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Sensitization to docetaxel in prostate cancer cells by green tea and quercetin☆,☆☆

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Abstract

Chemotherapy with docetaxel (Doc) is a standard treatment for metastatic and castration-resistant prostate cancer. However, chemoresistance and side effects of Doc limit its clinical success. We investigated whether natural products green tea (GT) and quercetin (Q), a flavonoid from apples and onions, will enhance the efficacy of Doc in androgen-independent (AI) prostate cancer cells. Two cell lines including LAPC-4-AI and PC-3 were treated *in vitro* with 40 μM of (−)-epigallocatechin gallate (EGCG), 5 μM of Q, 2 or 5 nM of Doc alone or in combination. The mixture of EGCG+Q+Doc increased the antiproliferative effect by threefold in LAPC-4-AI cells and eightfold in PC-3 cells compared to Doc alone. EGCG, Q and Doc in combination significantly enhanced cell cycle arrest at G2/M phase and increased apoptosis in both LAPC-4-AI and PC-3 cells compared to Doc alone. The mixture increased the inhibition of PI3K/Akt and the signal transducer and activator of transcription (Stat) 3 signaling pathways compared to Doc alone, and decreased the protein expression of multidrug resistance-related protein. In addition, the combination with EGCG and Q increased the inhibition of tumor cell invasion and colony formation in both LAPC-4-AI and PC-3 cells compared to Doc alone, and decreased the percentage of $CD44^+/CD24^-$ stem-like LAPC-4-AI cells. In summary, GT and Q enhanced the therapeutic effect of Doc in castration-resistant prostate cancer cells through multiple mechanisms including the down-regulation of chemoresistance-related proteins. This study provides a novel therapeutic modality to enhance the efficacy of Doc in a nontoxic manner.

Keywords: Green tea polyphenol; Quercetin; Docetaxel; Prostate cancer; Combination

1. Introduction

Prostate cancer is the most commonly diagnosed male malignancy and the second-leading cause of cancer death among men in the United States [1]. Androgen deprivation therapy (ADT) remains the main treatment for advanced and metastatic prostate cancer [2]. However, despite initial response, nearly all patients on ADT progress to castration-resistant prostate cancer (CRPC) in 18–24 months and no curative treatments currently exist for CRPC [3]. Chemotherapy with docetaxel (Doc) is currently a standard treatment for metastatic and CRPC and remains a backbone in current drug development [4]. Doc, a member of the family of taxanes, is an analogue of paclitaxel, a naturally occurring mitotic inhibitor isolated from the bark of the Pacific yew tree *Taxus brevifolia*. Doc binds to microtubules causing mitotic arrest and ultimately cell apoptosis [5]. However, the development of chemoresistance to Doc is observed in most patients and limits its clinical success [5]. The up-regulation of multidrug resistance (MDR) phenotypes including p-glycoprotein and MDR-associated proteins (MRPs) may be one of the mechanisms of Doc resistance [5]. In addition, the alterations in signaling pathways may cause resistance to Doc-induced apoptosis [5]. For example, the overexpression of antiapoptotic gene Bcl-2 and activation of nuclear factor-kappa B (NFκB) and Akt activity are commonly observed in CRPC patients undergoing Doc treatment [4,6]. The median progression-free survival with Doc treatment remains around 6 months and overall survival less than 2 years [4]. In addition, some severe side effects are associated with Doc treatment including the suppression of bone marrow function leading to immunodysfunction and anemia [7]. Clearly, it is of high clinical significance to enhance the efficacy of Doc at lower doses in a less toxic manner and to reduce its side effects.

Abbreviations: AR, androgen receptor; AI, androgen-independent; ATP, adenosine triphosphate; CRPC, castration-resistant prostate cancer; GT, green tea; GTPs, green tea polyphenols; EGCG, (−)-epigallocatechin-3-gallate; FBS, fetal bovine serum; MRP, multidrug resistance-associated protein; mTOR, mammalian target of rapamycin; NFκB, nuclear factor-kappa B; PI3K, phosphatidylinositol 3-kinases; Q, quercetin; SCID, severe combined immunodeficiency; Stat, signal transducer and activator of transcription.

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Green tea (GT) is produced from the leaves of the plant *Camellia sinensis*. The major bioactive components of GT are GT polyphenols (GTPs), mainly including (−)-epigallocatechin, (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin and (−)-epicatechin-3-gallate, with EGCG as the most abundant and most bioactive component [8]. The anticancer activities of GTPs have been demonstrated in several cancers including the prostate, mammary gland, colon, pancreas, liver, esophagus and liver cancer [8,9]. GTPs target multiple signaling pathways in anticarcinogenesis including the inhibition of NFκB and PI3K/Akt pathways, the induction of apoptosis and cell cycle arrest [9–12]. Oral infusion of GTPs equivalent to a realistic dose for human consumption (4–6 cups of tea daily for an average adult human) significantly inhibited prostate cancer development and distant site metastasis in transgenic adenocarcinoma in mouse prostate (TRAMP) mouse models and increased their overall survival when GT was administered during tumor initiation [13]. A 1-year GT extract intervention in men with high-grade prostate intraepithelial neoplasia showed a lower prostate cancer incidence of 3% in the tea group consuming 600 mg/day GT extracts vs. 30% in the placebo group [14]. Likewise, in a preprostatectomy trial of a GT supplement, McLarty et al. [15] demonstrated a decrease in serum prostate-specific antigen levels and decreased prostate tissue vascular endothelial growth factor and hepatocyte growth factor concentrations. Nevertheless, the low bioavailability and extensive methylation of GTPs *in vivo* to less active metabolites limit the anticancer activity of GT [9,16]. We were able to demonstrate that the combined use of quercetin (Q) with GT significantly increased the bioavailability and cellular uptake of GTPs and decreased their methylation *in vitro* and *in vivo*, possibly through the inhibition of MRPs and catechol-*O*-methyltransferase (COMT), leading to a synergistically enhanced inhibition of xenograft prostate tumor growth in severe combined immunodeficiency (SCID) mice [17–19]. Q is a flavonoid found in most edible vegetables and fruits particularly in onions, apples, and red wine. The inhibitory effects of Q on MRPs, p-glycoprotein and COMT have been well documented [20–23]. Q itself has exhibited chemopreventive activities, especially in prostate cancer through multiple mechanisms including the induction of apoptosis and the inhibition of proliferation and insulin-like growth factor-1 pathway [24–27].

In respect to the multiple targeting activities of GTPs and Q in anticarcinogenesis particularly their activities on NFκB, PI3K/Akt pathways and MRPs, they can be ideal candidates to be combined with Doc to enhance the therapeutic effect in a nontoxic manner. In the present study, we investigated the combined therapeutic effect of the mixture of EGCG, Q and Doc in androgen-independent LAPC-4-AI and PC-3 prostate cancer cells. This study is anticipated to provide a novel modality to improve clinical practice in treatment of CRPC with enhanced drug efficacy and reduced side effects.

2. Materials and methods

2.1. Cell line and cell culture

The AI PC-3 human prostate cancer bone metastasis cell line was purchased from American Type Culture Collection (ATCC, Chicago, IL, USA). The localized prostate cancer LAPC-4 cell line is a gift from Dr. Charles Sawyers' laboratory at UCLA. Androgen-independent LAPC-4-AI cells were developed by culturing androgen-dependent LAPC-4 cells in medium supplemented with androgen free charcoal-stripped fetal bovine serum (FBS). The proliferation of parental LAPC-4 cells was decreased by 20% after 96-h culture in androgen-free medium with fresh medium changed every 2 days, compared to that in regular medium. However, the growth of LAPC-4-AI cells was not reduced in androgen free medium. The LAPC-4-AI cells were used in this study in addition to PC-3 cells. Both cell lines were cultured in RPMI 1640 medium, supplemented with 10% (vol/vol) of FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 incubator. Normal human prostate epithelial PrEC cells were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA) and maintained in prostate epithelial cell PrEGM medium (Lonza Walkersville, Inc.).

2.2. Cell proliferation assay

LAPC-4-AI and PC-3 cells were seeded into opaque-wall 96-well plates at a density of 8×10^3 per well. An inhibition curve was achieved for individual compound including EGCG, Q and Doc by incubation of both cell lines with multiple doses of each compound for 48 h. A dose that leads to 10%–30% cell growth inhibition by each compound was selected for the combination study. Cells were treated with the following: vehicle control (DMSO), 40 μM EGCG (Sigma-Aldrich, St Louis, MO, USA), 5 μM Q (Sigma-Aldrich), 5 nM Doc (Sigma-Aldrich), EGCG+Q, EGCG+Doc, Q+Doc or EGCG+Q+Doc for 24 and 48 h. In addition, the combined effect of the mixture was compared with a higher dose of Doc at 20 nM. Cell proliferation was measured with adenosine triphosphate (ATP) assay using the CellTiter-Glo Luminescent cell viability assay kit (Promega Corporation, Madison, WI, USA). To minimize the effect of hydrogen peroxide (H_2O_2) that may be formed by autoxidation and/or dimerization of EGCG and Q in cell culture medium [28], 50 U/ml of catalase was added to the medium prior to EGCG, Q and Doc in all the experiments in the present study. There were four wells for each of the treatments, and the experiment was repeated twice.

2.3. Cell cycle and apoptosis analysis

When 50%–60% confluent in T25 flasks, both LAPC-4-AI and PC-3 cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. Cells were trypsinized and monolayers attaching to the bottom were collected. The procedures for cell cycle and apoptosis analysis using a small cytometer Cellometer Vision (Nexcelom Bioscience LLC, Lawrence, MA, USA) were described previously [29,30] with minor modifications. Briefly, for cell cycle assay, cells were centrifuged and pellet was resuspended and fixed in cold methanol. The cells were centrifuged again, and pellet was stained in propidium iodide (PI) solution (Nexcelom Bioscience LLC) for imaging cytometry using Cellometer Vision. For apoptosis assay, cells were centrifuged and pellets were resuspended in Annexin V binding buffer and double-stained with Annexin V–FITC (Nexcelom Bioscience LLC) and PI (Nexcelom Bioscience LLC) for Cellometer analysis. A positive control was generated by heating cells in a 45°C water bath for 10 min. Nontreated cells were used as negative control. Both controls were processed with the samples. The fluorescence data generated by the Cellometer software were converted into FCS files and analyzed by De Novo FCS Express 4 software (Los Angeles, CA, USA). The experiment was performed in triplicate.

2.4. Western blot analysis of protein biomarkers

LAPC-4-AI and PC-3 cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. Total protein was extracted using RIPA buffer (Santa Cruz Technology, Santa Cruz, CA, USA). The procedure for Western blot analysis was described before [31]. Briefly, 50 μg of protein was separated on a 4%–12% Bis–Tris gel (Invitrogen, Carlsbad, CA, USA). Proteins were electrotransferred to nitrocellulose membranes. Membranes were incubated with primary antihuman antibodies for the detection of Bax (sc-493), Bcl-2 (sc-509), MDR-related protein (MRP1; sc-7773; Santa Cruz Technology), Akt (4685), p-Akt (Ser473, 4058), STAT3 (9132) and p-STAT3 (4058, Cell Signaling Technology, Danvers, MA, USA). GAPDH protein was used as loading control. Protein was visualized and analyzed using a ChemiDoc XRS chemiluminescence detection and imaging system (Bio-Rad Laboratories, Irvine, CA, USA).

2.5. Cell invasion assay

The ability of EGCG and Q to enhance the effect of Doc in inhibition of cell invasion was tested in LAPC-4-AI and PC-3 cells using transwell chamber assay. The chamber is 24-well plate based with an insert of 8-μm pore size polyethylene terephthalate membrane (Corning Life Sciences, Tewksbury, MA, USA). Cells were cultured until 50%–60% confluency and treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. The cells were starved in serum-free medium overnight. The transwell chamber insert was coated with 20 μl of 1:6 diluted Matrigel (BD, Cambridge, MA, USA) and incubated in 37°C for 20 min to solidify. After trypsinization 1×10^5 cells were collected, suspended in 200 μl serum-free medium and added on the upper well. Three hundred microliters of complete growth medium was added to the bottom. After a 20-h incubation, cells were fixed with 5% glutaraldehyde and stained with 0.5% toluidine blue as described previously [32]. Cells on the upper membrane were wiped off with a cotton swab. Invaded cells on the lower membrane were counted under a microscope at ×200 magnification. Three fields were randomly selected and counted for each well. The experiment was performed in triplicate.

2.6. Tumor cell colony formation assay

LAPC-4-AI and PC-3 cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. A 24-well plate was coated with 200 μl Matrigel per well and incubated in 37°C for 20 min. After trypsinization, 2×10^4 cells in 200 μl complete medium were added to each well. Cells were incubated for 5 days. Two hundred microliters of fresh medium was added every 2 days. Pictures were taken from three fields of each well under a microscope at ×100 magnification. The number of cell colony which contained at least 10 cells was counted. The experiment was performed in triplicate.

2.7. Flow cytometry analysis of CD44/CD24 surface markers

$CD44^+/CD24^-$ prostate cancer cells have been shown to possess stem cell characteristics and are more proliferative, clonogenic, tumorigenic and metastatic than $CD44^-/CD24^-$ cells [33]. We evaluated the ability of the combination treatment to modulate the cell expression of these surface markers using flow cytometry analysis. LAPC-4-AI and PC-3 cells were treated

with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. Cells were trypsinized and 1×10^6 cells were collected and suspended in 1 ml of fresh growth medium. The cells were double-stained with 20 μl of each of CD24–PE and CD44–FITC conjugates (Nexcelom Bioscience LLC, Lawrence, MA, USA) and incubated at 4°C on a rocker for 1 h. The cells were centrifuged at 2000 rpm for 5 min and resuspended in 500 μl PBS for flow cytometry analysis on a BD LSRFortessa X-20 Cytometer (BD Biosciences, San Jose, CA, US). The data were analyzed using FACSDiva 7.0 software (BD Biosciences). The experiment was performed in triplicate.

2.8. Statistical analysis

The statistical analyses were performed using SPSS software (Version 20.0; Chicago, IL, USA). Data were presented as mean±standard deviation (S.D.). Comparison of means was performed by one-way analysis of variance with Tukey's posttest for paired comparisons. Differences were considered significant if $P<.05$.

3. Results

3.1. Enhanced antiproliferative effect

Both EGCG and Q significantly increased the antiproliferative effect of Doc in LAPC-4-AI and PC-3. The strongest antiproliferative effect was achieved by treatment with a mixture of the three chemicals (Fig. 1). In LAPC-4-AI cells, treatment with EGCG and Q with Doc 5 nM inhibited proliferation to the same extend as Doc 20 nM alone. At 48 h, the growth of LAPC-4-AI cells was inhibited by 19% (EGCG), 18% (Q), 21% (5 nM Doc), 36% (EGCG+Q), 41% (EGCG+Doc), 40% (Q+Doc) and 57% (EGCG+Q+Doc). PC-3 cells were less sensitive to Doc than LAPC-4-AI, but the combination treatment with EGCG, Q and Doc 5 nM exhibited a much stronger antiproliferative effect as compared to all individual treatments. At 48 h, PC-3 cell growth was inhibited by 5% and 11% by Doc at 5 nM and 20 nM, respectively. However, the combination of 5 nM Doc with EGCG and Q inhibited PC-3 cell growth by 42% (Fig. 1).

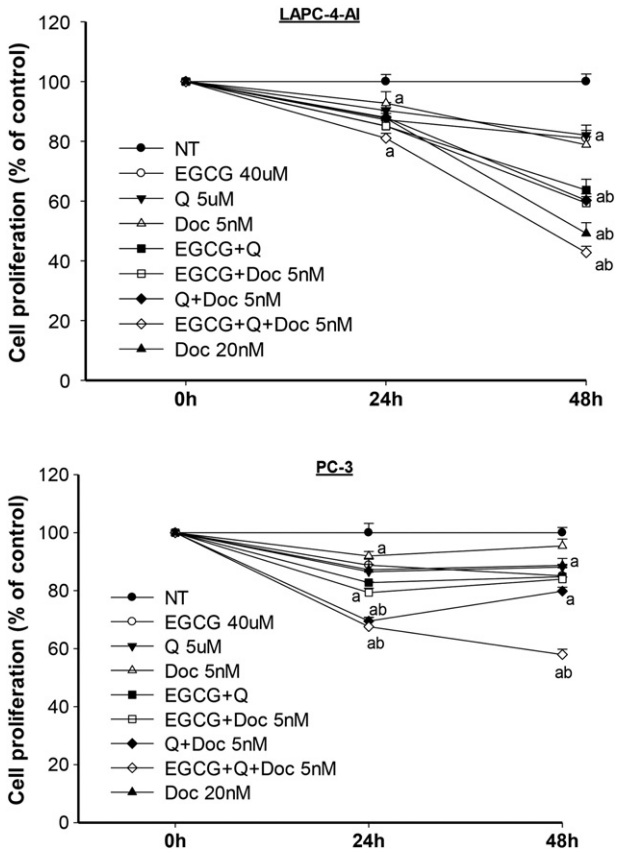

Fig. 1. EGCG and Q in combination with Doc enhanced the antiproliferative effect in AI LAPC-4-AI and PC-3 cells. Cells were treated with the indicated concentrations of EGCG, Q and Doc alone or in combination for 24 and 48 h. Cell proliferation was measured by ATP assay. Data are presented as mean±S.D. NT: nontreatment, DMSO control. Compared to (a) NT, (b) EGCG, Q or low dose of Doc, $P<.05$.

Table 1
Cell cycle distribution and apoptosis in LAPC-4-AI cells

Treatment	Cell cycle distribution (%)			Apoptosis (%)
	G0/G1	S	G2/M	
NT	71.8 ± 1.7^a	13.1 ± 2.1^a	14.9 ± 0.9^a	2.2 ± 0.2^a
EGCG+Q	63.4 ± 3.4^b	16.0 ± 2.1^{ab}	19.9 ± 2.1^b	17.6 ± 1.3^b
Doc 5 nM	60.0 ± 3.3^b	17.4 ± 2.6^{ab}	21.7 ± 2.6^b	7.6 ± 0.4^c
EGCG+Q+Doc	52.6 ± 2.2^c	18.4 ± 0.7^b	28.7 ± 1.4^c	15.1 ± 0.7^b

Androgen-independent LAPC-4-AI cells were cultured in T25 flasks until 50%–60% confluent. Cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. Cells were trypsinized and the monolayer attached to the bottom was collected for cell cycle and apoptosis analysis using a small cytometry system Cellometer Vision. Cells were stained with PI for cell cycle assay and double-stained with Annexin V–FITC and PI for apoptosis assay. The experiment was performed in triplicate and repeated twice with similar results. Values from one representative experiment were presented in mean±S.D. Values with different superscripts in each of the columns are significantly different. NT: nontreatment, DMSO control.

A combination index (CI) was calculated for the mixture of all three chemicals using the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) which is based on the widely accepted Chou–Talalay equation and mass–action law [34]. The value of CI less than 1 indicates a synergistic effect of a combination, equal to 1 additive and greater than 1 antagonistic [34]. The combination of a series of concentrations of EGCG (40–60 μM) and Q (5–10 μM) with Doc (2-5 nM) achieved CIs of 0.6–0.8 in LAPC-4-AI cells and 0.6–0.7 in PC-3 cells.

3.2. Effect on cell cycle arrest and apoptosis

The strongest effect observed on LAPC-4-AI cells by treatment with EGCG+Q was a eightfold increase in apoptosis, whereas the addition of Doc 5 nM had a stronger effect on cell cycle arrest in the G2/M phase, which was further increased by the combination treatment with EGCG, Q and Doc (Table 1). In PC-3 cells, treatment with EGCG and Q did not induce apoptosis as much as in LAPC-4-AI cells, but the combination treatment with all 3 compounds induced apoptosis threefold (Table 2).

3.3. Modulation on protein expression involved in apoptosis, proliferation and drug resistance

The combination treatment of EGCG, Q and Doc significantly increased the ratio of Bax/Bcl-2 protein expression compared to control in both LAPC-4-AI and PC-3 cells, mainly through decreasing the expression of

Table 2
Cell cycle distribution and apoptosis in PC-3 cells

Treatment	Cell cycle distribution (%)			Apoptosis (%)
	G0/G1	S	G2/M	
NT	72.9 ± 0.8^a	8.9 ± 1.1^a	17.9 ± 0.4^a	3.5 ± 0.2^a
EGCG+Q	66.3 ± 0.7^b	11.9 ± 0.2^b	21.5 ± 1.0^b	4.9 ± 0.3^b
Doc 5 nM	71.2 ± 0.5^a	10.0 ± 1.3^a	18.0 ± 0.2^a	5.5 ± 1.1^b
EGCG+Q+Doc	62.6 ± 0.5^c	11.8 ± 0.6^b	24.5 ± 1.1^c	10.1 ± 0.7^c

PC-3 cells were cultured in T25 flasks until 50%–60% confluent. Cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc for 48 h. Cells were trypsinized and monolayer attaching to the bottom was collected for cell cycle and apoptosis analysis using a small cytometor Cellometer Vision. Cells were stained with PI for cell cycle assay and double-stained with Annexin V–FITC and PI for apoptosis assay. The experiment was performed in triplicate and repeated twice with similar results. Values from one representative experiment were presented in mean±S.D. Values with different superscripts in each of the columns are significantly different. NT: nontreatment, DMSO control.

Bcl-2 (Fig. 2). The three chemicals in combination significantly increased the inhibition of the phosphorylation of both Akt and the signal transducer and activator of transcription (Stat) 3 compared to EGCG+Q or Doc alone in both cell lines. The mixture also increased the inhibition of Akt and Stat3 protein expression in LAPC-4-AI cells and PC-3 cells, respectively, compared to EGCG+Q or Doc alone. In LAPC-4-AI cells, only the combination of all three compounds significantly inhibited the protein expression of MRP1, while in PC-3 cells, EGCG+Q had a stronger effect on MRP1 protein expression compared to the treatment with all three compounds (Fig. 2).

3.4. Inhibition of tumor cell invasion

The invasion of LAPC-4-AI cells through Matrigel was inhibited by 53% (EGCG+Q), 50% (Doc 5 nM) and 74% (EGCG+Q+Doc) compared to control (Fig. 3). In PC-3 cells, Doc alone did not inhibit the cell invasion compared to control. However, both EGCG+Q and the combination of Doc with EGCG and Q significantly inhibited PC-3 cell invasion by 20% compared to control or Doc alone (Fig. 3).

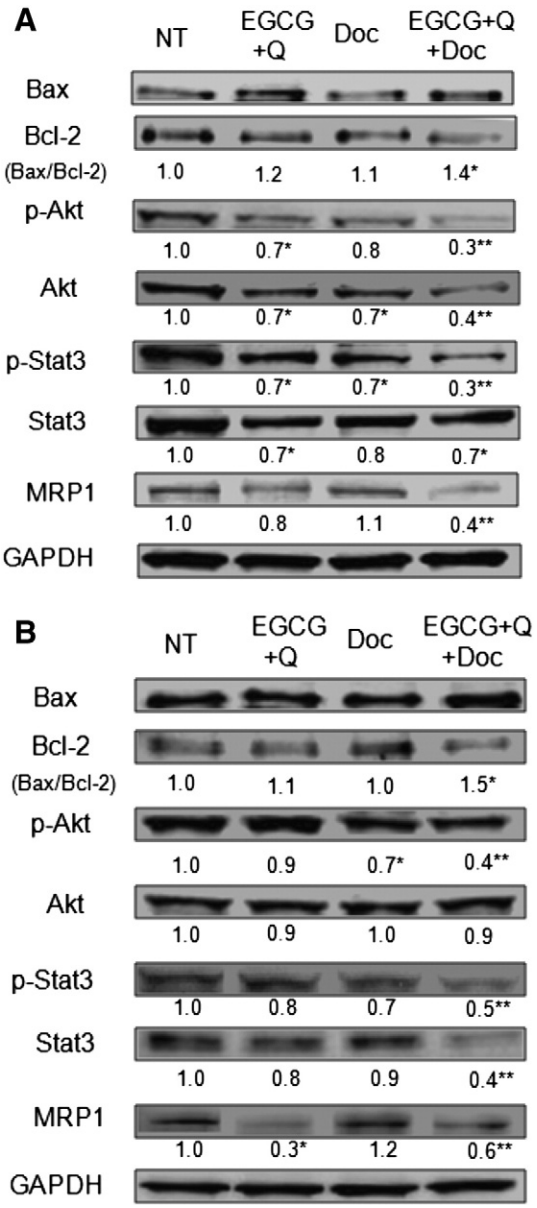

Fig. 2. Modulations on the expression and phosphorylation of proteins involved in apoptosis, proliferation and drug resistance. LAPC-4-AI (A) and PC-3 (B) cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc at same concentrations for 48 h. Protein expression and phosphorylation were analyzed with Western blot. Data are presented as mean values. *Compared to NT; **compared to NT, GT+Q or Doc group, $P<.05$. NT: nontreatment, DMSO control.

3.5. Inhibition of colony formation

The combination of EGCG, Q and Doc significantly enhanced the inhibition of tumor cell colony formation in LAPC-4-AI cells compared to EGCG+Q or Doc alone (Fig. 4). After 5 days, the formation of tumor colony was inhibited by 36% (EGCG+Q), 27% (Doc 5 nM) and 86% (ECGG+Q+Doc) in LAPC-4-AI cells. In PC-3 cells, treatment with EGCG+Q demonstrated a twofold stronger effect compared to Doc in inhibition of tumor colony formation (Fig. 4). The formation of tumor colony was inhibited by 66% (GT+Q), 32% (Doc) and 77% (GT+Q+Doc) in PC-3 cells after the 5-day incubation.

3.6. Modulation on surface marker expression

Doc alone slightly and not significantly decreased the percentage of $CD44^+/CD24^-$ cells in LAPC-4-AI cells compared to control (Fig. 5). Treatment with EGCG+Q did not change the expression of these surface markers. However, the combination of EGCG and Q with Doc significantly decreased the percentage of $CD44^+/CD24^-$ LAPC-4-AI

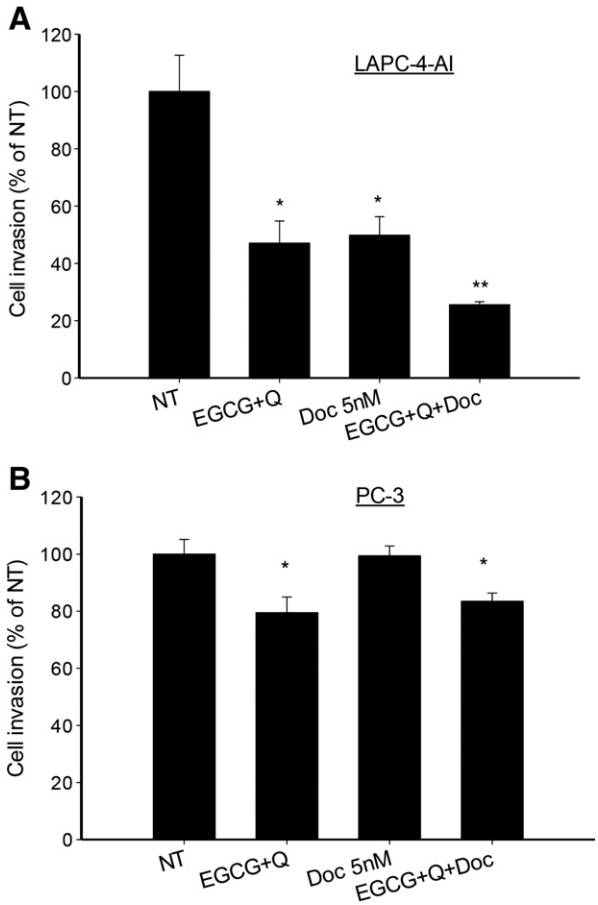

Fig. 3. EGCG and Q in combination with Doc enhanced the inhibition of tumor cell invasion. LAPC-4-AI (A) and PC-3 (B) cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc at same concentrations for 48 h. The cells were starved in serum-free medium overnight. Then the cells were seeded on the upper membrane of transwell chamber which was precoated with Matrigel. Complete growth medium was added to the bottom. After a 20-h incubation, cells on the lower membrane of chambers were stained and counted. Data are presented as mean±S.D. NT: nontreatment, DMSO control. *Compared to NT or Doc treatment, $P<.05$.

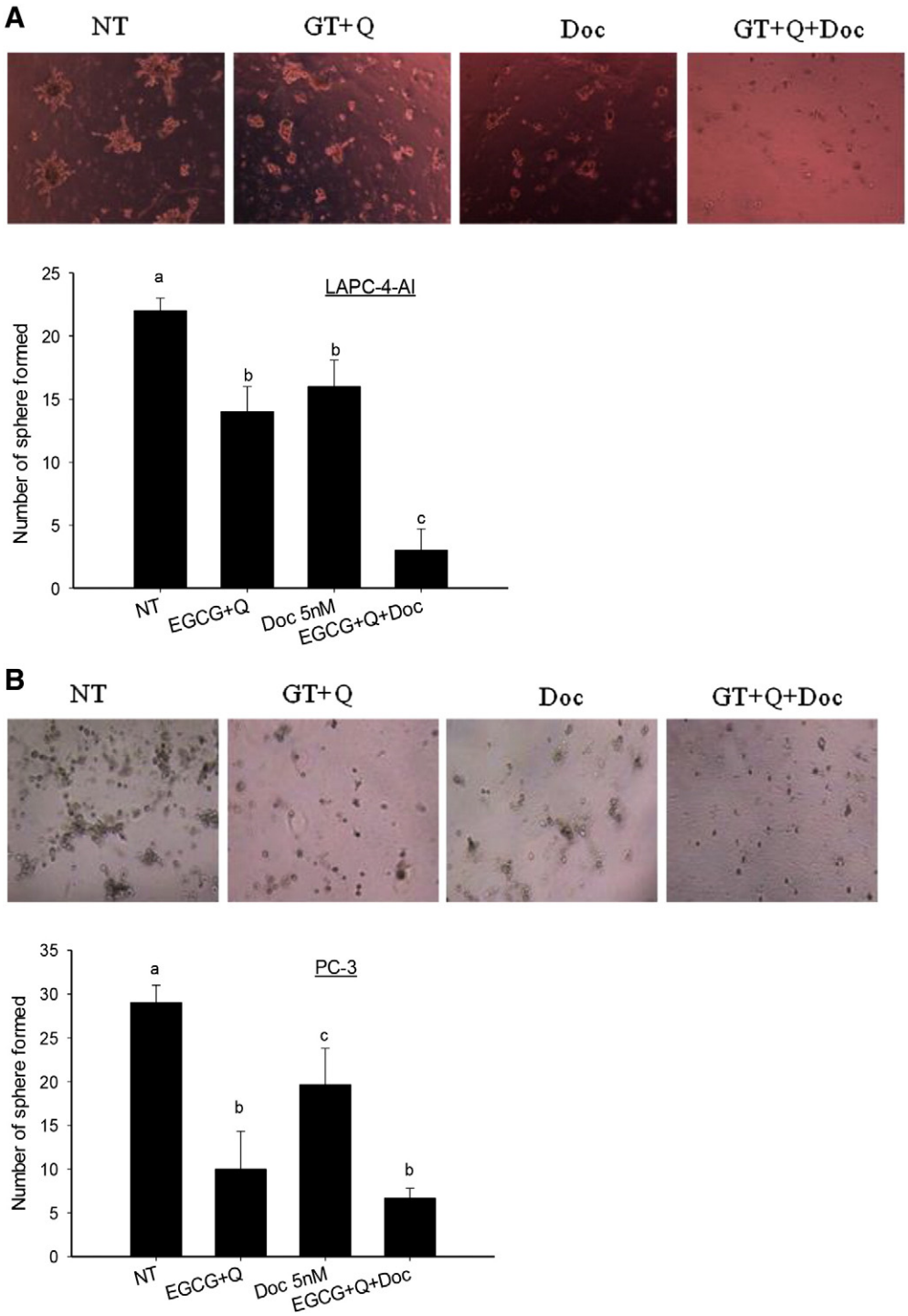

Fig. 4. EGCG and Q in combination with Doc enhanced the inhibition of tumor cell colony formation. LAPC-4-AI (A) and PC-3 (B) cells were treated with vehicle control, 40 μM EGCG +5 μM Q, 5 nM Doc or EGCG+Q+Doc at same concentrations for 48 h. Then the cells were seeded onto 24-well plate which was precoated with Matrigel. Fresh medium was changed every 2 days, and the cells were allowed to grow for 5 days. The number of colonies containing at least 10 cells was counted. Data are presented as mean±S.D. NT: nontreatment, DMSO control. Compared to NT, $^{*}P<.05$ and $^{**}P<.01$.

cells compared to control, EGCG+Q or Doc alone (Fig. 5). There was no effect observed in PC-3 cells with any treatment in modulation of these surface markers.

4. Discussion

The present study demonstrates that a novel regimen by combining natural products GT and Q with Doc significantly enhanced the therapeutic effect of Doc in CRPC cells. Both GT and Q were able to increase the antiproliferative effect of Doc, and the strongest effect was achieved by the combination of the three chemicals. Doc is currently a standard first-line treatment for CRPC usually used in combination with prednisone. An enhanced efficacy of Doc along with reduced side effects will provide significant benefits to CRPC patients to improve survival and quality of life. Several chemotherapy drugs have been tested for combination with Doc to improve CRPC

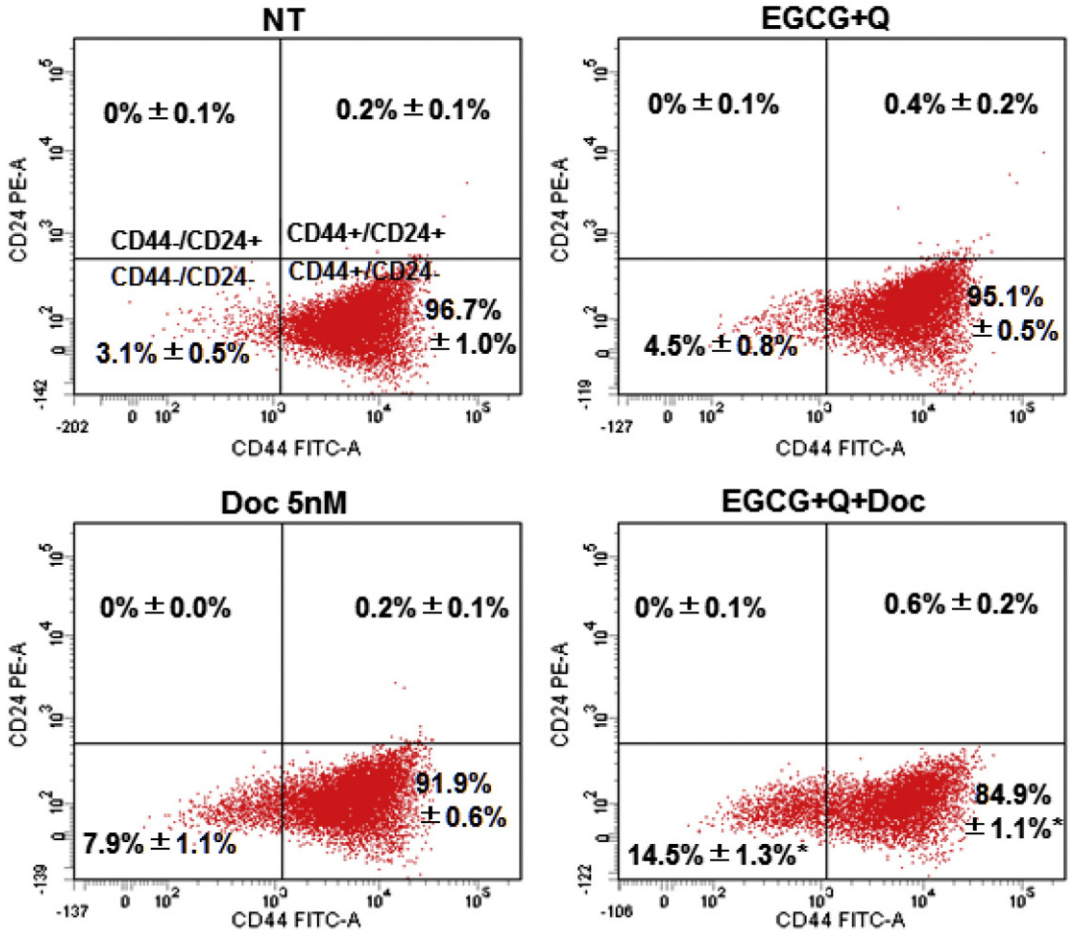

Fig. 5. EGCG, Q and Doc in combination decreased the percentage of CD44+/CD24$^-$ LAPC-4-AI cells. LAPC-4-AI cells were treated with vehicle control, 40 μM EGCG+5 μM Q, 5 nM Doc or EGCG+Q+Doc at same concentrations for 48 h. The monolayer cells attaching to the bottom were collected and double-stained with CD24$^-$PE and CD44-FITC conjugates for flow cytometry analysis using a BD LSRFortessa X-20 Cytometer. The experiment was performed in triplicate. Data are presented as mean values. NT: nontreatment, DMSO control. *Compared to NT, $P<.05$.

treatment [35]. However, no superiority to Doc/prednisone effect has been shown in phase III trials. In addition, these drug–drug combinations increase the challenge of adverse effects [35]. The anticancer activities of GT and Q have been well demonstrated in many preclinical studies [8,9]. Although results from human studies are not consistent, the majority of these studies support a preventive effect of GT in prostate cancer [9]. Both GT and Q target multiple signaling pathways involved in carcinogenesis, which may potentially provide a systemic control on cancer growth [8]. Since a cancer may have hundreds of gene mutations and dysfunctions and many pathways crosstalk with each other in tumor growth, it may not be able to control a cancer by targeting single or few signaling pathways. As a result, chemoresistance may appear during treatment, which is a major reason of the failure of chemotherapy drugs [36]. Natural products like GT and Q have been shown to selectively target cancer cells, while with minimum toxicity in normal cells [37]. The present study demonstrated that GT and Q in combination with Doc did not increase the toxicity in normal prostate epithelial PrEC cells compared to individual compounds (data not shown). The enhanced antiproliferative effect of the mixture was associated with an increased induction of apoptosis in both LAPC-4-AI and PC-3 cells compared to Doc alone. An increased ratio of Bax to Bcl-2 protein expression by Western blot analysis was associated with the observations from the fluorescence imaging of apoptosis using Cellometer. In addition, these three chemicals in combination enhanced the cell cycle arrest at G2/M phase in both LAPC-4-AI and PC-3 cells compared to EGCG+Q or Doc. These results suggest a promising nontoxic means by combination with GT and Q to enhance the efficacy of Doc.

Both GT and Q target multiple events and signaling pathways throughout the stages of tumor initiation, promotion and progression [10,38]. The combination treatment may increase the effect on these molecular targets by a sum of the activities of individual compounds. Regarding the important role of phosphatidylinositol 3-kinases (PI3K)/Akt pathway in cancer growth and progression as well as the development of drug resistance [3], we examined the combined effect of the mixture on this pathway. Akt functions upon phosphorylation by phosphorylated PI3K and activates its substrates, one being mTOR, leading to increased cell proliferation and survival [3]. The combination of GT and Q with Doc significantly increased the inhibition of the phosphorylation of Akt in both LAPC-4-AI and PC-3 cell lines compared to GT+Q or Doc alone. We further evaluated the inhibitory effect of the combination treatment on the protein expression of MRP1, a transport protein commonly found involved in the resistance to chemotherapy drugs [5]. The results demonstrated that GT and Q significantly decreased the level of MRP1 protein in both cells lines with or without the combination with Doc. These results suggest a promise of GT and Q to inhibit the development of chemoresistance during Doc treatment.

The invasion and colony formation of tumor cells play a critical role in development of metastasis; thus, they are important targets in cancer therapy [39]. The treatment with GT+Q+Doc exhibited the strongest effect on tumor cell invasion and colony formation in LAPC-

4-AI cells, and a stronger effect than Doc alone in PC-3 cells. An increased inhibition of the STAT3 signaling pathway by the combination treatment may partly contribute to the enhanced inhibitory effect on cell invasion and colony formation in both cell lines. The transcription factor STAT3 becomes activated by phosphorylation in response to cytokines and growth factors, and then it enters the nucleus to mediate the expression of various genes in regulation of cell growth, survival and motility [40]. A recent study showed that the inhibition of STAT3 by EGCG significantly inhibited cell motility, migration and invasion, and increased apoptosis in human pancreatic cancer cells [41]. In addition, we observed a decreased expression of CD44 surface protein in LAPC-4-AI cells by the combination treatment, which may also contribute to the reduced cell invasion and colony formation in LAPC-4-AI cells. $CD44+/CD24^{-}$ prostate cancer cells have been shown to possess stem cell-like characteristics [42]. These cells are more proliferative, clonogenic, tumorigenic and metastatic than $CD44-/CD24^{-}$ cells [33]. They are responsible for tumor initiation and formation and are predictive of poor prognosis in prostate cancer patients [42]. The ability of EGCG and Q to inhibit cell invasion and colony formation was also demonstrated by Tang et al. [43] in stem cell-like prostate cancer cells, where the combination of EGCG and Q synergistically enhanced the inhibitory effect. A recent study showed that EGCG in combination with paclitaxel significantly decreased the bone metastasis of prostate tumors in SCID mice after a 2-month treatment with EGCG (228 mg/kg, i.p.) plus paclitaxel (20 mg/kg, i.p.) biweekly, and significantly increased survival [44]. Based on the present results, we anticipate that a stronger combined effect will be achieved *in vivo* through the combination of both GT and Q with these taxanes.

In summary, the combination with natural products GT and Q significantly enhanced the therapeutic effect of Doc in AI prostate cancer cells through enhanced modulations on multiple signaling pathways and events involved in carcinogenesis and cancer therapy. Future *in vivo* animal studies will be important to confirm this novel therapeutic modality by combining GT and Q with Doc to enhance the efficacy of Doc in treatment of CRPC in a cost efficient and nontoxic manner.

Conflict of interest statement

None declared.

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