clin.* 2001;51:15–36.
- 2 Brinton LA. Epidemiology of cervical cancer – overview. Lyon: IARC Sci Pub; 1992. p. 3–23.
- 3 Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell.* 2002;9:459–470.
- 4 Altieri DC. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer.* 2003;3:46–54.
- 5 Bao R, Connolly DC, Murphy M, Green J, Weinstein JK, Pisarcik DA, et al. Activation of cancer-specific gene expression by the survivin promoter. *J Natl Cancer Inst.* 2002;94:522–528.
- 6 Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem.* 2002;277:3247–3257.
- 7 Rodel F, Hoffmann J, Distel L, Herrmann M, Noisternig T, Papadopoulos T, et al. Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res.* 2005;65:4881–4887.
- 8 Suzuki K, Koike H, Matsui H, Ono Y, Hasumi M, Nakazato H, et al. Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3. *Int J Cancer.* 2002;99:846–852.
- 9 Ravindranath MH, Muthugounder S, Presser N, Viswanathan S. Anticancer therapeutic potential of soy isoflavone, genistein. *Adv Exp Med Biol.* 2004;546:121–165.
- 10 Coldham NG, Sauer MJ. Pharmacokinetics of [(14)C] genistein

- in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicol Appl Pharmacol.* 2000;164:206–215.
- 11 Wei H, Bowen R, Cai Q, Branes S, Wang Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. *Proc Soc Exp Biol Med.* 1995;208:124–129.
  - 12 Shao ZM, Wu J, Shen ZZ, Barsky SH. Genistein exerts multiple suppressive effects on human breast carcinoma cells. *Cancer Res.* 1998;58:4851–4857.
  - 13 Davis JN, Sigh B, Bhuiyan M, Sarkar FH. Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutr Cancer.* 1998;32:123–131.
  - 14 Yan C, Han R. Genistein suppresses adhesion induced protein tyrosine phosphorylation and invasion of B16-BL6 melanoma cells. *Cancer Lett.* 1998;129:117–124.
  - 15 Arai N, Strom A, Rafter JJ, Gustafsson JA. Estrogen receptor beta mRNA in colon cancer cells: growth effects of estrogen and genistein. *Biochem Biophys Res.* 2000;270:425–431.
  - 16 Giovannucci E. Epidemiologic characteristics of prostate cancer. *Cancer.* 1995;75:1766–1777.
  - 17 Tham DM. Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab.* 1998;83:2223–2235.
  - 18 Akiyama T, Ogawara H. Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* 1991; 201:362–370.
  - 19 Markovits J, Larsen AK, Segal-Bendirdjian E, Fosse P, Sancier TM, Gazit A, et al. Inhibition of DNA topoisomerases I and II and induction of apoptosis by erbstatin and tyrphostin derivatives. *Biochem Pharmacol.* 1994;48:549–560.
  - 20 Cassidy A, Bingham S, Setchell KD. Biological effect of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr.* 1994;60:333–340.
  - 21 Wei H, Wei L, Frenkel K, Bowen R, Barnes S. Inhibition of tumorpromoter-induced hydrogen peroxide formation in vitro and in vivo by genistein. *Nutr Cancer.* 1993;20:1–12.
  - 22 Messina M, Barnes S, Setchell KD. Phyto-oestrogens and breast cancer. *Lancet.* 1997;350:971–972.
  - 23 Yanagihara K, Ito A, Toge T, Numoto M. Antiproliferative effect of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res.* 1993;53:5815–5821.
  - 24 Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, et al. Genistein, a dietary-derived inhibitor of in vivo angiogenesis. *Proc Natl Acad Sci U S A.* 1993;90:2690–2694.
  - 25 Sephens FO. Phytoestrogens and prostate cancer: possible preventive role. *Med J Australia* 1997;167:138–140.
  - 26 Hillman GG, Forman JD, Kucuk O, Yudelev M, Maughan RL, Rubio J, et al. Genistein potentiates the radiation effect on prostate carcinoma cells. *Clin Cancer Res.* 2001;7:382–390.
  - 27 Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem.* 1998;273:11177–11182.
  - 28 Asanuma K, Moriai R, Yajima T, Yagihashi A, Yamada M, Kobayashi D, et al. Survivin as a radio-resistance factor in pancreatic cancer. *Jpn J Cancer Res.* 2000;91:1204–1209.
  - 29 Li F, Altieri DC. The cancer anti-apoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. *Cancer Res.* 1999;59: 3143–3151.
  - 30 O'Connor DS, Grossman D, Plescia J, Li F, Zhang H, Villa A, et al. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc Natl Acad Sci U S A.* 2000;97: 12103–12107.
  - 31 Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. Flavonoids suppress androgen-independent human prostate tumor proliferation. *Nutr Cancer.* 2000;38:116–122.
  - 32 Shen JC, Klein RD, Wei Q, Guan Y, Contois JH, Wang TT, et al. Low-dose genistein induces cyclin-dependent kinase inhibitors and G(1) cell-cycle arrest in human prostate cancer cells. *Mol Carcinog.* 2000;29:92–102.
  - 33 Kumi-Diaka J, Sanderson NA, Hall A. The mediating role of caspase-3 protease in the intracellular mechanism of genistein-induced apoptosis in human prostatic carcinoma cell lines, DU145 and LNCaP. *Biol Cell.* 2000;92:595–604.
  - 34 Pennati M, Binda M, Colella G, Folini M, Citti L, Villa R, et al. Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression. *J Invest Dermatol.* 2003;120:648–654.
  - 35 Asanuma K, Kobayashi D, Furuya D, Tsuji N, Yagihashi A, Watanabe N. A role for survivin in radioresistance of pancreatic cancer cells. *Jpn J Cancer Res.* 2002;93:1057–1062.
  - 36 Rohayem J, Diestelkoetter P, Weigle B, Oehmichen A, Schmitz M, Mehlhom J, et al. Antibody response to the tumor-associated inhibitor of apoptosis protein survivin in cancer patients. *Cancer Res.* 2000;60:1815–1817.
  - 37 Andersen MH, Pedersen LO, Capeller B, Brocker EB, Becker JC, thor Straten P. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. *Cancer Res.* 2001;61:5964–5968.
  - 38 Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, et al. Generation of survivin-specific CD8+ T effector cells by dendritic cells pulsed with protein or selected peptides. *Cancer Res.* 2000;60:4845–4849.