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A component of green tea, (−)-epigallocatechin-3-gallate, promotes apoptosis in T24 human bladder cancer cells via modulation of the PI3K/Akt pathway and Bcl-2 family proteins

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Abstract

Bladder cancer is the fourth most common cancer in men and ninth most common in women. It has a protracted course of progression and is thus an ideal candidate for chemoprevention strategies and trials. This study was conducted to evaluate the chemopreventive/antiproliferative potential of (−)-epigallocatechin gallate (EGCG, the major phytochemical in green tea) against bladder cancer and its mechanism of action. Using the T24 human bladder cancer cell line, we found that EGCG treatment caused dose- and time-dependent inhibition of cellular proliferation and cell viability, and induced apoptosis. Mechanistically, EGCG inhibits phosphatidylinositol 3′-kinase/Akt activation that, in turn, results in modulation of Bcl-2 family proteins, leading to enhanced apoptosis of T24 cells. These findings suggest that EGCG may be an important chemoprevention agent for the management of bladder cancer.

Keywords: Bladder cancer; Akt; EGCG; Apoptosis; Bcl-2

Green tea, derived from the plant *Camellia sinensis*, is one of the most common beverages consumed worldwide, especially in China. Studies conducted on cell-culture systems and animal models as well as human epidemiological studies show that the polyphenols that are present in green tea could afford protection against a variety of cancer types [1–6]. It is generally agreed that many of the chemoprevention effects of green tea are mediated by polyphenols. The major catechins in green tea is (−)-epigallocatechin-3-gallate (EGCG) which accounts for 50–80% of catechin in green tea, representing 200–300 mg in a brewed cup of green tea [7]. Although studies have been reported on the growth inhibitory effect of green tea on human cancer cell lines such as prostate, colon and breast cancer [1], little has been reported about its inhibitory effect on human bladder carcinoma cells.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is an important intracellular mediator [8]. Akt acts downstream of PI3K to regulate many biological processes, such as proliferation, apoptosis, and growth and plays a critical role in controlling cell survival and apoptosis through phosphorylation of numerous cellular proteins, including Bad, the Forkhead family of transcription factors (which regulate FasL expression), caspase-9, and NFκB [9]. Studies have also suggested that Bcl-2 could be a crucial mediator downstream of PI3K/Akt signaling, whereas Akt has been shown to negatively regulate the activity of pro-apoptotic members of the Bcl-2 family [10,11]. Akt is activated in a wide variety of cancers including bladder cancer [12], and its activation results in enhanced resistance to apoptosis through multiple mechanisms. The link between activation of the PI3K/Akt pathway and cancer makes this

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pathway an attractive target for therapeutic strategies. Therefore, the development of cytotoxic drugs that target the PI3K/Akt pathway has become an increasingly important strategy for targeting cancer cells in various organs.

In this study, we show that in T24 bladder cancer cells EGCG, (a) inhibits the PI3K/Akt pathway, (b) downregulates antiapoptotic protein Bcl-2 and Bcl-xL, and (c) upregulates Bax and Bad, and (d) leads to the activation of caspase-3 and poly(ADPribose) polymerase (PARP).

Materials and methods

Reagents. EGCG was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The annexin V-FITC apoptosis detection kit was from Beckman Coulter (Fullerton, CA). Primary antibodies to Bcl-2, Bax, Bcl-xL, caspase-3, β-actin and poly(ADP-ribosyl) polymerase (PARP), and secondary antibodies were purchased from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to Akt, phosphorylated Akt (Ser473/Thr308), PIP3-dependent kinase 1 (pPDK1), and Bad were purchased from Cell Signaling Technology (Beverly, MA). The bicinchoninic acid protein assay kit was obtained from Pierce Biotechnology (Rockford, IL).

Cell culture. The human bladder cancer cell line T24 was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/L) at 37 °C in a humidified atmosphere containing 5% CO_2. EGCG was dissolved in PBS (pH 7.4) and used for the treatment of cells. For assessing morphological changes, 50–60% confluent cells were treated with different concentrations of EGCG, whereas PBS treated cells served as control. After 24 h of treatment, photographs were taken using a phase-contrast microscope at 200× magnification (Olympus, Japan).

Cell growth/cell viability assay. Proliferation of cells was determined by the MTT assay. Approximately 10,000 T24 cells were plated in each well of 96-well plates. After overnight incubation, the cells were treated with EGCG (0–80 μg/mL) for 12–48 h. At the various times following EGCG treatment, the medium was removed and MTT (20 μl of 5 mg/mL) was added to each well and incubated at 37 °C for 4 h. The plates were spun, and the purple colored precipitates of formazan were dissolved in 150 μl of dimethyl sulfoxide. Absorbance was measured at 490 nm using an ELISA plate reader. The reduction in viability of in EGCG-treated T24 cells was expressed as a percentage compared to non-EGCG treated control cells. Control cells were considered to be 100% viable.

Colony formation assay. The antiproliferative effect of EGCG on T24 cells was assessed by colony formation assay, as described previously [13]. Briefly, ~400 cells were plated into each well of 6-well plates in triplicate for 24 h. Thereafter, cells were treated with EGCG (1–40 μg/mL). The cells were kept in an incubator at 37 °C for 7 days. On day 8, the colonies were washed with PBS, fixed with formalin (10%), and stained with Giemsa [14]. The colonies that had $\geqslant$50 cells per colony were counted. The number of colonies formed in the presence of varying concentrations of EGCG was expressed as a percentage of untreated controls.

Detection of apoptotic cells by fluorescence staining and flow cytometry. Apoptosis was assessed by adding an acridine orange (100 mg/mL)/ethidium bromide (100 mg/mL) (1/1 v/v) mixture to the cell suspension and the percentage of cells undergoing apoptosis was determined with a Leica TCSSP Confocal Microscope (Wetzlar, Germany) as described [15]. Live cells have green fluorescence (with acridine orange) while dead cells fluoresce orange (with ethidium bromide). A quantitative assessment of apoptosis was made by determining the percentage of cells with nuclei that were highly condensed or fragmented. Annexin V and PI double staining was performed using the Annexin V-FITC Apoptosis Detection Kit as described by the manufacturer.

Western blot analysis. Western blots were used to analyze the expression of various proteins as described previously [16]. Briefly, cells were harvested at 24 h following EGCG treatment as described above, washed and lysed with lysis buffer (10 mmol/L Tris–HCl, 0.25 mol/L sucrose, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, and pH 7.5). Protein concentration in the resulting lysate was determined using the bicinchoninic acid protein assay. Appropriate amounts of protein (20–30 μg) were resolved by electrophoresis in 10–15% Tris–glycine polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and then incubated overnight with the appropriate primary antibody at dilutions specified by the manufacturer. They were next washed and incubated with the corresponding horseradish peroxidase conjugated secondary antibody at 1:1000 dilution in TBST. Bound secondary antibody was detected using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc., Rockford, IL, USA).

Statistical analysis. All values are expressed as means ± SD. Statistical significance was compared between various treatment groups and controls using the one-way analysis of variance (ANOVA). Data were considered statistically significant when P values were <0.05.

Results

EGCG inhibits T24 cell growth and clonogenic survival

The cytotoxic effect of EGCG on human bladder cancer T24 cells was determined with varying concentrations of EGCG and times (12–48 h) by MTT assay. As shown in Fig. 1A, the lowest concentration of EGCG that exhibited an effect on cell viability was 20 μg/mL for 36 h. Inhibition of cell viability by EGCG was dose and time dependent. Reduction in cell viability with EGCG treatment at concentrations of 20–100 μg/mL after 12 h ranged from 1% to 26%, whereas after 24, 36, and 48 h ranged from 4% to 61%, 9% to 83%, and 10% to 91%, respectively (Fig. 1A). The IC_{50} value for EGCG growth inhibition was estimated to be 60 μg/mL for 24 h. Based on these observations, we selected a dose range of 10–80 μg/mL and a time period of 24 h post-EGCG treatment for further mechanistic studies.

We also examined the effect of EGCG on the clonogenic survival of T24 cells. There was a drastic decrease in the ability of the T24 cells to form colonies with increasing doses of EGCG (10–40 μg/mL) (Fig. 1B). EGCG at dosages of 20 and 40 μg/mL completely inhibited the proliferation of cells with no colonies formed by the end of 7 days. These observations indicated that EGCG has antiproliferative and anticarcinogenic effects on T24 cells.

EGCG induces apoptosis in T24 cells

We next determined whether EGCG-mediated loss of T24 cell viability was the result of the induction of apoptosis. As shown in Fig. S1A, EGCG treatment resulted in induction of apoptosis in a dose-dependent manner (Fig. S1B). These data also show that EGCG treatment resulted in cell necrosis, which may be a secondary event in the apoptotic process. The pro-apoptotic effect of EGCG was confirmed by analyzing cell morphology (Fig. S1C) and by PI staining and the annexin V method (Fig. 2A). Phase-contrast photomicrographs taken 24 h after EGCG treatment revealed a dose dependent decrease in cell density. Changes in cell morphology and cell membrane blebbing, which are characteristics of apoptosis, were also detected.

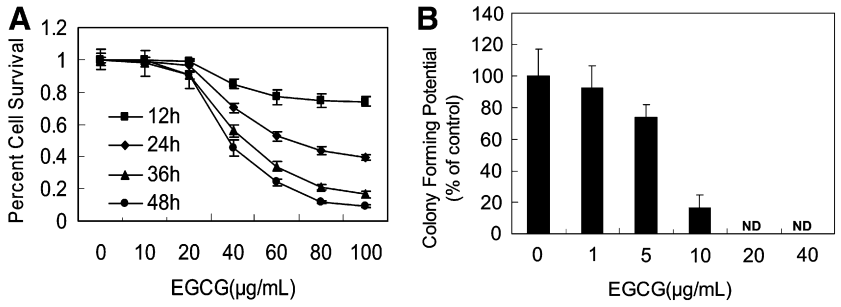

Fig. 1. EGCG inhibits cell viability and proliferation of human bladder cancer T24 cells in a dose- and time-dependent manner. (A) Dose- and time-dependent effect of EGCG on T24 cell viability. Viability of cells was determined by the MTT assay as described in Materials and methods. Reduced cell viability was observed with EGCG treatment (20–100 μg/mL) concentrations at 12, 24, 36, and 48 h. The data are presented as means ± SD ($n = 8$). (B) Cellular proliferation assayed by the clonogenic assay as described in Materials and methods. A value of 100 corresponds the number of colonies obtained with control (non-EGCG treated) cells. Treatment of T24 cells with ⩾10 μg/mL of EGCG almost completely inhibited colony formation. The experiment was done in triplicate, and each time point indicates the effect of EGCG on colony formation compared to controls (means ± SD). ND, not detectable.

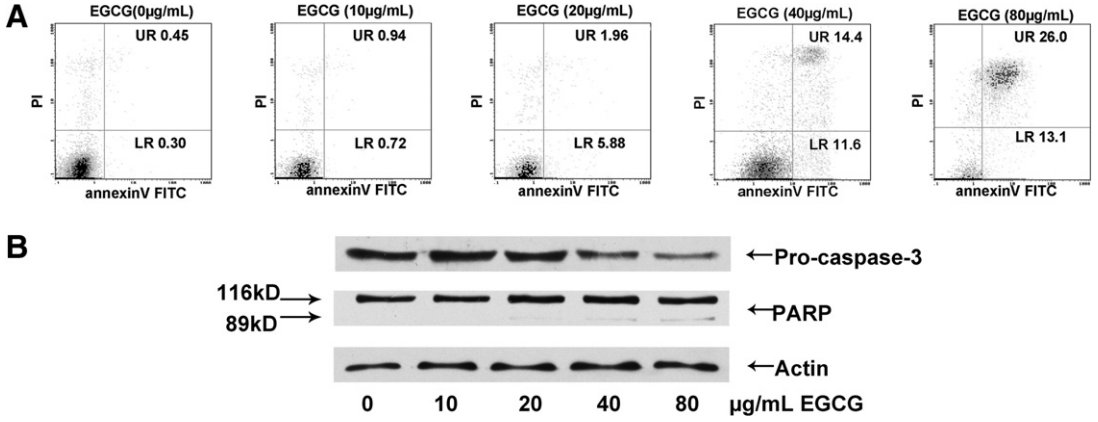

Fig. 2. EGCG treatment induces dose-dependent apoptosis in T24 human bladder cancer cells. (A) Flow cytometry of EGCG treated T24 cells using a double-staining method with FITC-conjugated annexin-V and PI. The LR quadrant indicates the percentage of early apoptotic cells (Annexin V-stained cells) and the UR quadrant the percentage of late apoptotic cells (Annexin V + propidium iodide-stained cells). (B) EGCG treatment activates caspase-3 and PARP in T24 human bladder cancer cells. The expression of proteins in treated cells was analyzed by Western blotting as detailed in Materials and methods. A representative blot is shown from three independent experiments with identical results.

We next quantified the extent of apoptosis by flow-cytometric analysis of EGCG-treated cells labeled with PI and annexin V. As shown by PI staining and the annexin V method, we found that EGCG caused a dosage dependent increase in T24 cell apoptosis (Fig. 2A). It was observed that treatment of T24 cells with 10 and 80 μg/mL of EGCG for 24 h increased the number of early apoptotic cells (LR) from 0.7% to 13.1%, respectively, in a dose-dependent manner compared to 0.3% in untreated control cells. The number of late apoptotic cells (UR) increased from 0.9% to 26.0% compared with 0.5% in non-EGCG treated cells. The total percent of apoptotic cells (UR + LR) increased from 0.8% in untreated T24 cells to 39.1% with 80 μg/mL of EGCG treatment for 24 h. Consistent with the phase-contrast and confocal microscopy data, flow cytometry using PI staining and annexin V revealed that treatment of T24 cells with EGCG resulted in a dose-dependent induction of apoptosis.

Activation of caspase-3 plays a central role in apoptosis by initiating cell death [17]. Caspase-3 has substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves poly(ADP-ribose) polymerase (PARP). It has been demonstrated that the proteolytic cleavage of PARP, is a biochemical event during apoptosis [18,19]. For these reasons, we determined the effect of EGCG on the expression of these proteins. As shown in Fig. 2B, the 89 kDa cleaved PARP fragment was detected in EGCG-treated samples. Fig. 2B also shows that the level of procaspase-3 was diminished at low doses (20 μg/mL) of EGCG, indicating activation of this caspase. Taken together, Western blot analysis indicated that treatment of T24 cells with EGCG resulted in a dose-dependent activation of caspase-3 and PARP (Fig. 2B) proteins 24 h after EGCG treatment. Thus the significant induction of apoptosis caused by EGCG confirms the observed reduction in cell viability and the anticarcinogenic effect on T24 human bladder cancer cells.

The antiproliferative effect of EGCG on T24 cells is mediated via modulation of the PI3K/Akt pathway

We next investigated whether or not PI3K/Akt was involved in EGCG-mediated apoptosis of T24 cells. As

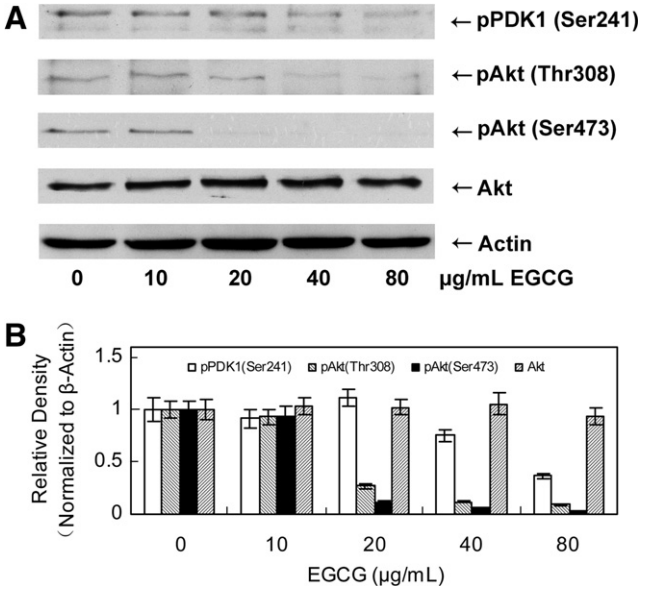

Fig. 3. EGCG reduces the expression of phospho-PDK1, phospho-Thr308-Akt, and phospho-Ser473-Akt. Cells were treated with different concentrations of EGCG for 24 h as indicated. The expression of proteins in treated cells was analyzed by Western blotting as detailed in Materials and methods. (A) Representative blots are shown from three independent experiments with identical results. (B) Protein expression levels of phospho-PDK1, phospho-Thr308-Akt, phospho-Ser473-Akt, and total Akt were normalized to that of actin and are presented as the fold change compared to controls.

shown in Fig. 3A and B, EGCG treatment resulted in an appreciable down-regulation of protein expression of phospho-PDK1, phospho-Thr308-Akt, and phospho-Ser473-Akt without an effect on total Akt expression in T24 cells. EGCG inhibited phosphorylation of PDK1, which phosphorylates Akt. Since basal levels of Akt phosphorylation are high in T24 cells, possibly due to mutation and inactivation of the PTEN tumor suppressor gene [20]; inhibition of Akt phosphorylation by EGCG may be attributed to inactivation of cell survival pathways resulting in apoptosis. These observations suggest that the PI3K/Akt pathway could be involved in the antiproliferative/pro-apoptotic response of bladder cancer cells to EGCG.

EGCG treatment results in modulation of the levels of Bcl-2 family proteins in a manner that promotes apoptosis in T24 cells

Studies have suggested that Bcl-2 could be a crucial mediator downstream of PI3K/Akt signaling, whereas Akt has been shown to negatively regulate the activity of pro-apoptotic members of the Bcl-2 family [10,11]. Therefore, we examined the effect of EGCG treatment on Bcl-2 family proteins in T24 cells. As shown in Fig. 4A, EGCG treatment of T24 cell lines resulted in a decrease in antiapoptotic Bcl-2 and a concomitant increase in pro-apoptotic Bax proteins. The ratio of Bax/Bcl-2 was significantly increased in a dose-dependent manner with EGCG treatment (Fig. 4B), suggesting that T24 cell apoptosis occurs with the involvement of Bcl-2 family proteins. Therefore, we looked at Bcl-2 family proteins (pro-apoptotic protein Bad and anti-apoptotic protein Bcl-xL) in EGCG treated T24 cells. Our data clearly show that EGCG caused an increase in Bad and a decrease in Bcl-xL protein levels (Fig. 4C and D). Thus there was an overall shift in the ratio of anti-apoptotic and pro-apoptotic proteins following EGCG treatment.

Discussion

In this study, we evaluated the chemopreventive/therapeutic potential of EGCG against bladder cancer and its mechanism of action. Consistent with earlier observations

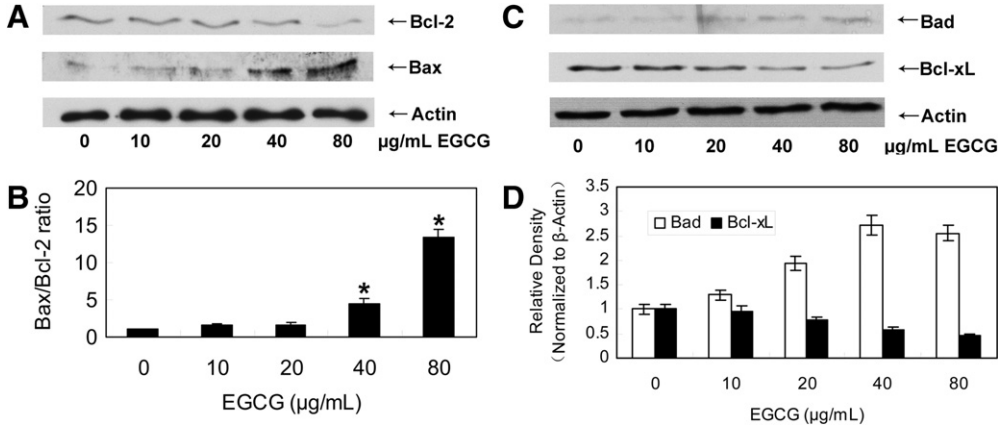

Fig. 4. EGCG modulates expression of apoptotic genes in T24 cells. Cells were treated with different concentrations of EGCG for 24 h as indicated. The expression of proteins in treated cells was analyzed by Western blotting as detailed in Materials and methods. (A) Representative Western blots for Bcl-2, Bax, and Actin. (B) Bcl-2 and Bax expression levels were normalized to actin and are presented as the Bax/Bcl-2 ratio (means ± SD) from three independent experiments. $^{*}P < 0.05$ compared to control (non-EGCG). (C) Representative Western blots for Bcl-xL, Bad, and Actin. (D) Protein expression levels of Bcl-xL and Bad were normalized to that of actin and are presented as the fold change compared to controls.

[21], we found that EGCG inhibits the proliferation and viability of bladder cancer cells by inducing apoptosis.

It is well established that hyperactivation of AKT kinases is a common event in many human cancers including bladder cancer [12,22], and this activation results in tumor cell survival and enhanced resistance to apoptosis through multiple mechanisms [23]. Our data show that EGCG treatment resulted in a significant dose-dependent inhibition of constitutively elevated levels of phosphorylated PDK1 and phosphorylated (active) Akt (at Ser473 and Thr308) in T24 cells. Since Akt is a downstream target of PI-3 kinase, the observed inhibition of Akt phosphorylation suggests that EGCG also inhibits PI-3 kinase. This argument is supported by previous studies showing that EGCG is an inhibitor of PI-3 kinase and serine/threonine protein kinases [24,25]. Accumulating evidence indicates a role for the PI-3 kinase pathway in bladder cancer. The PI-3 kinase/Akt pathway is being investigated as a target the development of bladder cancer therapies. Our study has clearly shown that EGCG downregulates the activation (phosphorylation) of Akt and, therefore, could be useful in the prevention and treatment of bladder cancer.

Activation of the PI3K/Akt pathway leads to increased expression of Bcl-2 [10,11], therefore we assessed the effect of EGCG on Bcl-2 family proteins. Our data show treatment of T24 cells with EGCG, (a) downregulates Bcl-2 and Bcl-xL proteins, and (b) upregulates levels of pro-apoptotic members of this family, i.e., Bax and Bad. Overall, there is a shift in the ratio between the anti-apoptotic and pro-apoptotic proteins following EGCG treatment. It has been suggested that the ratio of Bax/Bcl-2 proteins expression plays a significant role in transducing the apoptotic signal [26]. As shown in Fig. 4B, the ratio of Bax/Bcl-2 was significantly increased dose dependently after EGCG treatment. Our data also show that EGCG treatment significant reduced levels of phosphorylated (active) Akt in T24 cells.

Alteration in the ratio of Bax/Bcl-2 stimulates the release of cytochrome *c* from mitochondria into cytosol. Cytosolic cytochrome *c* then binds to Apaf-1 and leads to the activation of caspase-3 and PARP [27]. Activated caspase 3 is the key mediator of cell apoptosis cleaving intracellular proteins vital for cell survival and growth, such as PARP. In our *in vitro* system, EGCG activated caspase-3 (Fig. 2B), leading to PARP cleavage (Fig. 2B) and the induction of apoptosis in EGCG treated T24 cells.

In conclusion, the data from our study indicate that EGCG induced apoptosis in human bladder cancer T24 cells. This is mediated through the PI3K/AKT pathway which involves Bcl-2 family proteins and activation of caspase-3 and PARP. The results of our study provide evidence that EGCG may be an effective chemopreventive agent for human bladder cancer. However, further studies are needed to establish a cause–effect relationship between the PI3K/Akt pathway and EGCG effect. Furthermore, studies are also needed to define the upstream signaling involved in the inhibition of the PI3K/Akt pathway by EGCG in human bladder cancer cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.003.

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