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ORIGINAL ARTICLE

Blueberry as a Potential Radiosensitizer for Treating Cervical Cancer

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Abstract Cervical cancer (CC) is a leading cause of death in women worldwide. Radiation therapy (RT) for CC is an effective alternative, but its toxicity remains challenging. Blueberry is amongst the most commonly consumed berries in the United States. We previously showed that resveratrol, a compound in red grapes, can be used as a radiosensitizer for prostate cancer. In this study, we found that the percentage of colonies, PCNA expression level and the OD value of cells from the CC cell line SiHa were all decreased in RT/Blueberry Extract (BE) group when compared to those in the RT alone group. Furthermore, TUNEL+ cells and the relative caspase-3 activity in the CC cells were increased in the RT/BE group compared to those in the RT alone group. The anti-proliferative effect of RT/BE on cancer cells correlated with downregulation of pro-proliferative molecules cyclin D and cyclin E. The pro-apoptotic effect of RT/BE correlated with upregulation of the pro-apoptotic molecule TRAIL. Thus, BE sensitizes SiHa cells to RT by inhibition of proliferation and promotion of apoptosis, suggesting that blueberry might be used as a potential radiosensitizer to treat CC.

Keywords Blueberry extract · Radiation · Apoptosis · Cervical cancer

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Introduction

Amidst the current treatment regimens available today in cancer therapy including surgery, chemotherapy and radiation, treatment failure rates remain an issue. While many cancer patients are now living longer with their illness, multiple forms of cancer are steadily on the rise. In fact, more than one million people in the United States will be diagnosed with cancer this year alone [1]. An example of a cancer that has seen a recent rise in occurrence is cervical cancer (CC), which remains one of the deadliest malignancies in American women. According to the American Cancer Society, it is estimated that over 12,900 new cases of invasive CC will be diagnosed, and over 4000 women will die from the disease in 2016 alone [1]. Furthermore, according to the World Health Organization, CC is the most common gynecological tumors with an estimation of more than one million women worldwide currently living with CC [1]. With this in mind, there remains a critical need for novel, cost-effective treatments. Recent studies have focused on the use of diet in the fight against cancer. There has been in depth study demonstrating the actions of phytochemicals in the attenuation of carcinogenesis, the initiation of targeted cell death and apoptosis, as well as decreasing the risk of recurrence [2]. In particular, Blueberry has been shown to display profound effects in the attenuation of multiple forms of cancer including prostate cancer, non-small cell lung cancer, liver cancer, colon cancer, and breast cancer via induction of apoptosis, programed cell death and the modulation of cellular response mechanisms [3–13].

Aside from common bioactive compounds in fruit such as vitamins, minerals, sugars, organic acids, blueberries also contain phenolic compounds including flavonoids, tannins, stilbenoids, phenolic acids and lignans [14]. Anthocyanin is one of the flavonoids which is plentiful in berries [15]. Anthocyanin has been proven to have antioxidant, anti-

inflammatory and chemoprotective properties, and has also been shown to suppress proliferation, angiogenesis, as well as induce cancer cell apoptosis [15]. While blueberry consumption seems to be beneficial in cancer patients at all stages in disease, to our knowledge there has been minimal research into the use of blueberry as an adjuvant to chemotherapy. One study showed that a combination of anthocyanidins derived from blueberry with withaferin A enhanced the activity of chemotherapeutic drugs against human lung cancer cells [16]. While radiation therapy for cervical cancer is an effective alternate, its toxicity remains challenging which is why we aim to find an effective adjuvant therapy to address this issue.

Our previous studies have shown that resveratrol, a compound originally found in grapes which is also contained in blueberries, can be used as a radiosensitizer for prostate cancer [17]. With established evidence that blueberry modifies cancer at multiple stages, as well as cancer treatment by enhancing chemotherapeutic drugs [5–11, 13, 16, 18–24], it is presumable that blueberry extract (BE) will be effective when used in combination with radiation to potentiate the obliteration of cancer cells. Blueberry extract is less active than whole blueberry due to separation, but there is no significant difference in antioxidant activity when comparing the same amount of each substance since BE is more concentrated with flavonoids and phenolic acids [6, 25]. To date, no studies have been conducted to address the effects of BE on cancers. Here, we investigated the effects of BE on cancer cells from the common CC cell line SiHa when in combination with radiation and its possible molecular mechanisms.

Materials and Methods

Tumor Cell Line

The human cervical cancer cell line, SiHa was acquired from American Type Culture Collection, Manassas, VA. The cells were preserved in DMEM acquired from Invitrogen, Carlsbad, CA, and supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin also acquired from Invitrogen. The cultures were grown in a humidified 5% CO_2 incubator at 37 °C. The cell cultures were grown until reaching 70% confluence, and subsequently subjected to the designed experimental treatment regimens.

Treatment with Blueberry Extract and Radiation Therapy

SiHa cells at 70% confluence were treated with Badmonkey Botanicals 50 mg/ml BE for 24 h, followed by radiotherapy (RT) at 4 Gy, or mock treatment. The dosage of RT, the concentration of BE, as well as the duration of cellular incubation were based on our pilot experiments [17, 26–28]. All RT was executed using XRAD 320 Biological Irradiator at 320 Kv, 12.5 mA, and 50 cm focus-to-surface distance at a 280 cGy/min dose rate with an aluminum filter. SiHa cells were irradiated at room temperature in 75 cm^2 culture flasks [17, 28]. Upon completion of RT, SiHa cells were cultured for 48 h before harvest.

Clonogenic Survival Assay

Clonogenic survival assay (CSA) was performed as previously described [28–31]. 48 h after RT, SiHa cells were harvested from their culture flasks with TrypLE express, acquired from Invitrogen, and subsequently suspended in phosphate-buffered saline (PBS) and tallied with a hemocytometer. CSA was performed by plating 1000 cells into 60-mm Corning petri dishes in triplicate and incubated at 37 °C in a humidified 5% CO2 incubator. At day 5, fresh, seeded media was added. At day 9, following incubation, SiHa cells were fixed with 10% formaldehyde and stained with 0.05% crystal violet. The number of colonies were tallied and expressed as a percentage of total colonies in controls.

Immunohistochemistry (IHC)

Protocol for IHC staining of PCNA, CycE, and TRAIL was previously described [32, 33]. The dilution used for all primary antibodies (Ab) was 1:200, while the dilution used for the secondary antibodies was 1:500. Quantification of the PCNA+ cells was conducted by manually counting, randomly selected 3–5 high power fields with the help of MetaMorph 6.3r6 image analysis software. Average staining intensity for the proteins within the SiHa cell-covered area was also measured using MetaMorph image analysis software. Results were expressed as the average integrated immunostaining intensity of 3 slides ± SEM, relative to the intensity of the control cells.

RT-PCR

SiHa cells were washed with PBS and homogenized in TRIzol purchased from Invitrogen. RNA was extracted, and concentration was determined by Nanodrop. 1 μg RNA was reverse transcribed as previously described [32, 33]. GAPDH was used as a housekeeping gene to verify that the same amount of RNA had been amplified. The primer sequences used in this study were previously described [32, 33].

TUNEL Staining

Apoptosis was determined by TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) using a Chemicon Apoptag kit as previously described [28, 29]. To quantify the sum of apoptotic cells, all SiHa cells

within 5–6 randomly selected high power fields were manually counted at ×400 magnification, using MetaMorph image analysis software. The TUNEL+ cells were subsequently expressed as a percentage of total cells.

Measurement of Caspase-3 Activity

The cellular biological activity for another apoptotic marker caspase-3 in SiHa cells was also measured using a BioVision caspase-3/CPP32 colorimetric assay kit and this has been previously described [28, 29].

Statistics

All experiments were repeated three times, minimum. Statistical analysis of the data was performed using an unpaired, two-tailed Student's *t* test. A P value <0.05 was considered statistically significant.

Results

Effect of XRT/BE on Inhibition of Cervical Cancer Cell Proliferation

SiHa cells at 70% confluence were treated with 50 mg/ml BE for 24 h, followed by RT at 4 Gy, or mock treatment as described in the Material and Methods section. 48 h following RT, cell survival was evaluated by clonogenic survival assay. The percentage of SiHa colonies were significantly lower subsequent to RT/BE treatment when compared to the controls treated with medium alone or RT alone (Fig. 1a, $p < 0.05$). The anti-proliferative effect of RT/BE on SiHa cells was further echoed by staining for PCNA (Fig. 1, b-c). These findings were reinforced with utilization of a Quick Cell Proliferation Assay Kit to analyze cellular proliferation (Fig. 1d). These results show a strong indication that BE synergizes with RT to decrease survival of SiHa cells.

Effect of RT/BE Decreased Expression of pro-Proliferative Molecule Cyclin E and Cyclin B in SiHa Cells

Next, we asked why BE synergizes with RT to decrease survival of SiHa cells. mRNA expression of the major pro-proliferative molecules cyclin B, cyclin D, cyclin E, cdk2, and cdk4, as well as the anti-proliferative molecules p18, p21, p27, and p53 were determined first by RT-PCR in the XRT/BE group and the RT group (Fig. 2). The mRNA expression level of cyclin E was significantly lower in the RT/BE group as compared to that in RT group (Fig. 2, $p < 0.05$). This finding was further echoed by IHC staining for cyclin E. (Fig. 3). The mRNA expression level of cyclin D was also significantly lower in the RT/BE group as compared to that in RT group. Interestingly, the mRNA expression level of anti-proliferative molecule p21 was also significantly lower in the RT/BE group as compared to that in RT group and the reason for this is still unknown. These results suggest downregulation of the pro-proliferative molecule cyclin D and cyclin E correlates to an inhibitory effect of RT/BE on the proliferation and survival of SiHa cervical cancer cells.

RT/BE Induces Apoptosis of SiHa Cells

In addressing why BE synergizes with RT to decrease survival of SiHa cells, besides the contribution of inhibition of cell proliferation, the contribution by increased apoptosis induced by RT/BE could not be excluded. To address this possibility, apoptosis of SiHa cells after treatment was evaluated by the method of TUNEL staining (Fig. 4). A significant higher number of TUNEL+ cells was observed in the XRT/BE group in comparison to RT group (Fig. 4, $p < 0.05$). This result was further reinforced by examining relative caspase-3 activity in SiHa utilizing a caspase-3 activity kit (Fig. 4). These results indicate that XRT/BE induces apoptosis in SiHa cells, which may be another reason for the inhibitory effects observed in XRT/BE on survival of SiHa cells.

RT/BE Increases the Expression of pro-Apoptotic Molecule TRAIL in SiHa Cells

We further investigated the possible molecular mechanisms where XRT/BE induces apoptosis in SiHa cells. The mRNA expression of the pro-apoptotic molecules Fas, FasL, TRAILR1, TRAIL, and Bax as well as the anti-apoptotic molecules FLIP, Bcl-2, and survivin in SiHa cells was measured by RT-PCR. The level of mRNA expression of TRAIL was significantly higher in the RT/BE group in comparison to RT group (Fig. 5, $p < 0.05$). The finding from the IHC staining of TRAIL further solidified this finding (Fig. 3). These results indicate an upregulation of the pro-apoptotic molecule TRAIL in the SiHa cells treated with RT/BE might contribute in full, or at least in part, to the observed increase in cellular apoptosis in XRT/BE group.

Discussion

In this study, we showed that BE had synergistic effects in combination with RT to inhibit the survival of the Siha CC cells by inhibition of proliferation and the promotion of apoptosis. The anti-proliferative effect of RT/BE on the SiHa cells correlated with the downregulation of pro-proliferative molecules cyclin D and cyclin E. The pro-apoptotic effect of RT/BE correlated with the upregulation of pro-apoptotic molecule TRAIL. To our knowledge, this is the first study to

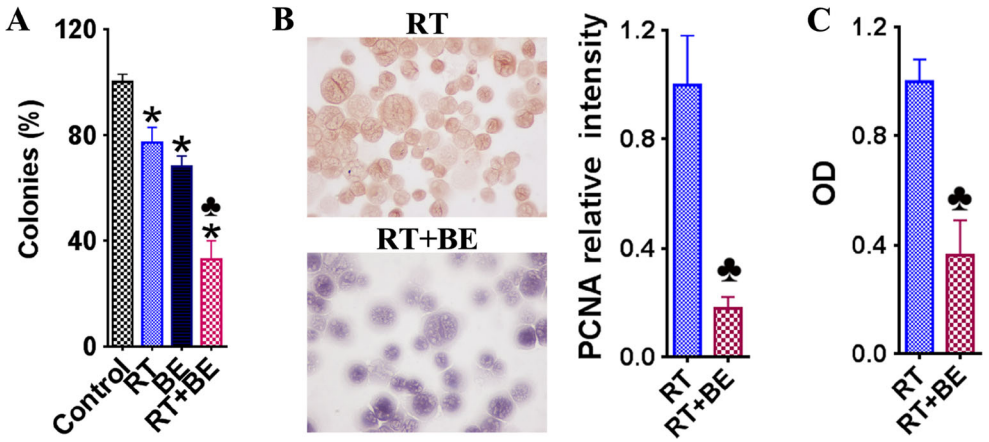

Fig. 1 Synergistic effect of RT/BE on growth inhibition of SiHa cells. **a** The clonogenic survival assay results for SiHa cells treated with or without RT in the presence or absence of BE are shown. The number of colonies were counted and expressed as a total percentage of colonies in the controls (medium alone). **b** Representative IHC results for PCNA derived from SiHa cells treated with RT/BE or RT alone. PCNA+ cells (red) in 5–6 randomly selected high power fields from three slides were counted using MetaMorph software and summarized. **c** Representative result determined by a proliferation kit. Results are expressed as the mean OD + SEM in each group, and are representative of two independent experiments. A significant difference in the percentage of colonies, PCNA+ cells or OD in each group compared to the controls is indicated by the asterisk ($p < 0.05$). A significant difference in the percentage of colonies, PCNA+ cells or OD in RT/BE group compared to that in RT group is indicated by the club ($p < 0.05$). Original magnification in B: ×400

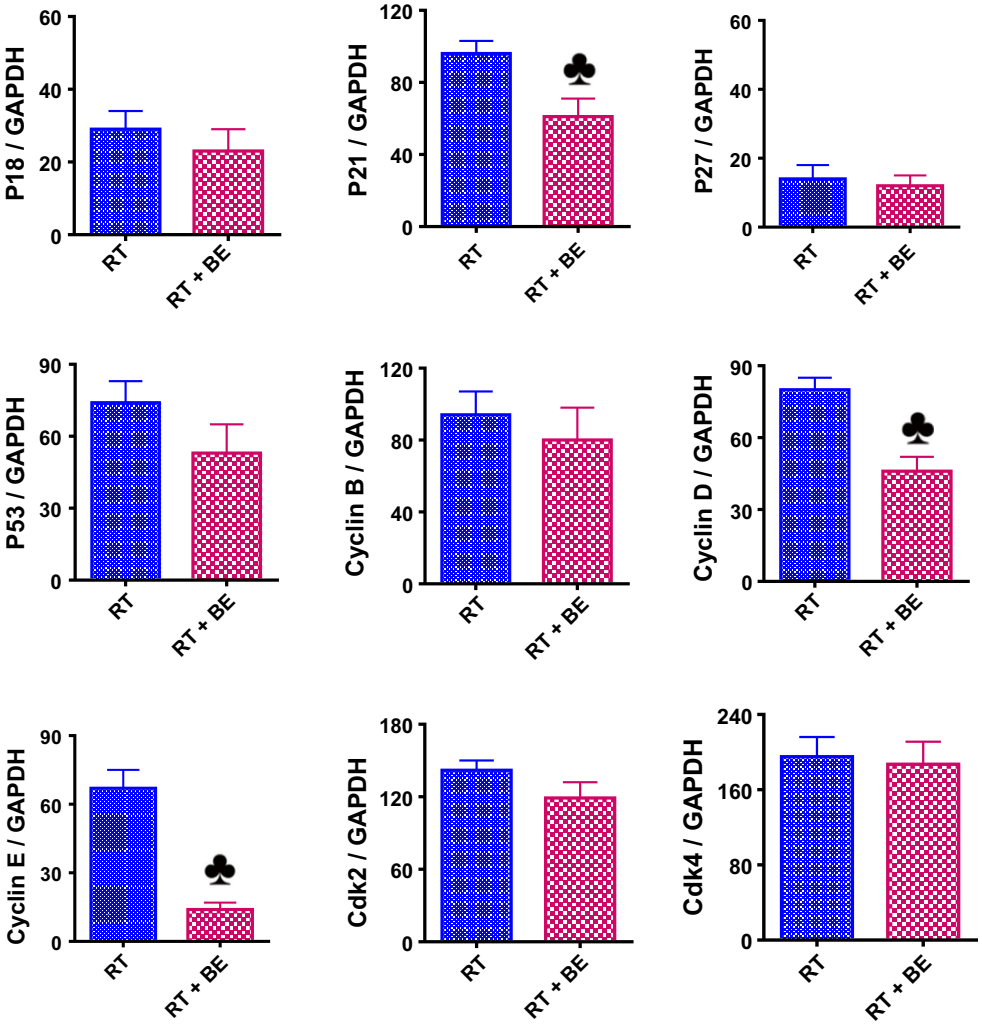

Fig. 2 Effect of RT/BE on expression of pro- and anti-proliferative molecules evaluated by RT-PCR. mRNA was extracted as described in the Methods section. Experiments were done in triplicate, and the results were expressed as the mean ratio of pro- and anti-proliferative molecule densitometric Units/GAPDH + SEM (×100), and are representative of two independent experiments. A significant difference in mRNA expression between SiHa cells treated with RT/BE and those treated with RT alone is indicated by the club ($p < 0.05$)

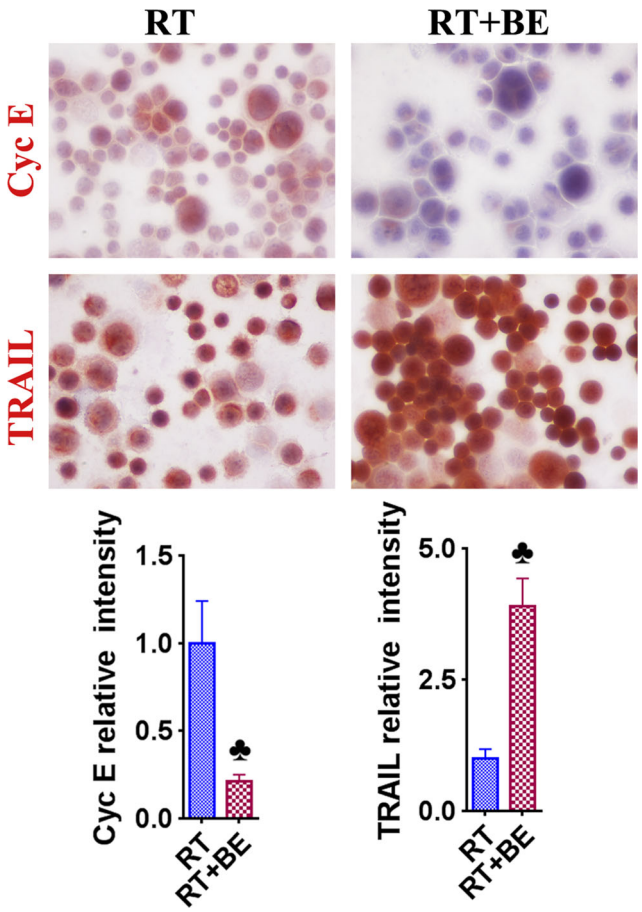

Fig. 3 Effect of XRT/BE on expression of cyclin E and TRAIL evaluated by IHC. Representative pictures of IHC are shown. The relative staining intensity in 3–5 randomly selected high power fields of three slides from each group was analyzed by MetaMorph analysis software. The results are expressed as the average integrated staining intensity of 3 slides +SEM, relative to control cells. A significant difference in staining intensity between cells treated with RT/BE and those treated with RT alone is indicated by the club ($p < 0.05$). Images shown are representative of two independent experiments. Original magnification: ×400

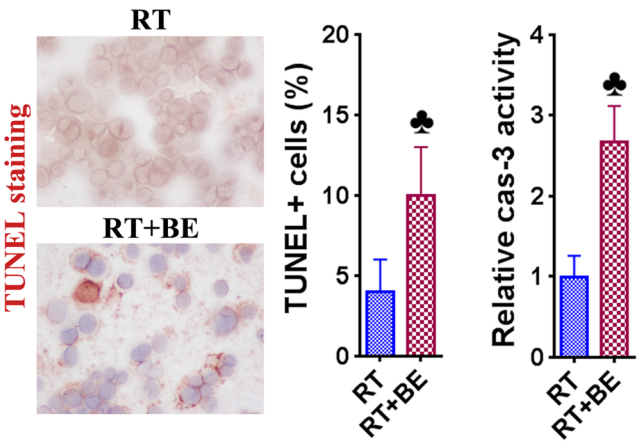

Fig. 4 RT/BE increases apoptosis of SiHa cells**. a** representative TUNEL staining is shown. **b** TUNEL+ cells in 3–5 randomly selected high power fields of three slides were tallied. **c** Cellular caspase-3 activity was measured as described in the Methods section. Results are expressed as mean activity relative to controls + SEM. Assays were completed in triplicate. A significant difference in the percentage of TUNEL+ SiHa cells or the relative caspase-3 activity in SiHa cells treated with RT/BE and those treated with RT alone is indicated by the club ($p < 0.05$). Images shown are representative of two independent experiments. Original magnification: A: ×400

demonstrate enhanced effects of RT when in combination with BE on the attenuation of proliferation and destruction of cancer cells. This is also the first study to decipher the underlying molecular mechanisms by which this occurs.

Due to its proven efficacy, cisplatin is the most widely accepted radiation sensitizer in the treatment of cervical cancer, and concomitant cisplatin-based chemoradiotherapy is the current standard of care for advanced-stage cervical cancer [34]. Studies have demonstrated synergistic effects between concurrent weekly cisplatin administration and radiation therapy through a variety of mechanisms, including increased production of reactive oxygen species, and increased interactions with reactive intermediates induced by radiotherapy; facilitation of lipid peroxidation; the initiation of p53 signaling, and decreasing expression of anti-apoptotic proteins [35]. Cisplatin has also been shown to inhibit DNA self-repair and induce G2 arrest in cervical cancer cells following radiation-induced DNA damage [23]. Interestingly, the relationship between cisplatin and radiation is not one-sided; in fact, radiation has been shown to increase the cellular uptake of cisplatin [36]. Although cisplatin has had great success in the treatment of cervical cancer, its dose-dependent side effects, and the propensity of cancer cells to form resistance to cisplatin-based therapies has limited both its use and efficacy [37]. Cisplatin resistance has been shown to be related to a reduction in the intracellular accumulation of the platinum compounds and activation of epithelial–mesenchymal transition [38]. Cisplatin resistance has been proven to be related to a reduction in the intracellular accumulation of the platinum compounds and activation of epithelial–mesenchymal transition [38]. So, unless an enhancer for cisplatin is discovered, a new radiation sensitizer is needed. Cisplatin can inhibit CC growth and induce apoptosis through upregulation of p21, p53 and Bax, which was a mechanism of action detected in BE [39, 40]. Molecular markers of cellular proliferation, such as cyclins, which act as positive regulators of cell cycle progression by binding to and activating cyclin-dependent kinases, are increased in rapidly proliferating cells (i.e. cancer cells) [41]. Cyclins D and E are two classes of cyclin that function at the *G1/S-phase* checkpoint and work in combination to phosphorylate pRb, allowing cellular progression into *S-phase* [42, 43]. In our study, we showed that the RT/BE group expressed a marked downregulation in both cyclin D and cyclin E when compared to the RT alone group. This same decrease was also observed in PCNA, which is an essential component of DNA replication machinery, functioning as the accessory protein for

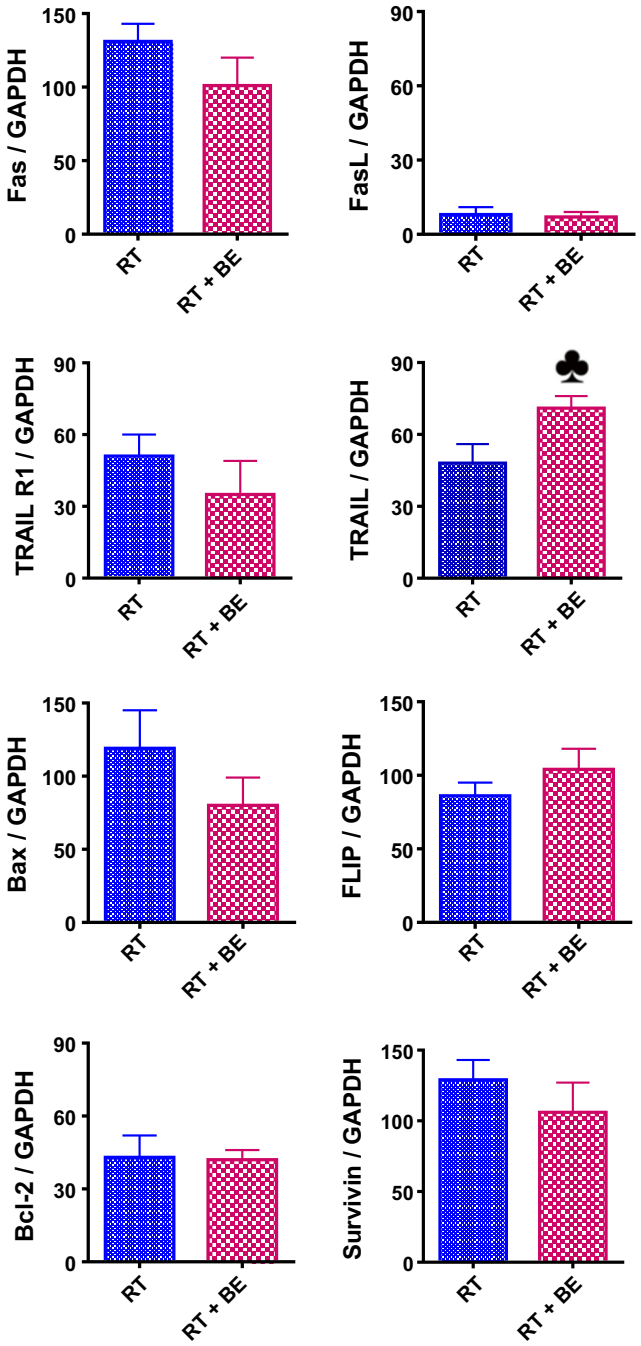

Fig. 5 Effect of RT/BE on expression of pro- and anti-apoptotic molecules evaluated by RT-PCR. mRNA was extracted as described in the Methods section. Experiments were conducted in triplicate and the results are expressed as the mean ratio of pro- and anti-apoptotic molecule densitometric Units/GAPDH + SEM (×100), and are representative of two or three independent experiments. A significant difference in mRNA expression between cells treated with RT/BE and those treated with RT alone is indicated by the club ($p < 0.05$)

DNA polymerase δ [44]. The observed decrease in PCNA, cyclin D, and cyclin E are significant since they demonstrate the efficacy of BE as a radiosensitizing agent and hints at a possible role in future treatment of CC. In this study, cyclin D is the target for BE because it was downregulated in the presence of BE in combination with RT. Cyclin D is also the target of other chemicals including some cytokines. We have reported that resveratrol sensitizes prostate cancer to radiation by downregulation of cyclin D [28] and it has been shown that IL-35 promotes growth of pancreatic cancer through the upregulation of cyclin D [45]. In this study, we found that p21 was also downregulated in the presence of BE in combination with RT. p21 is an important anti-proliferative molecule and thus its downregulation would in fact, favor cellular proliferation [46]. The detailed reason for this is still unknown, but similar effects were observed in our previous study [28]. One possible explanation might be a compensatory or adaptive response by cancer cells in an attempt to resist dangerous pro-apoptotic signals.

It has been found that at times, the events that lead to the activation of oncogenes or the loss of tumor suppressor genes, which should lead to unregulated cellular proliferation, might instead activate signal transduction pathways that block the proliferation of aberrant cells. These pathways lead to apoptosis, or programmed cell death to overcome the adaptive survival mechanisms observed in cancer cells. For example, the extrinsic pathway of apoptosis can be induced in cancer cells by the proapoptotic ligand TRAIL. TRAIL binds selectively to the death receptors TRAILR1 or TRAILR2, activating them, which eventually leads to the activation of caspases and induction of apoptosis [47]. Interestingly, this study showed a significant increase of pro-apoptotic molecules TRAIL and the activity of caspase-3 in the RT/BE group in comparison to the RT alone group. Perhaps the most significant observation made was the increase in caspase-3 activity, which signifies frank apoptosis. This was confirmed by the two-fold increase in TUNEL+ cells observed in the study. The TRAIL/TRAILR pathway is one of the critical pathways by which cancer cells undergo apoptosis. Interestingly these pathways seem to be a target for BE, because TRAIL was upregulated in the presence of the combination of BE with RT. Importantly, the TRAIL/TRAILR pathway has also been shown to be targeted by cytokines such as IL-9 and IL-32. In previous studies, we reported the inhibition of melanoma cell growth by IL-9 through the upregulation of TRAIL [48] as well as the inhibitory effects expressed by IL-32 on melanoma cell growth through the upregulation of TRAILR [49].

In summary, BE augments radiation therapy in CC by inhibiting cellular proliferation and promoting apoptosis in vitro. The data collected from our study demonstrate the potential of BE as a radiation sensitizer for CC. Future in vivo studies are warranted using RT/BE in the treatment of CC as well as other malignancies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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