Chinese-German Journal of Clinical Oncology July 2009, Vol. 8, No. 7, P420–P425
DOI 10.1007/s10330-009-0066-y

Role of survivin in apoptosis induced by grape seed procyanidin extract in human bladder cancer BIU87 cells

Jie Liu[1], Qianyuan Zhuang[1], Weiyi Zhang[2], Yonghua Wang[1], Xianguo Chen[1], Ejun Peng[1], Fei Liu[1]

[1] Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[2] Institute of Integrated Traditional Chinese Medicine and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Received: 9 April 2009 / Revised: 5 May 2009 / Accepted: 25 May 2009

Abstract ***Objects:*** The aim of this study was to research the effect of grape seed procyanidin extract (GSPE) on cell apoptosis in human bladder cancer BIU87 cells and investigate its molecular mechanism. ***Methods:*** BIU87 cells were treated with different concentrations of GSPE and cultured for 24 h *in vitro* while untreated group as control, MTT[3- (4,5-dimethylthiazole-2-yl) -2, 5-diphenyltetrazolium bromide] assay, Hoechst 33258 stainning, flow cytometry, RT-PCR and Western blot were used to detect the apoptotic induction effect of GSPE on BIU87 cells. ***Results:*** We found that GSPE induced cell apoptosis in BIU87 cells by a dose-dependent manner. Semiquantitated RT-PCR and Western blot analyses indicated that GSPE increased the expression of caspase-3 and decreased the expression of survivin ($P < 0.01$). ***Conclusion:*** GSPE induces apoptosis in BIU87 cells *in vitro*, and the effect maybe related with its down-regulation of survivin.

Key words apoptosis; bladder cancer; grape seed procyanidin extract (GSPE); survivin

Bladder cancer is the most common malignant tumor in urinary system, and the sixth most common malignancies in developed countries, where most bladder tumors are transitional cell carcinomas (TCCs) [1]. Approximately 75% of uroepithelial TCCs are superficial (Tis, Ta, and T1), most of the superficial cases are treated by transurethral removal of the tumor conservatively, followed by adjuvant intravesical therapy without removal of the bladder [2]. As a main therapeutic regimen, adjuvant intravesical therapy is used extensive on patients with superficial urothelial carcinoma of the bladder in clinic to treat tumor and preclude the recurrence of bladder cancer, but the toxicities, set effects and drug resistances depress the therapic effect of the drugs [3–5]. Of the superficial tumors, 50%–70% recur one or more times, but do not progress into invasive disease, and 10%–30% progress to invasive and potentially lethal disease after transurethral resection [2]. Thus, to decrease recurrence and progress of bladder cancer, it is critical for urologic workers to find a new and safe drug agent for intravesical therapy.

Procyanidins are a class of polyphenolic compounds which distributed in plants extensively, and derived from common dietary foods (fruits, beans and chocolate, etc.) and beverages (fruit juices, wine and tea, etc.) [6–10]. Grape seeds are a particularly rich source of procyanidins, and the procyanidins are typical from the other derives [11]. Grape seed procyanidin extract is mixture of several polyphenols mostly contain dimers, trimers, tetramers and other oligomers of (+)-catechin and (-)-epicatechin, mainly oligomers [12, 13]. Recent cell culture studies have shown that treatment of human oral squamous cell cancer CAL27 and SCC25, human prostate cancer DU145 and LNCaP, human colorectal cancer HT29, LoVo and CaCo2 cells with grape seed procyanidin extract results in an induction of cell apoptosis [14–20]. Lost of the fine balance between cell proliferation and apoptotic death contributes to increase in cellular mass and tumor progression. The agent, however, is not be reported in bladder cancer cells. Thus, the aim of this study was to demonstrate pro-apoptotic effect of grape seed procyanidin extract (GSPE) in human bladder cancer cell line BIU87, and delineate the mechanism of this effect.

Meterials and methods

Cell and reagents

Human bladder cancer cell line BIU87 was obtained from Institute of Urology, Peking University. GSPE (pu-

Correspondence to: Qianyuan Zhuang. Email:qyzhuang@tjh.tjmu.edu.cn

rity ≥ 95%, Nanjing Qingze Herbal Technology Development Co. Ltd, China) was dissolved in RPMI 1640 (without fetal bovine serum) as a 10 mg/mL stock solution and diluted by RPMI 1640 as desired concentrations, stored at –20 ℃ in light-tight containers until used. Fetal bovine serum (Gibco, USA), RPMI 1640 (Gibco, USA), Trizol (Invitrogen, USA), Hoechst 33258 apoptosis staining kit (Beyotime Institute of Biotechnology, China), Annexin-V-FITC/PI apoptosis assay kit (Jingmei Biotech Co. Ltd, China), anti-caspase-3, anti-survivin and anti-β-actin were procured from Santa Cruz Biotechnology (Santa Cruz, USA), ECL reagent kit (Pierce, USA).

Cell culture

Medium for BIU87 cell line was supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin (10,000 units/mL)–Streptomycin (10,000 μg/mL) solution. Cell cultures were maintained in 25 cm^2 cell culture flasks or six-well cell culture plates (Iwaki, Japan) at 37 ℃ and 5% CO_2 in humidified chambers. When BIU87 cell was plated to 80%–90% confluence, cells were fed with fresh medium and were treated with either RPMI 1640 alone (control group) or different concentrations of GSPE which were diluted by RPMI 1640 (as described above), and cultured for 24 h to get the cells for following tests. Three separate, independent replications of each test were performed.

MTT assay for cellular viability

Cell viability was determined using the MTT [3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma, USA] assay. The cells were plated at 1×10^4 cells per well 200 μL of complete culture medium in 96 well microtiter plates, and treated with designed concentrations (12.5, 25, 50, 100, 200 μg/mL) of GSPE till 80%–90% confluence. Each concentration of GSPE was repeated in 6 wells. After incubation for specified times at 37 ℃ in a humidified incubator, MTT reagent [20 μL, 5 mg/mL in PBS (10 nmol/L, pH 7.45)] was added to each well and incubated for 4 h. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μL). Absorbance (A) was recorded on a microplate reader at a wavelength of 490 nm. The effect of GSPE on growth inhibition was assessed as the percentage of inhibition in cell growth where untreated cells were taken as 100% viable. Percent viability was calculated as [value of drug-treated group (A)/control group (A)] × 100.

Apoptosis assessment by Hoechst 33258 staining

After treatment with GSPE, BIU87 cells on six-well plates were immersed in 0.5 mL of methanol for 10 min, followed by rinse with PBS for 3 min twice. Then cells were stained with 1 mg/mL Hoechst 33258 compounds in a dark chamber at room temperature for 5 min and rinsed for 3 min twice in PBS again. Cells were analyzed by fluorescence microscopy (Leica, German). The apoptotic cells were featured as pyknotic and fragmented nuclei emitting intense fluorescence.

Apoptosis analysis by flow cytometry

To quantify GSPE induced apoptosis of BIU87 cells, annexin V and PI staining was performed followed by flow cytometry. After treatment of cells with different concentration of GSPE for 24 h, both floating and attached cells on six-well plates were collected by digestive juice (0.25% trypsinization plus 0.02% EDTA) and washed with 4 ℃ PBS twice, and subjected to annexin V and PI staining using apoptosis assay kit following the step-by-step protocol provided by the manufacture. In brief, cells were resuspended by 250 μL of bind buffer and adjusted the density for 1×10^6. For each sample, 100 μL cell suspension was aspirated and added 5 μL of annexin V-FITC and 10 μL of PI. After 15 min of incubation in the dark at room temperature, each sample was diluted with 400 μL of PBS to obtain a final volume appropriate for flow cytometry.

RNA isolation and semiquantitative RT–PCR

Total RNA of cells on 25 cm^2 culture flasks were extracted by Trizol reagent kit (Invitrogen, USA) according to the protocols provided by the manufacturer. The quality of each total RNA sample (including its concentration and purity) was checked and controlled by measurement of the optical density of each sample. Up to 10 μL with dimethyl pyrocar-bonate (DEPC)-treated water, 1 μg of RNA and 300 pmol of oligo-dT_{15} (Takara Bio, Japan) were added together and were heated at 65 ℃ for 5 min, 200 units of M-MLV reverse transcriptase (Takara Bio, Japan), 1 μl of 10 mM dNTP (Promega, USA), 20 units of RNAsin (Promega, USA), and 4 μL of 5×M-MLV RT buffer (Promega, USA) were then added to the mixture and the final volume was brought up to 20 μL with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 ℃ for 1 h and 72 ℃ for 10 min.

PCR amplification was performed in a reaction volume of 25 μL containing 1 μL of the appropriate cDNA, 1 μL of each set of primers at a concentration of 10 pM, 2.5 μL of 10 × RT buffer, 1 μL of 2.5 mM dNTP, and 1 μL of Taq DNA polymerase (TaKaRa, Japan). Specific primers for human caspase-3, survivin, and β-actin as a loading control: caspase-3 (size of PCR product: 422 bp): sense 5'-GAC ATC TCG GTC TGG T-3', antisense 5'-CAG GTG CTG TGG AGT A-3'; survivin (size of PCR product: 439 bp): sense 5'-GGC ATG GGT GCC CCG ACG TTG-3', antisense 5'-CAG AGG CCT CAA TCC ATG GCA-3' [21]; β-actin (size of PCR product: 580 bp): sense 5'-ATG ATA

TCG CCG CGC TCG TC-3', antisense 5'-CGC TCG GTG AGG ATC TTC A-3' [22]. The reaction condition of PCR (caspase-3) was as follows (β-actin primers quodvided in Liang [21], while survivin in Ikeguchi [22]): initial denaturation at 94 ℃ for 5 min; 30 cycles of denaturation at 94 ℃ for 45 s, annealing at 50 ℃ for 45 s, and extension at 72 ℃ for 1 min on a Mastercycler gradient (Eppendorf– Netheler–Hinz GmbH, Germany). PCR products were electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide. Gene expression was presented by the relative yield of the PCR product from target sequences to that from the β-actin gene.

Western blot analysis

Following desired treatments, BIU87 cells were collected by scraping and washed with ice-cold PBS for two times. The cell pellets were homogenized in extraction buffer (50 mM Tris-HCl, 0.1% SDS, 150 mM NaCl, 100 μg/mL pheny-lmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1% Nonidet P-40, and 0.5% sodium orthovanadate), then incubated at 4 ℃ for 30 min and centrifuged 20 min at 12,000 g/min. Concentration of total protein in the supernatant was quantified by Bradford assay. For immunoblot analyses, 50 μg of protein lysates per sample were resolved over 15% SDS- PAGE gels and transferred onto a nitrocellulose membrane. Followed by blocking with 5% non-fat milk powder (w/v) in TBS (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4 ℃. The membranes were incubated with anti-caspase-3 (1:500), anti-survivin (1:500), and anti-β-actin (1:500) respectively, at 4 ℃ overnight. After binding of horseradish peroxidase (HRP)-coupled goat anti-rabbit/mouse IgG (Santa Cruz, USA; 1:5000) at room temperature for 2 h, antigens were visualized by enhanced chemiluminescence (ECL-kit, Pierce; USA). All results were representative of three independent experiments.

Statistics analysis

Data were expressed as mean ± SD (standard deviation). All experiments were performed in triplicate. Statistical significance was evaluated by one-way ANOVA using SPSS 11.5 software. Differences were considered significant when the *P* value was less than 0.05.

Results

Inhibition of BIU87 cell growth by GSPE

The effect of GSPE on cell viability was determined employing a MTT assay. BIU87 cells were cultured with GSPE at final concentrations of 12.5, 25, 50, 100 and 200 μg/mL for 24 h, after which MTT assays were then carried out. The cells cultured in GSPE-free media were used as the control. As shown in Fig. 1, the viability of the BIU87 cells incubated with GSPE at concentrations of 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL for 24 h was 99.55 ± 0.50% (n = 18, P > 0.05), 99.15 ± 0.63% (n = 18, P > 0.05), 86.98 ± 1.48% (n = 18, P < 0.01), 68.18 ± 2.06% (n = 18, P < 0.01) and 51.42 ± 1.78% (n = 18, P < 0.01) of the control value, respectively. A trend of decreasing viability with increasing GSPE concentration was significantly observed at 50, 100 and 200 μg/mL in BIU87 cells. The results of the MTT assay showed that GSPE exerts a dose-dependent cytotoxic effect on the bladder cancer BIU87 cells. For further mechanistic studies a dose of 50, 100 and 200 μg/mL GSPE was selected.

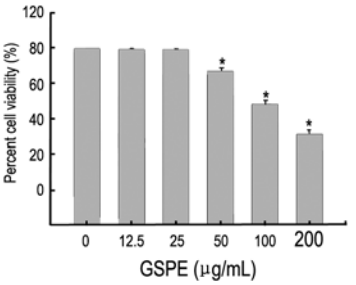

Fig. 1 Effect of GSPE on the viability of BIU87 cells. Cells were treated with specified concentrations of GSPE for 24 h, and cell viability was determined by MTT assay as described in materials and methods. The experiments were repeated at least three times. The results were presented as the mean± SD. * represents P < 0.01 compared to the control

Induction of apoptosis by GSPE in BIU87 cells

Apoptosis was confirmed by analyzing the nuclear morphology of GSPE-treated BIU87 cells. Nuclear morphology was evaluated with membrane-permeable blue Hoechst 33258. Fig. 2 showed representative Hoechst 33258 fluorescence photo-micrographs of cultured BIU87 cells treated with or without GSPE, respectively. In control cultures, nuclei of BIU87 cells appeared with regular contours and were round and large in size, none of BIU87 cells with smaller nuclei and condensed chromatin were seen. In contrast, most of GSPE-treated BIU87 cells appeared hyper-condensed (brightly stained) and fragmented nuclei. The numbers of apoptotic nuclei containing condensed chromatin increased significantly with the raise of incubation concentration.

For the quantification of apoptotic BIU87 cells, annexin V and PI were used for staining. Annexin V^+ and PI^- cells were designated as apoptotic. As shown in Fig. 3, compared with the RPMI 1640-treated vehicle control cells, which almost no apoptotic cells were detected, 24 h of GSPE treatment at 50, 100, and 200 μg/mL doses resulted in 8.7, 28.2, and 40.6% apoptotic cells, respectively. When the results were analyzed in terms of annexin V plus PI staining of the cells, a dose dependent effect of GSPE was evident (P < 0.01, data were not shown).

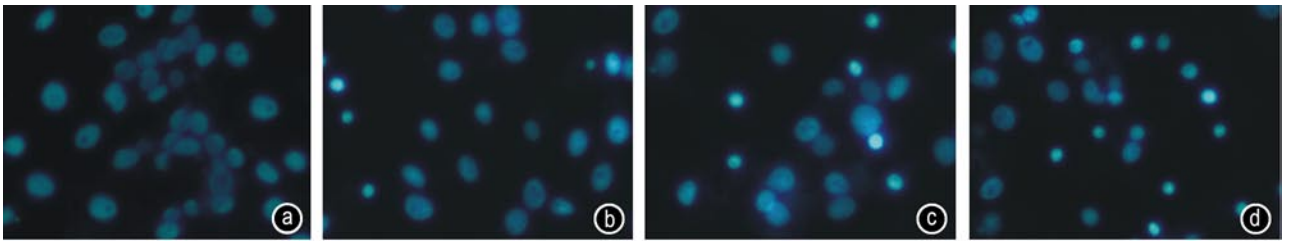

Fig. 2 Morphological changes in the nuclei (typical of apoptosis) of BIU87 cells induced by GSPE. Cells with condensed chromatin were defined as apoptotic BIU87 cells. (a) Control; (b) BIU87 cells treated with 50 μg/mL GSPE; (c) BIU87 cells treated with 100 μg/mL GSPE; (d) BIU87 cells treated with 200 μg/mL GSPE

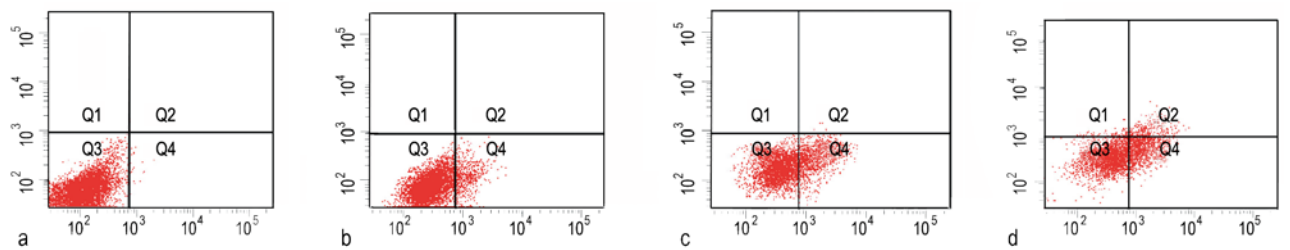

Fig. 3 GSPE induced apoptosis of BIU87 cells verified by flow cytometric analysis using Annexin V/PI staining method. Early apoptotic cells, represented by annexin V+ and PI- cells (lower right quadrant), 24 h of GSPE treatment at 0, 50, 100, and 200 μg/mL doses resulted in 0.3 (a), 8.7 (b), 28.2 (c), and 40.6% (d) early apoptotic cells, respectively. Data were representative of three independent experiments

Effects of GSPE on the expression of caspase-3 and survivin in BIU87 cells

To evaluate the possible mechanisms of GSPE induced apoptosis in BIU87 cells, RT–PCR and Western blot assays were established for caspase-3 and survivin. As shown in Fig. 4, the mRNA expression level of caspase-3 was increased while the mRNA expression level of survivin was decreased in a dose-dependent manner by GSPE treatment in BIU87 cells for 24 h. Semiquantitative RT-PCR indicitated that the difference between the groups of different concentrations were significant ($P < 0.01$; Fig. 4).

We also detected the protein expression of caspase-3 and survivin in BIU87 cells by Western bolt analysis after being treated with different concentrations of GSPE for 24 h. Similar to caspase-3 and survivin mRNA expression, Western blot analysis indicated that the protein expression level of caspase-3 was increased and the protein expression level of survivin was decreased in a dose-dependent manner Fig. 5. The levels between different groups were significant ($P < 0.01$; Fig. 5).

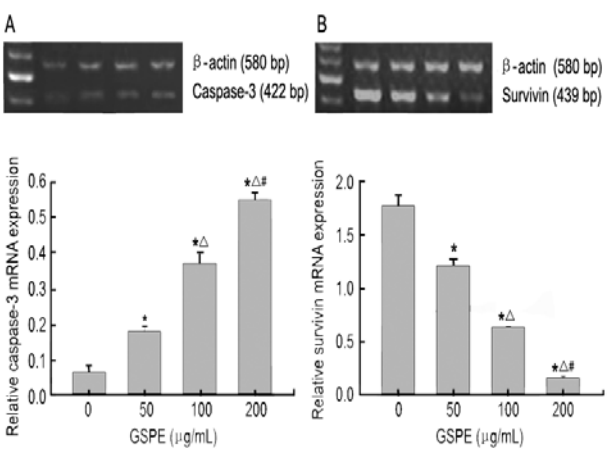

Fig. 4 Expression of caspase-3 (A) and survivin mRNA (B) in response to GSPE treatment in BIU87 cells. Cells were treated with GSPE for 24 h, harvested and total RNA was isolated and analyzed by RT–PCR for caspase-3 and survivin mRNA expression. The housekeeping gene β-actin was used as a control. Results were representative of data obtained from three seperated experiments. The details were described under materials and methods. * $P < 0.01$ compared to the control; $^{\Delta}$ $P < 0.01$ compared to 50 μg/ml group; $^{\#}$ $P < 0.01$ compared to 100 μg/mL group. The mRNA expression level of caspase-3 was increases while survivin was decreased in a dose-dependent manner by GSPE treatment.

Discussion

Almost 75% of the transitional cell carcinomas in bladder is superficial, which always followed an adjuvant intravesical therapy conventionally after transurethral removal of the tumor to preclude the recurrence and invasion. For most of the chemotherapy drugs used in clinic had toxicities and set effects in some extent, it is critical to find a new and safe drug agent for intravesical therapy.

Several studies have shown that some microchemicals present in the diet and several fruits and vegetables

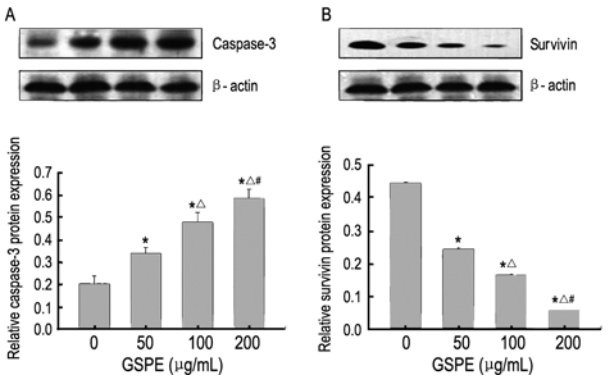

Fig. 5 Expression of caspase-3 (A) and survivin (B) protein in response to GSPE treatment in BIU87 cells. Cells were treated with specified concentrations of GSPE for 24 h, harvested and total cell lysates were prepared. The details were described under materials and methods. A typical immunoblot from three separate experiments with similar results was shown here. The protein expression level of caspase-3 was increased while survivin was decreased in a dose-dependent manner by GSPE treatment. * $P < 0.01$ compared to the control; $^{\Delta}$ $P < 0.01$ compared to 50 μg/mL group; $^{\#}$ $P < 0.01$ compared to 100 μg/mL group

are the most desirable class of agents for the prevention and/or intervention of various cancers [23–25]. Among these chemicals, GSPE have received increasing attention in recent years for its strong effects of antioxidant and antitumor. Recent studies *in vitro* and *in vivo* had shown great antitumor effects on most human cancers (such as breast cancer, lung cancer and gastric adenocarcinoma, etc.) by the treatment of GSPE [13, 14–20, 26–29]. The agent, however, enhances the growth and viability of normal human gastric mucosal cells [28], and indicates a lack of toxicity [30, 31].

Apoptosis, a process of programmed cell death, is critical for the development and maintenance of healthy tissues. Dysregulation of cell death pathways occur in many types of human cancer. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, plays an important role in inhibition of apoptosis and binds specifically to the terminal effector cell death proteases, caspase-3 and -7 to attend the procedure of apoptosis in tumor cells [32–34]. It is present during embryonic and fetal development, but undetectable in normal differentiated adult tissue *in vivo* [35]. In various human cancers including bladder cancer, survivin is strongly overexpressed [36–38] and has been established as a diagnostic, prognostic and therapeutic marker [39–44].

In the present study, after treatment of GSPE for 24 h, morphological characterization (hyper-condensed and fragmented nuclei) of apoptotic BIU87 cells could be seen by Hoechst 33258 staining, and the number of apoptotic BIU87 cells increased with the concentrations of GSPE by flow cytometry. The mRNA and protein expression of caspase-3 increased while survivin decreased with a dose-depended manner of the treatment of GSPE. Those indicate that down-regulate the expression of survivin which correlated with apoptosis may be involved in GSPE induced apoptosis in BIU87 cells.

Taken together, this study provided first evidence that GSPE inhibited cell proliferation by apoptosis on human bladder cancer BIU87 cells which was associated with down-regulation of the expression of survivin. This ability of GSPE to induce apoptosis imply its potential to be a chemotherapeutic agent. Although the precise molecular mechanism of the effect induced by GSPE remains unclear, it might be a potent useful antitumor agent for adjuvant intravesical therapy against human bladder cancer.

References

1. Landis SH, Murray T, Bolden S, *et al*. Cancer statistics. CA Cancer J Clin, 1999, 49: 8–31.
2. de Vere White RW, Stapp E. Predicting prognosis in patients with superficial bladder tumors. Oncology, 1998, 12: 1717–1723.
3. Hassen W, Droller MJ. Current concepts in assessment and treatment of bladder cancer. Curr Opin Urol, 2000, 10: 291–299.
4. Rischmann P, Desgrandchamps F, Malavaud B, *et al*. BCG intravesical instillations: recommendations for side-effects management. Eur Urol, 2000, 37: 33–36.
5. Koya MP, Simon MA, Soloway MS. Complications of intravesical therapy for urothelial cancer of the bladder. J Urol, 2006, 175: 2004–2010.
6. de Pascual-Teresa S, Santos-Buelga C, Rivas-Gonzalo JC. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. J Agric Food Chem, 2000, 48: 5331–5337.
7. Bagchi D, Bagchi M, Stohs SJ, *et al*. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology, 2000, 148: 187–197.
8. Gu L, Kelm M, Hammerstone JF, *et al*. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. J Agric Food Chem, 2002, 50: 4852–4860.
9. Gu L, Kelm MA, Hammerstone JF, *et al*. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. J Nutr, 2004, 134: 613–617.
10. Seeram NP, Adams LS, Hardy ML, *et al*. Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. J Agric Food Chem, 2004, 52: 2512–2517.
11. Ricardo Da Silva JM, Darman N, Fernandez Y, *et al*. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J Agric Food Chem, 1991, 39: 1549–1552.
12. Monagas M, Gómez-Cordovés C, Bartolomé B, *et al*. Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon. J Agric Food Chem, 2003, 51: 6475–6481.
13. Mittal A, Elmets CA, Katiyar SK. Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. Carcinogenesis, 2003, 24: 1379–1388.
14. Chatelain K, Phippen S, McCabe J, *et al*. Cranberry and grape seed

extracts inhibit the proliferative phenotype of oral squamous cell carcinomas. Evid Based Complement Altern Med, 2008 Jul 23. [Epub ahead of print].
15. Singh RP, Tyagi AK, Dhanalakshmi S, *et al*. Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3. Int J Cancer, 2004, 108: 733–740.
16. Tyagi A, Agarwal R, Agarwal C. Grape seed extract inhibits EGF-induced and constitutively active mitogenic signaling but activates JNK in human prostate carcinoma DU145 cells: possible role in antiproliferation and apoptosis. Oncogene, 2003, 22: 1302–1316.
17. Agarwal C, Singh RP, Agarwal R. Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release. Carcinogenesis, 2002, 23: 1869–1876.
18. Kaur M, Agarwal R, Agarwal C. Grape seed extract induces anoikis and caspase- mediated apoptosis in human prostate carcinoma LNCaP cells possible role of ataxia telangiectasia mutated–p53 activation. Mol Cancer Ther, 2006, 5: 1265–1274.
19. Kaur M, Singh RP, Gu M, *et al*. Grape seed extract inhibits in vitro and In vivo growth of human colorectal carcinoma cells. Clin Cancer Res, 2006, 12: 6194–6202.
20. Engelbrecht AM, Mattheyse M, Ellis B, *et al*. Proanthocyanidin from grape seeds inactivates the PI3-kinase/PKB pathway and induces apoptosis in a colon cancer cell line. Cancer Lett, 2007, 258: 144–153.
21. Liang J, Pan Y, Zhang D, *et al*. Cellular prion protein promotes proliferation and G1/S transition of human gastric cancer cells SGC7901 and AGS. FASEB J, 2007, 21: 2247–2256.
22. Ikeguchi M, Ueta T, Yamane Y, *et al.* Inducible nitric oxide synthase and survivin messenger RNA expression in hepatocellular carcinoma. Clin Cancer Res, 2002, 8: 3131–3136.
23. Park EJ, Pezzuto JM. Botanicals in cancer chemoprevention. Cancer Metastasis Rev, 2002, 21: 231–255.
24. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science, 1997, 278: 1073–1077.
25. Birt DF, Pelling JC, Nair S, *et al*. Diet intervention for modifying cancer risk. Prog Clin Biol Res, 1996, 395: 223–234.
26. Ye X, Krohn RL, Liu W, *et al*. The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells. Mol Cell Biochem, 1999, 196: 99–108.
27. Agarwal C, Sharma Y, Zhao J, *et al*. A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation. Clin Cancer Res, 2000, 6: 2921–2930.
28. Zhao J, Wang J, Chen Y, *et al*. Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation–promotion protocol and identification of procyanidin B5-3'-gallate as the most effective antioxidant constituent. Carcinogenesis, 1999, 20: 1737–1745.
29. Bomser JA, Singletary KW, Wallig MA, *et al*. Inhibition of TPA-induced tumor promotion in CD-1 mouse epidermis by a polyphenolic fraction from grape seeds. Cancer Lett, 1999, 135: 151–157.
30. Yamakoshi J, Saito M, Kataoka S, *et al*. Safety evaluation of proanthocyanidin- rich extract from grape seeds. Food Chem Toxicol, 2002, 40: 599–607.
31. Ray S, Bagchi D, Lim PM, *et al*. Acute and long-term safety evaluation of a novel IH636 grape seed proanthocyanidin extract. Res Commun Mol Pathol Pharmacol, 2001, 109: 165–197.
32. Altieri DC, Marchisio PC. Survivin apoptosis: an interloper between cell death and cell proliferation in cancer. Lab Invest, 1999, 79: 1327–1333.
33. Tamm I, Wang Y, Sausville E, *et al*. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res, 1998, 58: 5315–5320.
34. Shin S, Sung BJ, Cho YS, *et al*. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry, 2001, 40: 1117–1123.
35. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, Survivin, expressed in cancer and lymphoma. Nat Med, 1997, 3: 917–921.
36. Kitsukawa S, Aoyagi T, Noda K, *et al.* Quantitative analysis of Survivin mRNA expression in bladder transitional cell carcinomas. Hinyokika Kiyo, 2008, 54: 101–106.
37. Wang Y, Zhu Z, Zeng F, *et al.* Expression and prognostic significance of Survivin in the progression of bladder transitional cell cancer. J Huazhong Univ Sci Technolog Med Sci, 2007, 27: 444–447.
38. Kitamura H, Torigoe T, Honma I, *et al*. Expression and antigenicity of survivin, an inhibitor of apoptosis family member, in bladder cancer: implications for specific immunotherapy. Urology, 2006, 67: 955–959.
39. Smith SD, Wheeler MA, Plescia J, *et al*. Urine detection of survivin and diagnosis of bladder cancer. JAMA, 2001, 285: 324–328.
40. Shariat SF, Casella R, Khoddami SM, *et al*. Urine detection of Survivin is a sensitive marker for the noninvasive diagnosis of bladder cancer. J Urol, 2004, 171: 626–630.
41. Schultz IJ, Kiemeney LA, Karthaus HM, *et al*. Survivin mRNA copy number in bladder washings predicts tumor recurrence in patients with superficial urothelial cell carcinomas. Clin Chem, 2004, 50: 1425–1428.
42. Als AB, Dyrskjot L, von der Maase H, *et al*. Emmprin and survivin predict response and survival following cisplatin-containing chemotherapy in patients with advanced bladder cancer. Clin Cancer Res, 2007, 13: 4407–4414.
43. Fuessel S, Kueppers B, Ning S, *et al*. Systematic in vitro evaluation of survivin directed antisense oligodeoxynucleotides in bladder cancer cells. J Urol, 2004, 171: 2471–2476.
44. Fuessel S, Herrmann J, Ning S, *et al*. Chemosensitization of bladder cancer cells by survivin-directed antisense oligodeoxynucleotides and siRNA. Cancer Lett, 2006, 232: 243–254.