

Growth inhibitory effects of quercetin on bladder cancer cell

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1. ABSTRACT

Quercetin, a flavonoid found in many fruits and vegetables, belongs to an extensive class of polyphenolic compounds. Previous studies reported that quercetin inhibits the proliferation of various cancer cells and tumor growth in animal models. We investigated the growth inhibition and colony formation of quercetin on three bladder cancer cells (EJ, J82 and T24). The expression of tumor suppressor genes and oncogenes such as P53, Survivin, PTEN, as well as the methylation status of these genes was also evaluated. We observed that quercetin induced apoptosis in bladder cancer cells in a time- and dose-dependent manner. Quercetin (100 micromolars) significantly inhibited EJ, T24 and J82 cell growth accompanied by an increase in the G0/G1 phase. In all cell lines, quercetin decreased the expression of mutant P53 and Survivin proteins. However, there was no change in the level of PTEN protein. Moreover, the DNA methylation levels of the estrogen receptor (Er-beta), P16^{INK4a} and RASSF1A were strongly decreased (from 35 to 70%) in the quercetin-treated group compared to the control. In conclusion, our study suggested that quercetin inhibits

growth, colony formation and hypermethylation of bladder cancer cell lines. Quercetin-induced apoptosis might be associated with a decrease in mutant P53 and Survivin proteins.

2. INTRODUCTION

At present, bladder cancer continues to be one of the most common genitourinary malignancies and its occurrence ranks first place in the various tumors among men in China. According to WHO statistics for 2000, bladder cancer is the seventh most commonly occurring cancer among men in the world. The American Cancer Society estimated that in 2003 there were approximately 57,400 new cases of bladder cancer and 12,500 deaths from bladder cancer in the United States (1). Transitional cell carcinoma (TCC) accounts for >90% of bladder cancer cases. The majority of TCC tumors are superficial at the time of diagnosis and most of these (60-70%) have a propensity for recurrence after initial transurethral resection of bladder cancer. Some patients (15-25%) are at high risk

for progression to invasive bladder cancer. Tumors or cancer cells are generally characterized by an aberrant hypermethylation in many of their CpG islands, resulting in inappropriate transcriptional repression and gene expression. Many of such inactivated genes generally encode for proteins involved in tumor initiation, development and progression (2-3).

The efficacy of various currently available therapeutic strategies for bladder cancer is not always sufficient, especially for advanced disease, recurrent superficial cancer, and treatment-resistant carcinoma *in situ*. Intravesical administration of BCG after transurethral resection is by far the most effective treatment for superficial bladder cancer. However, side effects of BCG therapy are common, and approximately one third of patients fail to respond (4, 5). Adjuvant chemotherapies have been used to prevent the recurrence of bladder TCC, but their efficacies are variable due to the side effects of anticancer agents and the drug resistant property of the target cells. Because of various side effects of chemical therapeutic drugs, many researchers tried to find natural drugs with little toxicity, and made great progress. Therefore, it is vital to find a new drug with little toxicity using intravesical administration and it is very important to use adjuvant treatment for bladder cancer.

The bioflavonoid, quercetin, a polyphenolic compound widely distributed in the plant kingdom, has been shown to inhibit the proliferation of cancer cells (6, 7, 8). The antitumor effects of plant flavonoids have been reported to induce cell growth inhibition and apoptosis in a variety of cancer cells and protects against cancer in various animal models (9). It has been shown that quercetin treatment caused cell cycle arrests such as G2/M arrest in breast and laryngeal or G1 arrest in colon and gastric cancer cells as well as in leukaemic cells (10-15). Moreover, quercetin-induced apoptosis may result from the induction of stress proteins, disruption of microtubules and mitochondria, release of cytochrome c, and activation of caspases (16-19).

The regulation of apoptosis is a complex process that involves various cellular genes. P53 is a cellular gatekeeper for cell growth and division; it can regulate cell cycle arrest, apoptosis, and DNA repair in a variety of cells (18, 20). The p53 downstream effectors include p21, which participates in cell cycle arrest, and bax, which triggers apoptosis (18, 19). Inhibition of p53 function may cause a decrease of DNA repair and an increase of genomic instability (21, 22). Also, the p53 protein mediates the G1 and G2/M checkpoints, which trigger p21 to participate in cell cycle arrests (23). It has been shown that mutations in the p53 gene are the most common genetic defect in human bladder cancer (24). Of particular significance, alterations in p53 are linked to cancer progression and response to radiation and chemotherapy (25).

Several cellular proteins such as Survivin and PTEN are known to act as antagonists for maintaining the cell alive. Survivin is expressed in human cancer cells (26-28). It may exhibit anti-apoptotic effects and inhibit the

activity of caspases in cancer cells (28-30). It has been shown that Survivin may promote mitotic progression (31-32) in cancer cells. In bladder cancer, its expression correlates with tumor grade, recurrence risk, and survival. The PTEN gene, located on chromosome 10, is a phosphatase in the phosphatidylinositol 3'-kinase (PI3'K)-mediated signal transduction pathway. PTEN inhibits the activation of Akt, a serine-threonine kinase involved in proliferative metabolic and anti-apoptotic pathways, and has tumor suppressive properties (33-34).

Although there are many studies that investigate the effects of quercetin on other cancer cells and elucidate related molecular mechanisms, we did not find quercetin used in bladder cancer cells. While it has been shown that quercetin treatment exhibits growth inhibitory effects on other cancer cells correlated with the expression of P53, Survivin and PTEN (35-36), there is no study that investigates the effect of quercetin on bladder cancer cells. So, we hypothesized that the anti-cancer effects of quercetin, as manifested by its ability to reduce viability of bladder cancer cells, suppressing colony formation and inducing apoptosis, are mediated by its ability to regulate the expression of various genes controlling tumor suppression and oncogenesis.

The present study was undertaken to investigate the effect of quercetin on the proliferation and colony formation ability of human bladder cancer cells. Furthermore, we examined the effect of quercetin on the incidence of apoptosis, the methylation status of three genes found in the pathways impaired by hypermethylation (P16, Er-beta and RASSF1A) and the expression of proteins (P53, Survivin and PTEN).

3. MATERIALS AND METHODS

3.1. Reagents

Hoechst 33258, quercetin, and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Anti-p53 (DO-1), anti-Survivin (FL-142), anti-PTEN and Horseradish peroxidase-conjugated goat anti-mouse IgG antibodies or goat anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The SP9000 immunocytochemical staining kit was purchased from Beijing Zhongshan Jinqiao Biotechnology (Beijing, China). PRMI 1640 medium was from Gibco BRL (Invitrogen Co, Carlsbad, CA). Fetal bovine serum was from Hangzhou Sijiqing Biotechnology (Hangzhou, China).

3.2. Cell culture and treatment

The EJ, T24 and J82 cell lines were derived from the urology institution of Lanzhou University. These bladder carcinoma cell lines were cultured in complete culture (CC) medium, consisting of RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 micrograms/ml streptomycin, L-glutamine (0.03%, w/v), and sodium bicarbonate (2.2%, w/v). The cells were cultured at 37 °C and 5% CO₂ in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH). To study the effects of quercetin on cell morphology, EJ, T24 and J82 cells were seeded onto 96-

Effect of quercetin in bladder cancer cells

well plates at a density of 5×10^3 /well in the growth medium. Confluent cells were washed with serum-free RPMI 1640 (SRF) medium and then incubated further with SRF medium for 6 h. Cells were then treated with various concentrations of quercetin (12.5, 25, 50, 75 and 100 micromolars) in SRF medium for 14 h. Photographs were taken using the inverse microscope (Canon, Tokyo, Japan).

3.3. Cytotoxicity assay

To study the effects of quercetin on cell proliferation and viability, all cell lines (5×10^3 /well) were plated in 96-well plates and incubated in CC medium. After 24 h, cells were washed once with SRF medium and treated with 0, 12.5, 25, 50, 75, 100 micromolars quercetin in SRF medium. Cell proliferation and cell viability were determined after 24 h or 48 h of treatment by incubation in CC medium containing 0.5 mg/ml MTT for 4 h. The surviving cells converted MTT to formazan that generates a blue-purple color when dissolved in dimethyl sulfoxide (DMSO). The color intensity was measured at 570 nm using a plate reader (OPTImax, Molecular Dynamics). Experiments were repeated at least three times, and the data were expressed as mean \pm SD.

3.4. Cell growth assay

EJ cells (3×10^3) were suspended in CC medium and plated in 24-well plates. After 16–18 h of incubation, cells were treated with 0, 12.5, 25, 50, 75, 100 micromolars quercetin for 24 h in SRF medium. After the treatment, the cells were washed twice with PBS and re-cultured in CC medium. Subsequently, the cells were incubated for various times before they were counted by a hemocytometer.

3.5. Colony formation assay

Approximately 400 EJ cells were cultured in 60mm-Petri dishes at 37 °C and 5% CO₂ in a humidified incubator. Twenty four hours later, cells were treated with different concentrations of quercetin (0, 12.5, 25, 50, 75 and 100 micromolars) for another 24 h. Then, cells were washed twice with PBS and re-cultured in CC medium for 14 days. The cells were harvested for colony formation. Colonies were fixed with methanol, treated with Giemsa stain, and counted by using image analysis software. Photographs were taken using a digital camera (Canon, Tokyo, Japan).

3.6. Detection of apoptosis by two different methods

3.6.1. Hoechst 33258 staining

EJ, T24 and J82 cells were plated onto cover-slips in a 60mm Petri dish in CC medium for 24 h and followed by a treatment with 100 micromolars quercetin for another 24 h. Then, the cells were washed with PBS and fixed by 70% alcohol for 30 min. Fixed cells were washed with PBS three times for 5 min. Hoechst 33258 was added to 20 microliters, incubated for 30 min at room temperature, and observed by fluorescence microscope (Olympus BH, Japan).

3.6.2. TUNEL staining

EJ, T24 and J82 cells were cultured on cover-slides. After 24 h, cells were washed once with SRF medium and treated with 100 micromolars quercetin in fresh SRF

medium for another 48 h. Cells were fixed with phosphate buffer saline (PBS) containing 4% formaldehyde for 1 h at room temperature. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Wuhan boshide biotechnology). The detection of apoptosis was performed as described by the manufacturer. Slides were visualized under microscope (Olympus BH-2, Japan) and the apoptotic nuclei were stained in brown.

3.7. Cell cycle analysis

Cells (1×10^6) were suspended in CC medium and plated in plastic bottles (100 cm³). After 16–20 h incubation, the cells were treated with 0 and 100 micromolars quercetin for 24 h in SRF medium. At the end of treatment, the cells were trypsinized, collected and fixed with ice-cold 70% ethanol overnight at -20 °C. After centrifugation, the cell pellets were treated with 4 microgram/ml propidium iodide solution containing 100 microgram/ml RNase and 1% Triton X-100 for 30 min. Subsequently, the samples were analyzed in a FACS calibur system (BD Biosciences) using CellQuest software. The percentage of cell cycle phases was analyzed by the ModFit LT software (Version 2.0, BD Biosciences).

3.8. Methylation-specific polymerase chain reaction (MSP-PCR)

The methylation status of P16, Er-beta and RASSF1A genes was evaluated only on T24 cells which have been already shown hypermethylated for these genes. Cells were plated in the presence of quercetin (0 or 100 micromolars) and incubated for 3 days. Later, the cells were collected and submitted for the MSP-PCR technique. The protocol was carried out as described by Herman et al. (1996) (37). Briefly, DNA was isolated from samples and treated with sodium bisulfite, which is used to convert unmethylated cytosines to uracils, which are recognized as thymine by Taq polymerase, while 5-methylcytosines remained unaltered. DNA was purified with the Wizard DNA Purification System (Promega) followed by the amplification of bisulfite-modified DNA of the gene promoter. Primer pairs (described in Table 1) were purchased from Invitrogen Co (Carlsbad, CA, USA) and the PCR conditions were as followed: 95 °C, 15 min (Hot start) followed by 40 cycles (95 °C-30 s; 65 °C-30 s; 72 °C-40 s) with a final extension of 10 min at 72 °C. The PCR products were analyzed by the electrophoresis in 4% agarose gel, stained with ethidium bromide and visualized under UV illumination. The band intensities were analyzed using the Quantity One software, version 4.5.0 (Bio-Rad Laboratories, Hercules, CA).

3.9. Immunocytochemical staining

The cells were cultured on cover-slips, which were kept in a 60mm-Petri dish for 16–20 h before treatment. After treatment with or without 100 micromolars quercetin for 24 h, the cells were washed with isotonic PBS (pH 7.4) and then fixed in 4% paraformaldehyde solution in PBS for 1 h at 37 °C. Then, the cover-slips were washed three times with PBS, and nonspecific binding sites were blocked in PBS containing 10% normal goat serum, 0.3%

Table 1. Primer sequences used for methylation-specific PCR

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
P16 ^{INK4a}	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAACCACAACCATAA
	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACC GCGACCTAA
RASSF1	U: TTTGGTTGGAGTGTGTTAATGTG	U: CAAACCCCACTAAACAA
	M: GTGTAAACGCGTTGCGTATC	M: AACCCCGCGAACTAAACGA
Er-beta	U: TTGTTAGGAGGTAGTTGTAAGTGT	U: CCTACAACTCAAACCTCAAACA
	M: TAGGAGGTAGTTGTAAGCGC	M: ACAACTCAAACCTCCGAACG

M, represents methylated-specific primers; and U, unmethylated primers. RASSF1A, Ras Associated domain Family protein 1 isoform A; Er-beta, Estrogen receptor beta

Triton X-100 for 1 h. The cells were incubated with rabbit polyclonal anti-human Survivin antibody (1:200) and mouse monoclonal anti-mutant P53 and PTEN (1:200) in PBS containing 0.3% Triton X-100 and 10% normal goat serum overnight at 4 °C, respectively. Cells were subsequently washed three times with 0.3% Triton X-100 in PBS followed by an incubation with biotinylated goat-anti-rabbit and goat-anti-mouse IgG (1:250) in PBS containing 0.3% Triton X-100 and 10% normal goat serum. After 2–3 h of incubation at 37 °C, cells were washed three times with 0.3% Triton X-100 in PBS. The amplification of avidin-biotin-horseradish peroxidase complex was carried out (Avidin-Biotinylated enzyme complex/horseradish peroxidase, 1:100) at room temperature and the 3,3'-diaminobenzidine tetrachloride dihydrate was used as a detection system. The cells were washed three times with 0.3% Triton X-100 in PBS.

3.9. Statistical analysis

Data are presented as mean \pm SD (or SE) from at least three separated experiments. Statistical differences were evaluated using the one-way ANOVA and considered significant when $p < 0.05$.

4. RESULTS

4.1. Quercetin affects the cell viability

Human EJ, T24 and J82 bladder cancer cells were treated with 12.5, 25, 50, 75 and 100 micromolars of quercetin for 24 and 48 h, respectively. Cell viability and cell proliferation were assessed by the MTT assay. As shown in Figure 1, quercetin caused a dose- and time-dependent reduction in cell viability. This inhibition occurred irrespective of the cell line. An approximate 50% reduction in cell viability was seen at a dose of 100 micromolars after 48 h of incubation.

4.2. Quercetin inhibits cell growth and colony formation

In order to further confirm the growth inhibitory effect of quercetin-treated on bladder cancer cells, EJ cells were selected to detect growth curve status, which were plated (1×10^3) in 24-well plates and treated with quercetin (0, 25, 50, 100, 150, 200 micromolars) for 24h followed by 14 days of culture without treatment. Cell numbers were counted using a hemocytometer. As shown in Figure 2A, the level of colony formation of EJ cells was significantly inhibited by the increased concentrations of quercetin. The counting of colony formation number showed a significant dose-dependent reduction by quercetin (344.67 \pm 7.41, 253.67 \pm 4.50, 223.67 \pm 2.62, 196 \pm 1.63, 103.3 \pm

7.36 and 3.3 \pm 0.47 for 0, 12.5, 25, 50, 75 and 100 micromolars, respectively) (Figure 2B). There was a significant difference among groups ($p < 0.05$) and the concentrations of 150 and 200 micromolars quercetin completely blocked cell growth (data not shown).

4.3. Apoptosis induced by quercetin

To determine whether quercetin reduced cell number by inducing apoptosis, Hoechst 33258 assay and TUNEL were performed. Using Hoechst staining, an increased level of nuclear fragmentation and apoptotic bodies was detected in cells treated with quercetin (100 micromolars) (Figure 3A). The results of the *in situ* detection of apoptosis (TUNEL), represented in Figure 3B, shows a great number of brown-stained nuclei in the quercetin-treated group. These stained-nuclei correspond to apoptotic cells.

4.4. Quercetin affects the cell cycle

To further determine the possible involvement of quercetin in the regulation of the cell cycle, the effect of quercetin on bladder cancer cells was analyzed by flow cytometry. The results show that quercetin (100 micromolars) increased the G0/G1 fraction and decreased the G2/M fraction in EJ and T24 cells (Figure 4).

4.5. Quercetin decreases the DNA methylation levels of P16^{INK4a}, Er-beta and RASSF1A genes

The representative results of one experiment are summarized in Figure 5. As shown in the gel picture (Figure 5A), both control and treated groups contain the PCR products in the methylated and unmethylated genes. However, the signal intensity of methylated genes was strongly reduced in the presence of quercetin. In comparison with the control group (untreated cells), the reduction was about 35%, 57% and 73% for Erb, RASSF1A and P16^{INK4a}, respectively (Figure 5B).

4.6. Quercetin inhibits expression of mutant P53 and surviving proteins

To examine the expression of P53 and Survivin proteins in bladder cancer cells, EJ, T24 and J82 were plated onto cover-slips in a 60mm Petri dish in complete RPMI for 24h. These cells were treated with 100 micromolars quercetin for another 24h. After treatment, cells were subjected to immunocytochemical staining. As shown in Figure 6, the density of brown color represented the level of mutP53 and Survivin expression. Compared with the control, the expression of mutP53 and Survivin proteins in the treated cells was significantly decreased.

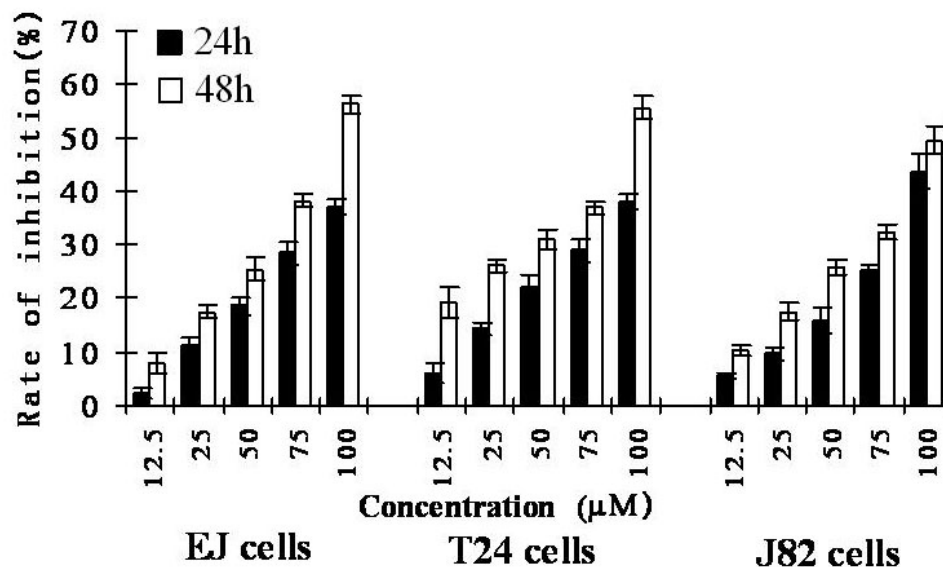


Figure 1. Inhibitory effect of quercetin on the proliferation of EJ, T24 and J82 cancer cells. Cells were treated with increasing concentrations of quercetin (12.5, 25, 50, 75 and 100 micromolars) for 24h and 48h. Cell survival was measured by MTT. Values are expressed as mean \pm SE of 4-6 experiments. For each cell line, there are significant differences between groups and within the same duration (24 and 48 h) of treatment ($p < 0.05$).

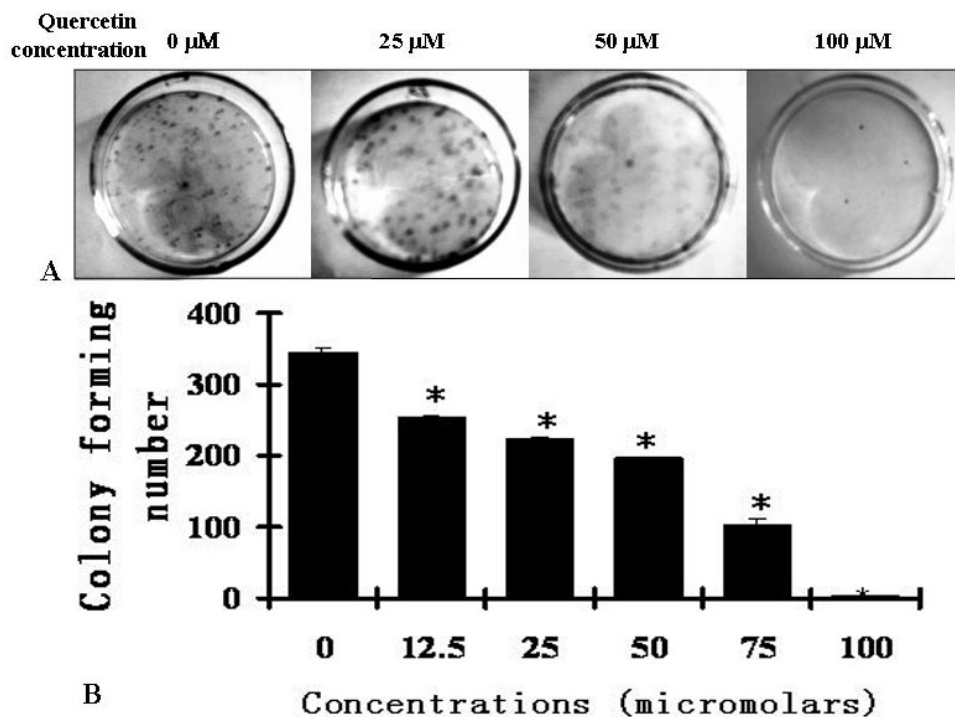


Figure 2. Effect of quercetin on EJ cell growth. (A) Approximately 400 EJ cells were plated in 60-mm Petri dishes and grown in complete RPMI 1640 (CC) medium. After 24h, cells were washed once with serum-free RPMI 1640 (SFR) medium and treated with different concentrations of quercetin in SFR. After 24h treatment, cells were washed once with SFR medium and cultured in complete RPMI 1640 for various times. (B) Cells were harvested for colony formation counting using Gene Snap Automatic Colony Counter Software. Colonies were defined as consisting of >16 cells. The bars represent the mean \pm SE of 4-6 experiments. Asterisk denotes significant differences between treatment groups ($p < 0.05$).

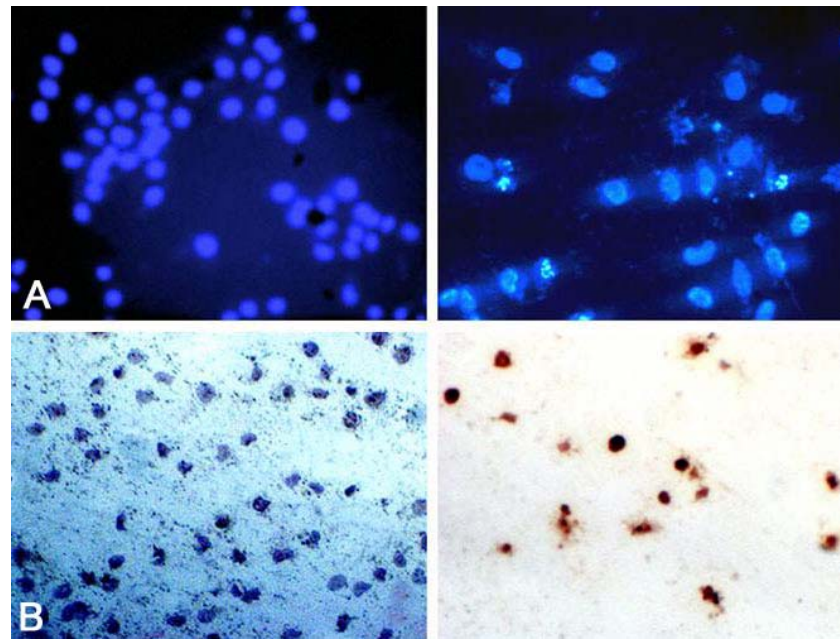


Figure 3. Detection of apoptosis in EJ cells by Hoechst-staining and TUNEL techniques. Cells were cultured on cover-slips and exposed to quercetin (0 or 100 micromolars) for 24 h. (A) Cells were washed, fixed, stained with Hoechst 33258, and then observed under a fluorescent microscope. (B) Cells were washed, fixed and submitted to the TUNEL technique. Slides of cells were visualized under a microscope (Olympus BH-2).

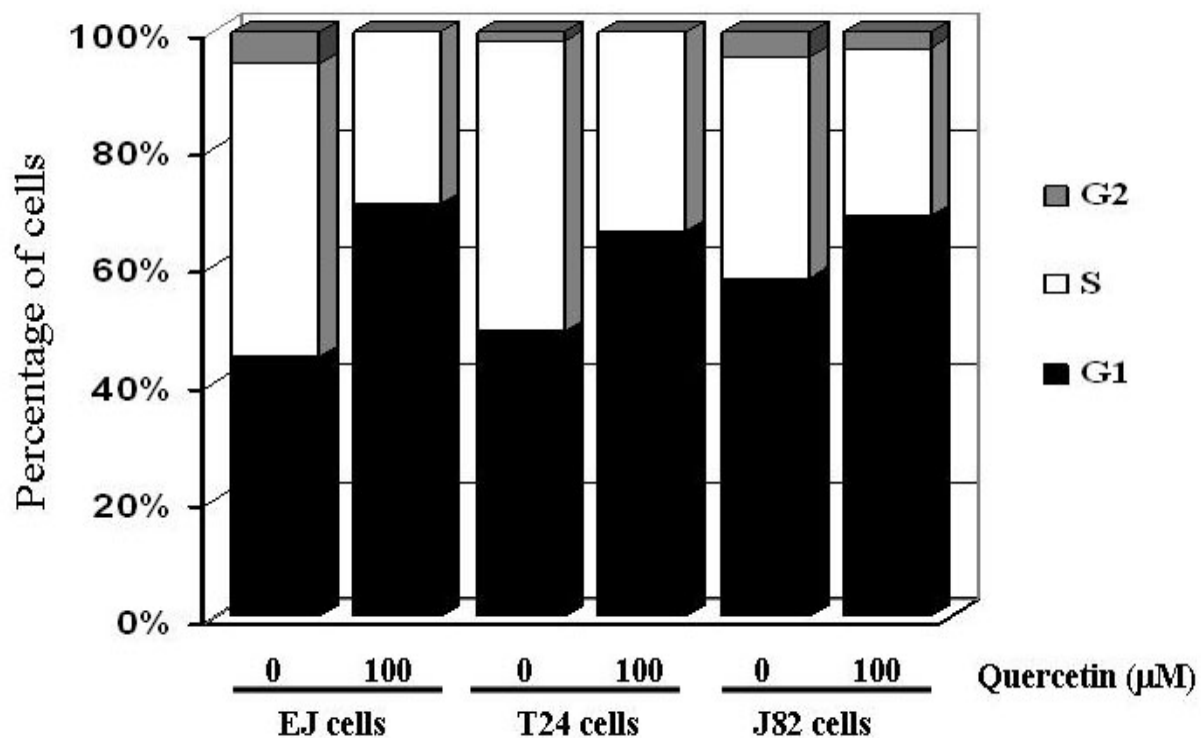


Figure 4. Effect of quercetin on EJ, T24 and J82 cell cycle progression. Cells were treated with quercetin (0 or 100 micromolars) for 24 h. Cells were harvested and stained with propidium iodide for cell cycle analysis using the flow cytometry. The data represent the mean \pm SE of 3 experiments.

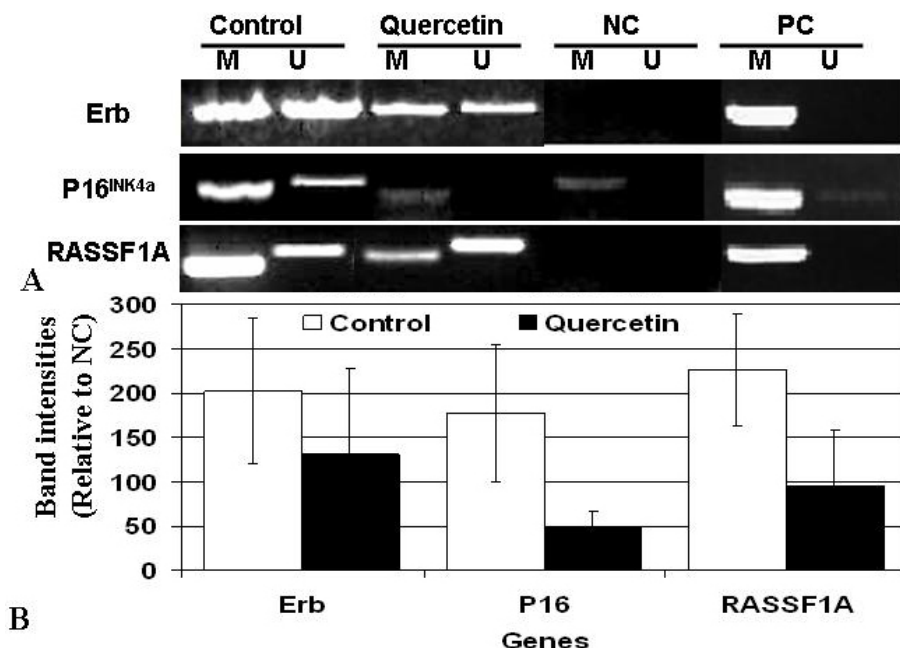


Figure 5. Effect of quercetin on hypermethylated genes in T24 cells. Cells were treated with quercetin (0 or 100 micromolars) for 72 h. The extracted DNA was subjected to the MSP assay using the primers for detecting methylated (M) or unmethylated (U) Er-beta (Erb), P16^{INK4a} and RASSF1A DNA. The RKO cells were used as a positive control (PC) for the presence of the three genes. Water was used instead of DNA as a negative control (NC). (A) The PCR products were separated on 4% agarose gel containing ethidium bromide and visualized under a UV light spectrophotometer. (B) Bands intensities of methylated genes (M) relative to negative control (NC) were then analyzed. The data represent the mean \pm SE of 3 experiments.

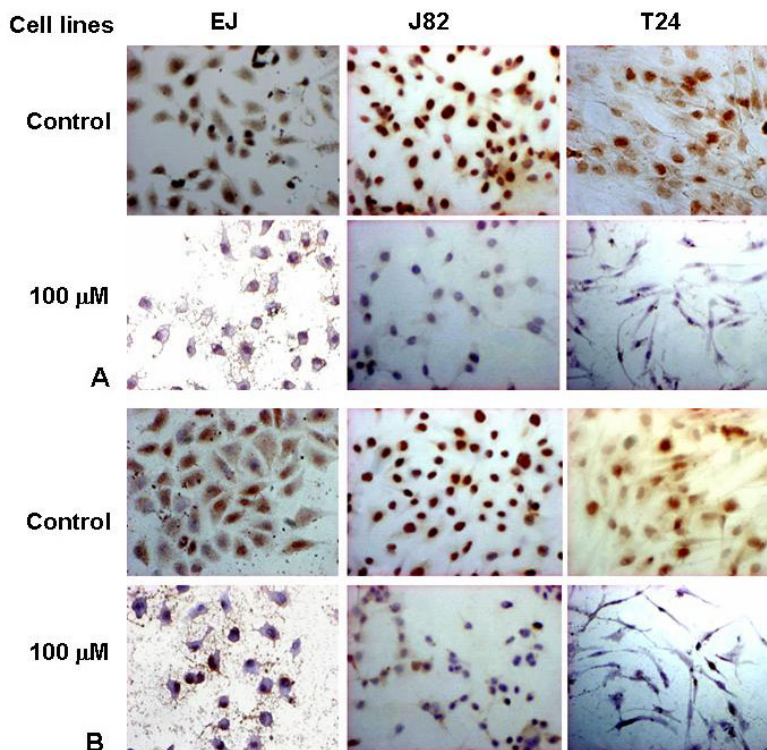


Figure 6. Detection of P53 and Survivin proteins by immunocytochemistry. Cells were treated with quercetin (0 or 100 micromolars) for 24 h. The brown-staining of cells is mainly located in the nuclei.

4.7. Quercetin does not affect the expression of tumor suppressor gene PTEN

In order to investigate the expression of PTEN, the three bladder cancer cells (EJ, T24 and J82) were plated onto cover-slips in a 60mm Petri dish in complete RPMI for 24h. These cells were treated with 100 micromolars quercetin for another 24h. Immunocytochemical staining was used to determine the expression of PTEN protein. Both control and treated cells showed a similar level in expression of PTEN protein (picture not shown).

5. DISCUSSION

The natural product, quercetin, belongs to an extensive class of polyphenolic flavonoid compounds, which are widely encountered in both fruits and vegetables. Citrus fruits, apples, onions, parsley, tea, and red wine are the primary dietary sources of quercetin. Also, olive oil, grapes, dark cherries and dark berries, (i.e. blueberries, blackberries, and bilberries) present a high content of flavonoids, including quercetin (38). Epidemiological studies have shown that the consumption of vegetables and fruits is associated with a low risk of cancer (36). Several studies have demonstrated the anti-proliferative and apoptotic effect of quercetin on different types of cancer cells. Protection against cancer development has also been reported in various animal models (39-42). Some researches have shown that flavonoids also act as enhancers of the effect of radiation (43). This present study shows that quercetin is able to inhibit the growth of human bladder cancer cells associated with an increase in apoptotic cells. To our knowledge, this study is the first to assess the effect of quercetin on bladder cancer.

The regulation of apoptosis is a complex process in which a number of cellular genes such as P53 are involved. P53 is known to regulate several events such as cell cycle arrest, apoptosis, and DNA repair in a variety of cells (18, 20). Mutations in the p53 gene are the most common genetic defect in human bladder cancer (24). Of particular significance, alterations in p53 are linked to cancer progression and response to radiation and chemotherapy (44). In this present work, the high expression of mutP53 protein observed in the studied cells (EJ, T24 and J82), is in agreement with a previous report (45). The decreased expression of mutP53 by quercetin suggests that the induced-apoptosis might be through a P53-dependent pathway. Further investigations are needed to find whether the downstream effectors of P53, such as P21 and bax are involved in quercetin-mediated apoptosis on bladder cancer cells.

Survivin belongs to the inhibitors of apoptosis (IAP) family, which have recently emerged as modulators of an evolutionarily conserved step in apoptosis. Survivin is present during embryonic and fetal development, but it is down-regulated in normal adult tissues. However, it becomes re-expressed in a variety of cancers (colorectal cancer, neuroblastoma, bladder cancer, non-small cell lung cancer, and breast cancer) (26-28). Because of these particularities (overexpression, ubiquitous), Survivin is recognized as a general target in cancer therapy (46). In

bladder cancer, its expression correlates with tumor grade, recurrence risk and survival. So, it has been suggested as a novel diagnostic/prognostic marker of bladder cancer. The rather poor responses to conventional treatment for bladder cancer require novel, specific therapy approaches. In this current study, the deletion or lower expression of Survivin protein occurred after quercetin treatment for 24h. The down-regulation of bladder cancer associated protein may represent a new option to specifically inhibit bladder cell growth and induce cell death.

The tumor suppressor gene phosphatase and tensin homolog (PTEN), located on chromosome 10, is a phosphatase in the phosphatidylinositol 3'-kinase (PI3K)-mediated signal transduction pathway (47). PTEN inhibits the activation of Akt, a serine-threonine kinase involved in proliferative metabolic and anti-apoptotic pathways, and has tumor suppressive properties (34-35). The PTEN gene has been recently identified as a tumor suppressor candidate, since it is implicated in multiple tumor types based on mutations or homozygous deletions of the gene in certain human cancers (48, 49). PTEN is deleted or mutated in sporadic cases of glioblastoma, breast cancer, kidney cancer, melanoma, and endometrial cancer (50-53). A previous report has shown a correlation between the inhibition of prostate cancer cell colony formation and up-regulation of PTEN gene after a quercetin treatment (35). These results contrast with those reported here. Indeed, there is no expression of PTEN protein either in untreated or quercetin treated cells in our study. This could suggest that the bladder cell lines do not express PTEN and thus, eliminates the possibility of involvement of PTEN in the quercetin induced-cell growth inhibition and apoptosis in bladder cancer cell lines used in the present study.

Hypermethylation of tumor suppressor genes is believed to play an important role in the initiation and progression of many cancers (2). DNA methylation is the only postsynthetic DNA modification that occurs in mammalian cells. The hypermethylation occurs mostly in the CpG islands of the promoter region of genes, and results to an inhibition or silencing of the gene transcription (54). The methylation of normally unmethylated CpG islands has been associated with a variety of human tumors (55, 56), and an alteration of methylation patterns can result in abnormal gene expression. Here, we studied the methylation status of three genes involved in tumor suppression through the cell cycle control (P16INK4a or CDKN2A and RASSF1a for Ras associated domain family protein 1 isoform A), and estrogenic transduction (estrogen receptor beta Er-beta or ESR2). These genes were found hypermethylated in T24 cancer cells, which is in agreement with other reports in various human cancers (57, 58). The partial demethylation of these genes by quercetin most likely restores their transcriptional activity, leading to the synthesis of cellular death-associated proteins. This result suggests the participation of P16, RASSF1A and Er-beta in the quercetin-induced cell growth arrest and apoptosis in T24 cells.

6. CONCLUSIONS

In summary, the natural phytochemical, quercetin, can inhibit the growth and proliferation of bladder cancer

cells and induce apoptosis, which is associated with a decrease in DNA methylation levels of P16, RASSF1A and Er-beta genes, and a reduction in the expression of mutP53 and Survivin protein. Quercetin is a potential chemopreventive and chemotherapeutic agent for bladder cancer. Further research is needed to investigate the downstream molecular mechanism associated with the demethylated-genes as well as those related to the cell cycle.

7. ACKNOWLEDGMENTS

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Abbreviations: PTEN: phosphatase and tensin homolog, RASSF1A: Ras Associated domain Family protein 1 isoform A, Erb: Estrogen Receptor Beta or ESR2

Key Words: Quercetin, Bladder cancer, DNA methylation, PTEN, Apoptosis, Cell cycle, p21, RASSF1A, Er-beta

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