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Anti-proliferative and proapoptotic effects of (−)-epigallocatechin-3-gallate on human melanoma: Possible implications for the chemoprevention of melanoma

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Melanoma accounts for only about 4% of all skin cancer cases but most of skin cancer-related deaths. Standard systemic therapies such as interferon (IFN) have not been adequately effective in the management of melanoma. Therefore, novel approaches are needed for prevention and treatment of this disease. Chemoprevention by naturally occurring agents present in food and beverages has shown benefits in certain cancers including nonmelanoma skin cancers. Here, employing 2 human melanoma cell lines (A-375 amelanotic malignant melanoma and Hs-294T metastatic melanoma) and normal human epidermal melanocytes (NHEM), we studied the antiproliferative effects of epigallocatechin-3-gallate (EGCG), the major polyphenolic antioxidant present in green tea. EGCG treatment was found to result in a dose-dependent decrease in the viability and growth of both melanoma cell lines. Interestingly, at similar EGCG concentrations, the normal melanocytes were not affected. EGCG treatment of the melanoma cell lines resulted in decreased cell proliferation (as assessed by Ki-67 and PCNA protein levels) and induction of apoptosis (as assessed cleavage of PARP, TUNEL assay and JC-1 assay). EGCG also significantly inhibited the colony formation ability of the melanoma cells studied. EGCG treatment of melanoma cells resulted in a downmodulation of anti-apoptotic protein Bcl2, upregulation of proapoptotic Bax and activation of caspases -3, -7 and -9. Furthermore, our data demonstrated that EGCG treatment resulted in a significant, dose-dependent decrease in cyclin D1 and cdk2 protein levels and induction of cyclin kinase inhibitors (ckis) $p16^{INK4a}$, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. Our data suggest that EGCG causes significant induction of cell cycle arrest and apoptosis of melanoma cells that is mediated *via* modulations in the cki-cyclin-cdk network and Bcl2 family proteins. Thus, EGCG, alone or in conjunction with current therapies, could be useful for the management of melanoma.

Key words: green tea; EGCG; melanoma; apoptosis; Bcl-2 family; caspase; cell cycle

Melanoma is a malignant proliferation of melanocytes, the pigment forming cells of the skin. The number of new melanomas diagnosed in the United States is increasing despite the nationwide emphasis on sun protection programs. Melanoma accounts for ~ 4% of all skin cancer cases and ~ 79% of skin cancer-related deaths in the USA. Since 1973, the incidence rate for melanoma (the number of new melanomas diagnosed per 100,000 people each year) has more than doubled. Annually, about 133,000 cases of melanoma are diagnosed worldwide. Current biochemotherapy regimens such as IL-2, IFN-α or the combination are found to be ineffective in improving survival in melanoma patients.[1] Therefore, novel approaches are needed for the management of melanoma. "Chemoprevention" could be one such approach. Chemoprevention, by definition, is a means of cancer control, where the occurrence of the disease can be entirely prevented, slowed or reversed by the administration of 1 or more naturally occurring and/or synthetic compounds.[2,3] Chemoprevention can be described under following categories: i) primary, preventing initial progression in high-risk group; ii) secondary, preventing premalignant conditions and iii) tertiary, preventing cured patients from developing secondary malignancies.[2]

Studies have shown that the polyphenolic fraction obtained from green tea or its major constituent, EGCG, imparts chemopreventive as well as chemotherapeutic effects in nonmelanoma skin cancers.[3–5] Melanoma cells have a poor ability to handle oxidative stress relative to normal melanocytes, which may be attributed to constitutive abnormalities in their melanosomes.[6] The abnormal redox-regulation in melanoma cells could be exploited for devising new approaches for the management of disease.[6] The biological responses of EGCG includes an antioxidant function; carcinogen modulation; inhibition of i) tumor growth, ii) cell proliferation, iii) invasion and metastasis, iv) angiogenesis and v) atherogenesis; cell cycle arrest and induction of apoptosis.[7] EGCG, a known receptor tyrosine kinase (RTK) inhibitor, is known to be a strong bivalent cation chelator as well, thus inhibiting the activity of receptor kinases by chelating the bivalent cations needed for the RTK function.[8,9] Since melanoma development is believed to be a result of oxidative stress in cutaneous melanocytes, we hypothesized that antioxidative properties of EGCG may prove useful for melanoma management.

Our study was designed to investigate the chemopreventive potential of EGCG against melanoma. Because defective regulation of apoptosis and cell cycle have been implicated in the development of cancer including melanoma, we hypothesized that EGCG will impart antiproliferative effects in melanoma cells by induction of apoptosis and cell cycle arrest mediated *via* the cki-cyclin-cdk network and/or Bcl2 family proteins. Our study suggested that EGCG resulted in an induction of apoptosis and cell cycle arrest of melanoma cells that was accompanied with i) downmodulation of Bcl2 with a concomitant upregulation of Bax, ii) activation of caspases -3, -7 and -9, iii) downmodulation of cyclin D1 and cdk2 and iv) induction of $p16^{INK4a}$, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$.

Material and methods

Cells and EGCG treatment

A-375 and Hs-294T melanoma cells were obtained from The American Type Culture Collection (ATCC, Manassas, VA) and NHEM from Cascade Biologies, Inc. (Portland, OR). Cells were maintained at standard tissue culture conditions as recommended by the vendors. Different concentrations of EGCG (>98% purity; available from Mastui Norin Co., Japan), in PBS, were used to treat the cell lines in their corresponding medium. The cells were treated with varying concentrations of EGCG (0, 1, 5, 10 and 20 μg/ml) for selected times (48–72 hr) in earlier experiments. In some later experiments, in order to keep the EGCG concentration closer to physiological achievable concentrations, a lower range concentration (0, 1, 2.5, 5 and 10 μg/ml) was used.

Cell viability

The effect of EGCG on the viability of cells was determined by trypan blue dye exclusion and MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assays. The cells were plated at 1×10^5 in 6-well plates and in 2 ml DMEM complete medium containing different concentrations of EGCG. After incubation for 48 hr, cells were collected and an aliquot of cell suspension was

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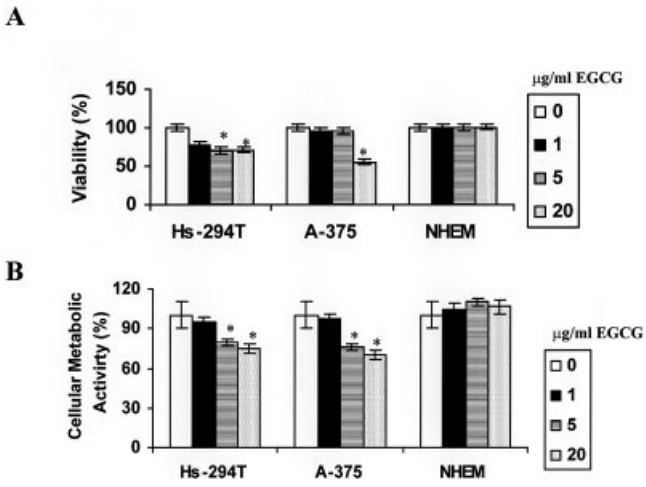

FIGURE 1 – (*a*) Effect of EGCG on the viability of melanoma cells. The cells were treated with specified concentration of EGCG for 48 hr and the viability of cells was determined by trypan blue exclusion assay. The data is shown as percent cell viability that is expressed as mean ± SEM from 3 experiments conducted in triplicate. A *p*-value <0.05 was considered statistically significant. (*b*) Effect of EGCG on the growth of melanoma cells. As detailed under Materials and methods, the cells were treated with EGCG and the viability of cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The data are expressed as percent reduction in cellular metabolic activity, and represent the means ± SE of 3 experiments where each treatment was performed in at-least 3 wells. A *p*-value <0.05 was considered statistically significant.

mixed with equal volume of trypan blue and loaded onto the slot of hemacytometer and counted. For MTT assay, cells were plated at 2×10^3 cells per well in 200 μl DMEM complete medium containing different concentrations of EGCG in a 96-well microtiter plate. After incubation for desired time, cell viability was determined using the MTT assay. Briefly, 4 μl of MTT reagent (5 mg/ml in PBS) was added to each well and incubated for 2 hr and the plate was centrifuged at 1,800 rpm for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. Formazan crystals were dissolved in 150 μl of DMSO and the absorbance was recorded on a microplate reader at of 540 nm wave length. The data is presented as percent reduction in cellular metabolic activity.

Soft-Agar colony formation assay

The effect of EGCG on colony formation ability of the melanoma cells was assessed by soft agar colony formation assay. This *in-vitro* proliferation assay is a measure of proliferating and dividing cells in culture. The assay was performed in 6-well plates; in each well, 2 ml of 0.5% agar (in culture medium) was layered in the bottom followed by 1 ml of 0.38% agar as the top layer. Approximately 2,000 cells were then plated over the top layer. The cells were treated with EGCG (0, 1 and 5 μg/ml) and maintained at 37°C in a humidified 10% CO_2 atmosphere. After 15 days, the number of colonies were counted under an inverted phase-contrast Olympus 1×70 microscope at ×4 magnification and photographed.

Measurement of apoptosis and cell cycle by flow cytometry

We used multiple methods to detect the apoptosis in EGCG treated melanoma cells.

TUNEL Assay. We used Apo-BrdU terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) kit (Molecular Probes, Inc., Eugene, OR) to measure the extent of apoptosis and cell cycle distribution by exploiting the fact that the DNA breaks expose a large number of 3′-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal deoxynucleotidyl transferase (TdT) to add deoxyribonucleotides in a template-independent fashion. Addition of BrdUTP to the TdT reaction serves to label these break sites. Once incorporated into the DNA, BrdU can be detected by FITC or alexa flour 488 dye conjugated anti-BrdU antibody and PI to simultaneously give cell cycle and apoptotic data using flow cytometry.

For this assay, the cells were treated with EGCG (0, 1, 5 and 10 μg/ml) and maintained at 37°C in a humidified 10 % CO_2 atmosphere. The cells were gently trypsinized and added to the culture media and pelleted by centrifugation. The pellet was washed with PBS, counted and the cells (1×10^6) were fixed overnight in ethanol (90%). The cells were washed and labeled with UTP-BrdU overnight, washed again with PBS and incubated with alexa flour 488 Anti-BrdU antibody followed by counterstaining with PI. Cells were analyzed using a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) in the UWCCC Flow Cytometry Facility in the University of Wisconsin. The analyses were performed using Cell Quest software (BD Biosciences, San Jose, CA) for apoptosis and ModFit LT software (Verity Software House, Topsham, ME) for cell cycle analysis.

JC-1 mitochondrial membrane potential detection assay. We used APO-LOGIC JC1 kit (Cell Technology Inc., Minneapolis, MN) for *in-situ* detection of mitochondrial membrane transition events in live cells, which provides an early indication of the initiation of cellular apoptosis. The collapse in the electrochemical gradient across the mitochondrial membrane ($\Delta\Psi$) was measured using a fluorescent cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, known as JC-1. In nonapoptotic cells, JC-1 exists as a monomer in the cytosol (green) and also accumulates as aggregates in the mitochondria (red). In apoptotic and necrotic cells, JC-1 exists in monomeric form and stains the cytosol green.

For this assay, the cells were treated with EGCG (0 and 10 μg/ml) and maintained at 37°C in a humidified 10% CO_2 atmosphere for 72 hr and were stained with JC-1 reagent in the medium for 15 min. The cells were gently trypsinized and added to the culture media and pelleted by centrifugation. The pellet was washed with PBS and analyzed using a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) in the UWCCC Flow Cytometry Facility in the University of Wisconsin. The analyses were performed using Cell Quest software (BD Biosciences, San Jose, CA) for apoptosis.

Immunocytochemistry

For this purpose, cytospin preparations of the treated and control melanoma cells were rinsed with PBS and fixed in 3.7% formaldehyde by immersing the cover slip in the solution for 10 min. The nonspecific adsorption of the antibodies was minimized by blocking with 1% BSA in PBS for 10 min. The cells were then incubated with primary antibody for the target protein for 45 min, washed with PBS for 3 times for 5 min each and then with appropriate biotinylated secondary antibody followed by avidin-HRP conjugate, DAB substrate and analysis by optical microscopy.

Immunoblot analyses

Immunoblot analyses were used to assess the effect of EGCG on the protein expression of various target proteins. The cells, following treatment, were washed with phosphate-buffered saline PBS (10 mM, pH 7.2) and total cell lysates were prepared using appropriate lysis buffer containing protease inhibitors. Appropriate amounts of protein (25–100 μg/ml) were mixed in 1:1 Laemmli buffer (2×) and then resolved over 8–14% tris-glycine polyacrylamide gel (depending upon the molecular weight of the protein to be assessed) and then transferred onto nitrocellulose membranes. The blots were blocked using 5% nonfat dry milk and probed using appropriate primary antibody and the secondary antibody conjugated with horseradish peroxidase (HRP). The proteins were detected by chemiluminescence using SuperSignal West Pico Chemiluminescent detection system from Pierce (Rockford, IL).

The antibodies used in our study were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) (p16, Bcl2, Bax, Bak and PCNA), SantaCruz (p21, p27, cleaved-PARP, Ki-67, β-actin caspase-3, -7 and -9) and Neomarkers, Inc. (Fremont, CA) (Cdk2 and Cyclin D2).

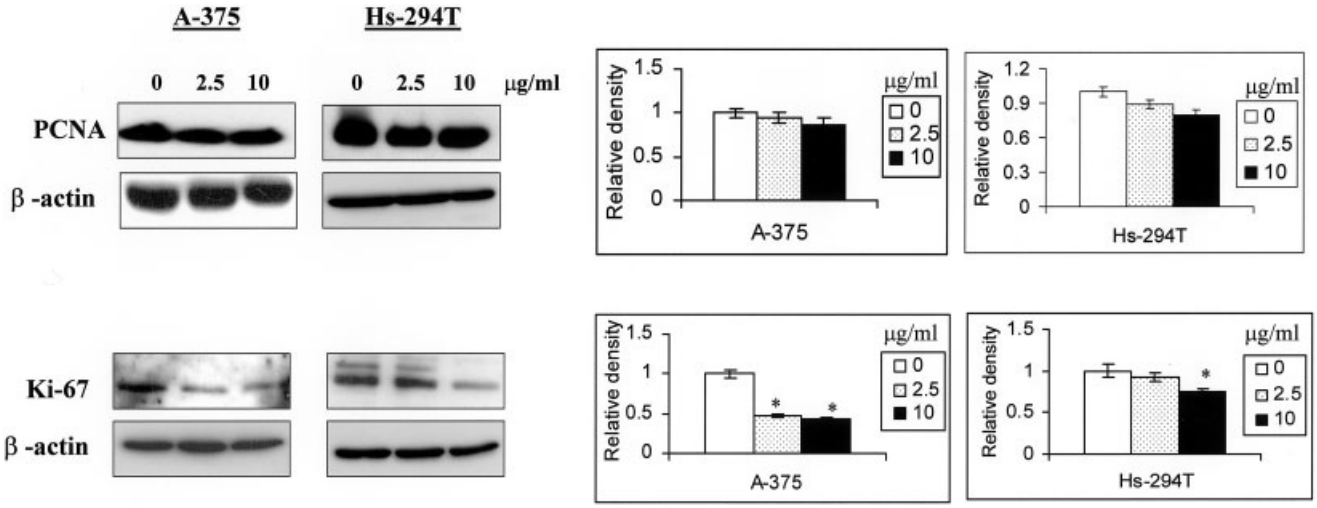

FIGURE 2 – Effect of EGCG on the protein levels of PCNA and Ki-67 in melanoma cells. As detailed under Materials and methods, the cells were treated with the specified concentrations of EGCG for 48 hr. The cells were harvested and the levels of PCNA and Ki-67 proteins were assessed by immunoblot analysis. Equal loading was confirmed by stripping the blot and reprobing it for β-actin. The bands shown here are from a representative experiment repeated 3 times with similar results. The quantification of protein was performed by densitometry analysis using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT) and the data (relative density normalized to β-actin) is expressed as mean ± SE of 3 experiments ($*p < 0.05$).

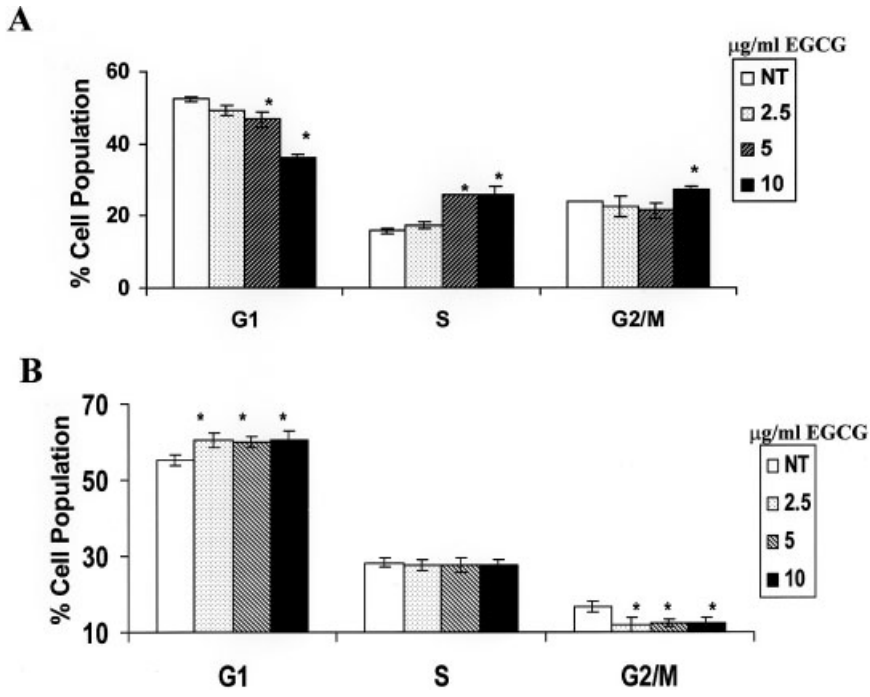

FIGURE 3 – Effect of EGCG on cell cycle distribution in Hs-294T metastatic melanoma cells (*a*) and in amelanotic A-375 melanoma cells (*b*). The growing cells were treated with the specified concentrations of EGCG for 72 hr. The effects of treatments on cell cycle distribution were assessed by Apo-Brdu TUNEL and counter stained for PI to measure the cellular DNA content as detailed under Materials and methods. The percent of cells in G_1, S, and G_2/M phases of the cell cycle are shown as mean ± SE of 3 experiments ($*p < 0.05$).

Results

EGCG causes a decrease in the viability and proliferation of melanoma cells

The antiprolifirative effects of EGCG on the viability and proliferation of A-375 and Hs-294T melanoma cells was studied using multiple techniques. As shown in Figure 1*a*, the trypan blue exclusion assay demonstrated that EGCG treatment resulted in a significant concentration-dependent decrease in the viability of both melanoma cell lines studied. Our data also demonstrated that EGCG treatment resulted in decreased cellular metabolic activity as assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 1*b*). Because it is believed that an effective agent for the management of melanoma should not be cytotoxic to normal cells, we also studied the effect of EGCG on normal human epidermal melanocytes (NHEM). Interestingly, as shown in the Figure 1*a*,*b*, EGCG treatment resulted in a decrease in cellular viability as well as cellular metabolic activity of melanoma cells but not in NHEM, even at lower EGCG concentrations (1–20 µg/ml). At higher concentrations of EGCG (40–80 µg/ml), which are probably physiologically nonachievable concentrations, a much higher inhibitory effect was observed (data not shown).

The decrease in the growth and viability function of melanoma cells by EGCG treatment could be attributed to the antiproliferative effects of EGCG. Therefore, employing immunoblot analysis and immunostained cytospin preparations (data not shown), we evaluated the effect of EGCG on the protein levels of PCNA and

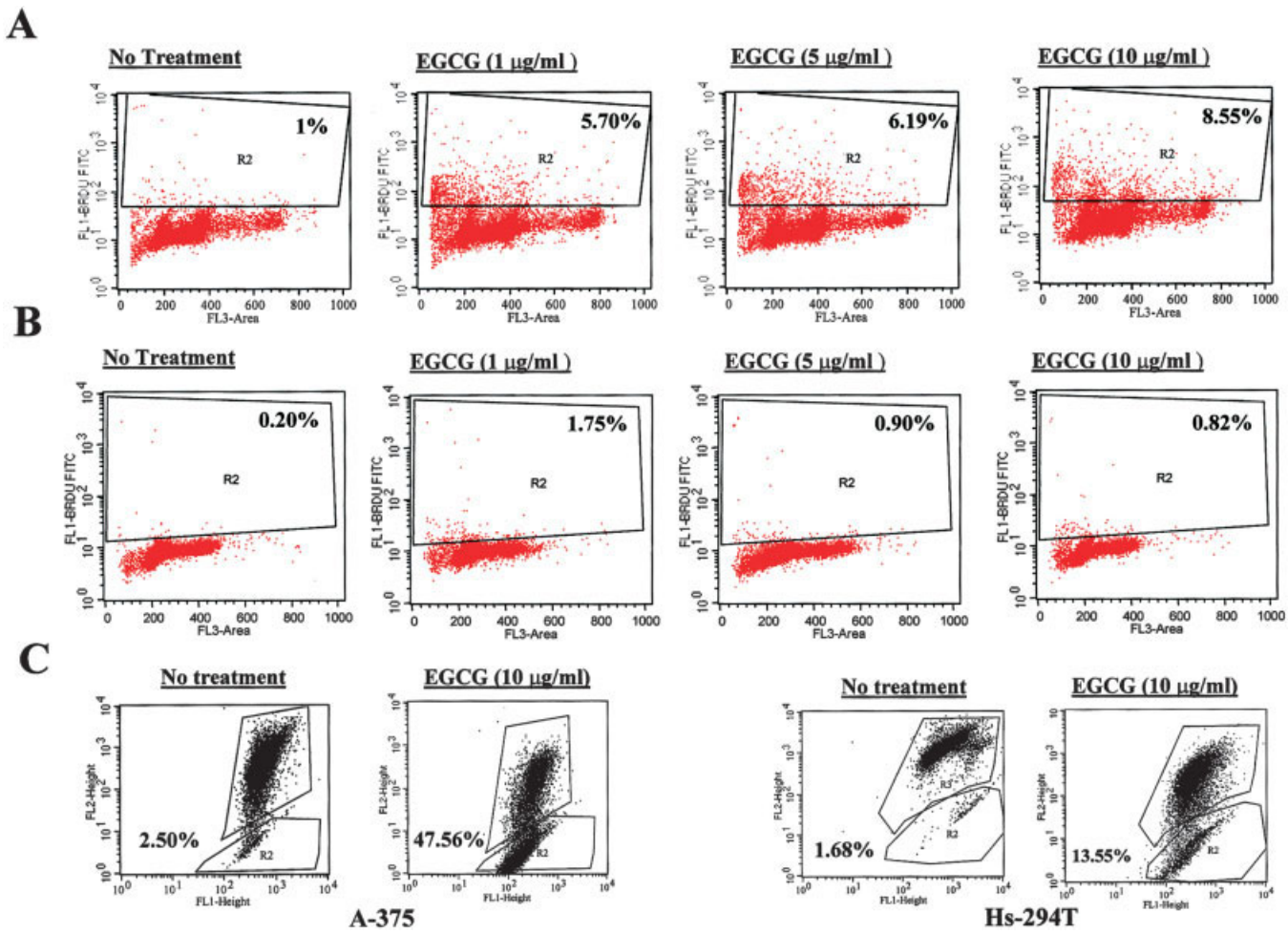

FIGURE 4 – Effect of EGCG on fragmentation of DNA in Hs-294T metastatic melanoma cells (*a*) and in amelanotic A-375 melanoma cells (*b*) and membrane potential (*c*). The growing cells were treated with the specified concentrations of EGCG for 72 hr to follow the extent of apoptosis by APO-BrdU TUNEL assay kit and APO-LOGIC JC-1 assay reagent. The fragmentation of DNA in apoptotic cells is measured by BrdU incorporation which is visualized by conjugation to an Alexa Fluor 488 dye-labeled anti-BrdU antibody. BrdU incorporation was analyzed with a flow cytometer followed by a computational analysis of cells staining positive for BrdU. The data are expressed as mean ± SE of 3 experiments ($*p < 0.05$). The results shown in panel (*a*,*b*) are from a representative experiment repeated 3 times with similar results. Details of the experiments are given in Materials and methods. (*c*) EGCG treated melanoma cells were labeled with Apo-Logic JC-1 reagent for the determination of mitochondrial membrane potential. After washing, cells were analyzed on a FACScan benchtop cytometer (Becton Dickinson, San Jose, CA). A dot plots of red fluorescence (FL2) *vs*. green fluorescence (FL1) showing live cells with intact mitochondrial membrane potential and dead cells with lost mitochondrial potential respectively. Details of the experiments are given in Materials and methods.

Ki-67, which are the cell proliferation markers expressed by actively proliferating cells and rapidly degrade as the cell enters the nonproliferative stage. As shown in Figure 2, our data demonstrated that EGCG treatment of both melanoma cell lines resulted in a significant down-modulation in Ki-67 protein levels. However, only a marginal inhibitory effect was observed on PCNA protein expression. These data suggested that the EGCG-mediated decrease in cell growth/cell viability is a result of decreased cellular proliferation.

EGCG causes an arrest of the cell cycle and induction of apoptosis in melanoma cells

To assess whether the anti-proliferative effects of EGCG are mediated *via* alterations in the regulation of cell cycle and apoptosis, we performed DNA cell cycle analysis (for effects on cell cycle regulation) and TUNEL assay (for DNA fragmentation). As revealed by DNA cell cycle analysis, EGCG-treatment resulted in a significant increase of cells in the S phase in Hs-294T cells at concentrations of 1–10 μg/ml EGCG (Fig. 3*a*). On the other hand, in A-375 cells, EGCG resulted in an increased accumulation of cells in G_1-phase of the cell cycle (Fig. 3*b*). These data were verified by analyzing BrdU incorporated melanoma cells using monoclonal anti BrdU-FITC antibody and PI staining and flow cytometry with similar results (data not shown).

To assess the effect of EGCG on DNA fragmentation in melanoma cells, we employed Apo-BrdU™ TUNEL assay, where DNA breaks expose 3′- hydroxyl ends and these serve as a starter for TdT mediated BrdUTP incorporation in a non template dependent fashion to the break sites that can be detected by anti-BrdU and immunolabeling. EGCG treatment resulted in a significant increase in apoptosis in Hs-294T (~ 10% apoptotic cells; Fig. 4*a*). To further confirm our data we used additional *in-situ* detection of apoptosis by measuring the effects on mitochondrial membrane potential ($\Delta\Psi$) using a fluorescent cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, known as JC-1. This technique effectively detects apoptosis even at very early stages. In healthy cells, JC-1 exists as a monomer in the cytosol (FL1 positive; green) and also accumulates as aggregates in the mitochondria (FL2 positive; red). In apoptotic and necrotic cells, JC-1 exists

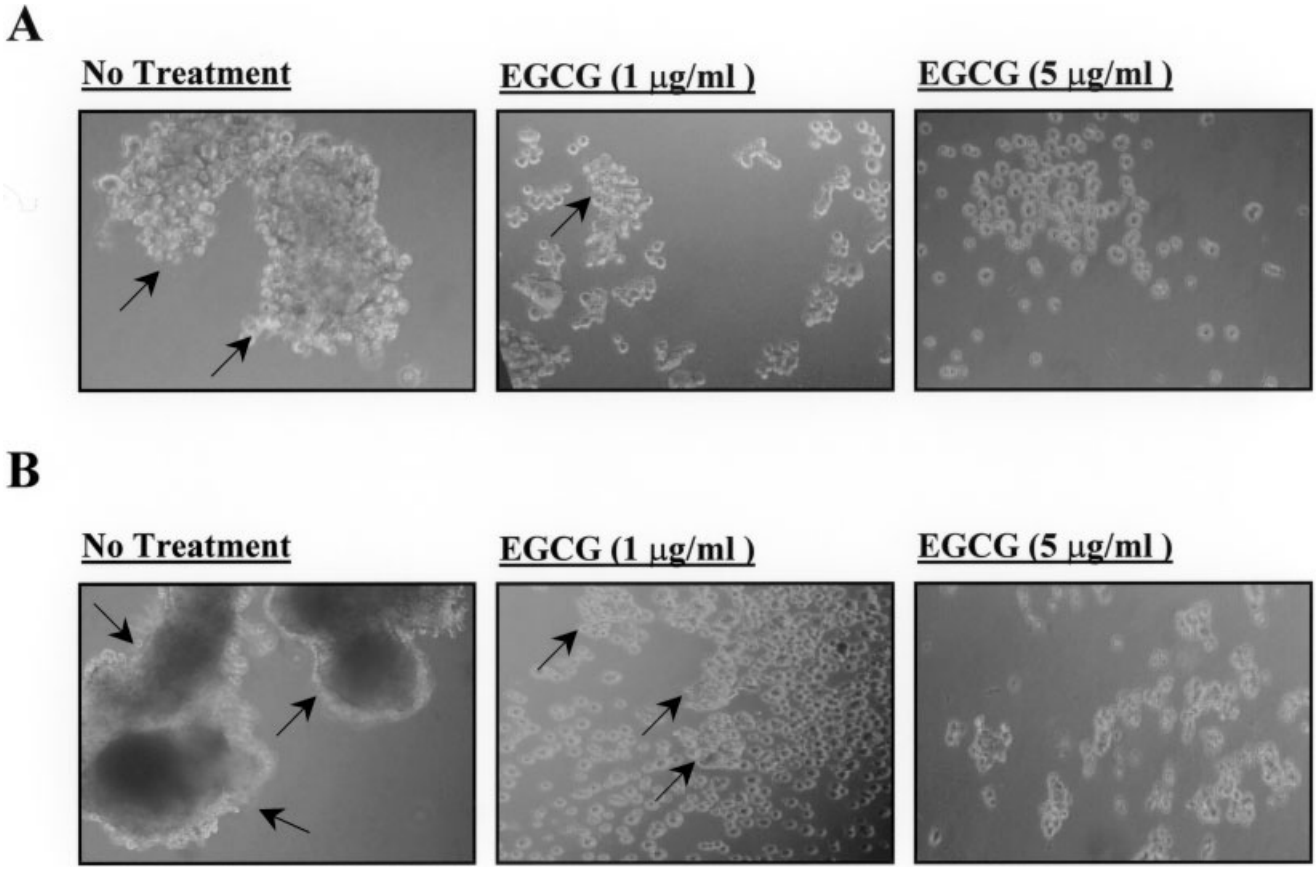

FIGURE 5 – Effect of EGCG on colony formation abilities of Hs-294T metastatic melanoma cells (*a*) and in amelanotic A-375 melanoma cells (*b*). The growing cells were plated on a thin layer of agar in culture medium and treated with EGCG. At 15 days post treatment, plates were assessed for size and colonies and photographed at ×4 magnification. Colonies (in a representative field) are indicated with arrows. The results shown are from a representative experiment repeated 3 times with similar results. Details of the experiments are given in Materials and methods.

exclusively in monomer form and produces a green cytosolic signal. As shown in Figure 4*c*, EGCG treatment of Hs-294 T cells and A-375 cells resulted in 14% and 47.5% apoptosis, respectively, as the FL1 positive population compared to no treatment control.

EGCG inhibits the clonogenic survival of melanoma cells

Because the colony formation ability of cancer cells is regarded to be a measure of proliferative/carcinogenic potential, we examined the effect of EGCG on clonogenic survival of Hs-294T and A-375 melanoma cells. We found that the melanoma cells used in our study demonstrated a tendency to make very large (but low number) colonies rather than multiple smaller colonies (Fig. 5). Interestingly, EGCG treatment resulted in significant inhibition in the ability of both the melanoma cell lines to make colonies. Thus, EGCG treatment (0, 1 and 5 μg/ml) resulted in a significant reduction in the number and size of the colonies of Hs-294T and A-375 cells at 15 days posttreatment as demonstrated by more loosened cells in the treated groups as opposed to large and tightly aggregated colonies in no treatment controls (Fig. 5*a*,*b*).

EGCG treatment results in an increase in Bax, decrease in Bcl-2 and increase in Bax/Bcl-2 ration in melanoma cells

Our results showed a marked reduction in cell growth and proliferation and an induction of apoptosis of melanoma cells by EGCG. To understand the mechanism of the antiproliferative effects of EGCG towards melanoma cells, we examined the effect of EGCG treatment on Bcl-2 family proteins in melanoma cells. The Bcl-2 family proteins play a critical regulatory role *via* its interacting pro- and anti-apoptotic members, which integrate a wide array of diverse upstream survival and distress signals to decide the fate of cells. Bax and Bcl-2 proteins are the key elements of this protein family. Therefore, we evaluated the effect of EGCG treatment on the levels of Bax and Bcl-2 protein. As shown in Figure 6*a*, EGCG treatment of melanoma cell lines resulted in a significant decrease in anti-apoptotic Bcl-2 in A-375 cells and marginal decrease in Hs-294T cells and significant increase in pro-apoptotic Bax proteins in both the cell types, thereby resulting in an increase in the Bax/Bcl-2 ratio that favors apoptosis.

EGCG treatment results in an activation of caspase-machinery in melanoma cells

The induction of apoptosis *via* modulations in Bcl-2 family proteins is known to be mediated *via* an activation of caspase-machinery, which is believed to play a key role in the execution of apoptosis. Therefore, we evaluated the involvement of various caspases during EGCG-mediated apoptotic death of melanoma cells. As shown by immunoblot analysis, it was observed that EGCG-treatment resulted in a significant increase in the active forms of caspase-3 and caspase-9 in A-375 cells, with only a marginal increase in caspase-7 (Fig. 6*b*). On the other hand, EGCG-treatment resulted in a significant increase only in caspase-7 expression in Hs-294T cells, with only marginal increases in caspase-3 and caspase-9 (Fig. 6*b*). Furthermore, EGCG treatment did not cause any change in the levels of caspase-8 in A-375 or Hs-294T cells (data not shown). EGCG-treatment also resulted in a significant cleavage of poly (ADP-ribose) polymerase (PARP), as evident from a significant increase in cleaved PARP

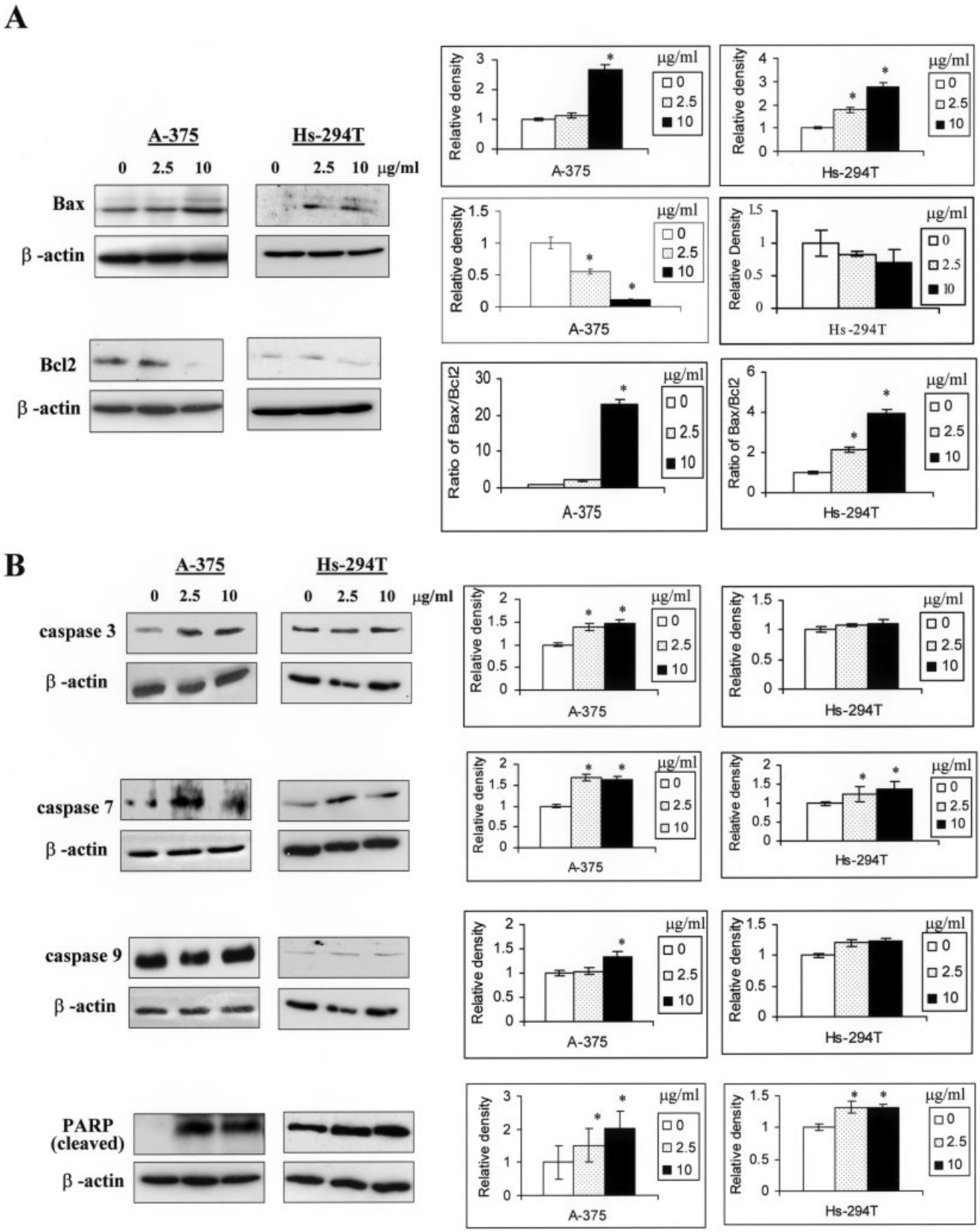

FIGURE 6 – Effect of EGCG on the protein levels of i) Bax and Bcl2 (*a*), ii) caspase -3, -7 and -9 and cleavage of PARP (*b*), iii) cyclin D1 and cdk2 (*c*), and iv) p27, p21 and p16 (*d*), in melanoma cells. As detailed under Materials and methods, the cells were treated with the specified concentrations of EGCG for 48 hr. The cells were harvested and the levels of specific proteins were assessed by immunoblot analysis. Equal loading was confirmed by stripping the blot and reprobing it for β-actin. The bands shown here are from a representative experiment repeated 3 times with similar results. The quantification of protein was performed by densitometry analysis using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT) and the data (relative density normalized to β-actin) is expressed as mean ± SE of 3 experiments (*$p < 0.05$).

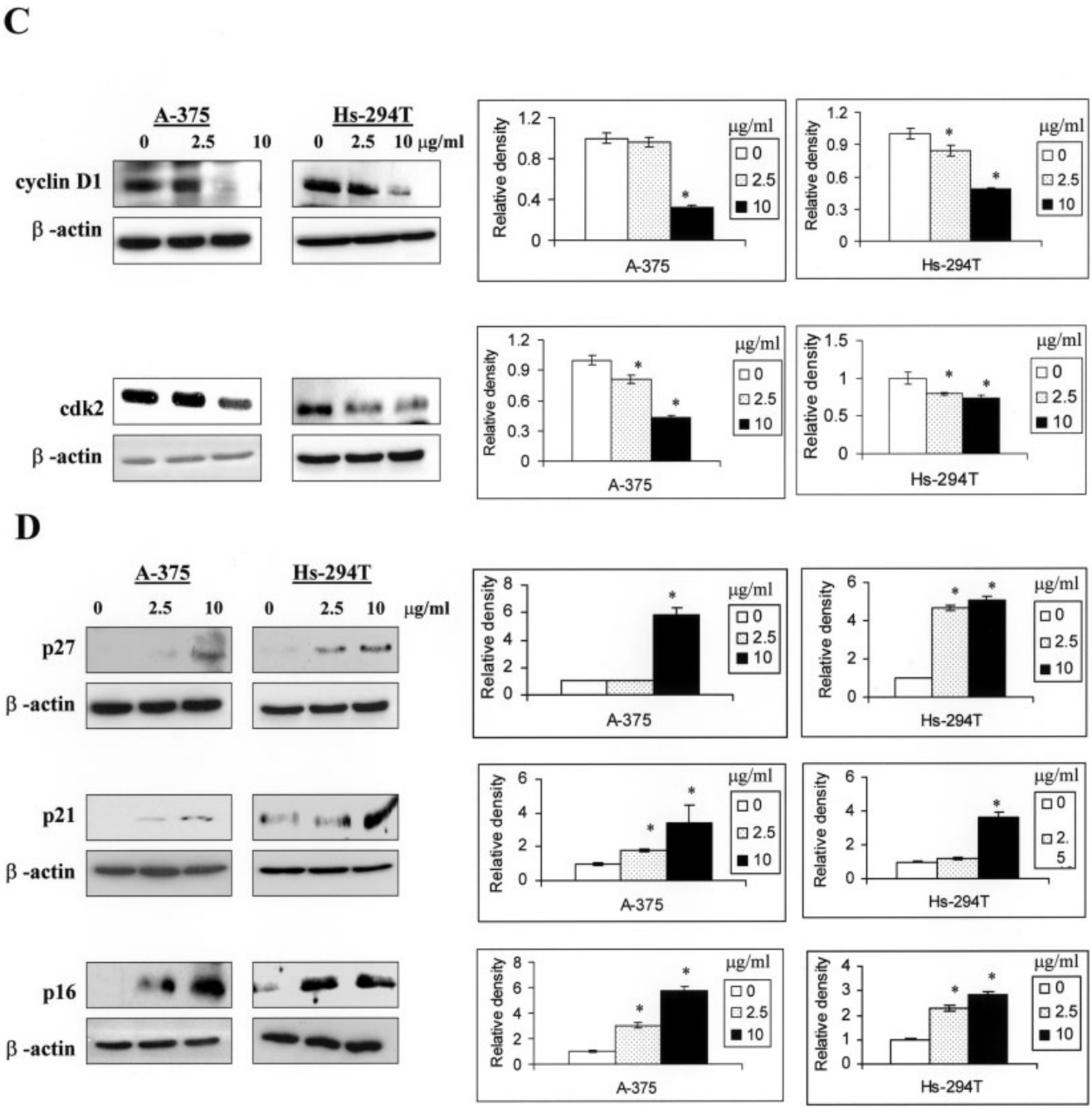

FIGURE 6 – CONTINUED.

fragments in both melanoma cell lines, consistent with the induction of apoptosis by EGCG (Fig. 6*b*).

The anti-proliferative response of EGCG for melanoma cells is mediated via *modulations in the cki-cyclin-cdk network*

Because our data demonstrated that the anti-proliferative effects of EGCG on melanoma cells are associated with cell cycle arrest, we studied the involvement of cyclin kinase inhibitor (cki)-cyclin-cyclin dependent kinase (cdk) machinery in the anti-proliferative response of EGCG. As shown by immunoblot analysis, EGCG treatment resulted in a significant downregulation of cyclin D1 and cdk2 proteins in both the melanoma cell lines studied (Fig. 6*c*). Furthermore, EGCG treatment resulted in a significant induction of the ckis viz. $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p16^{INK4}$, albeit at different concentrations (Fig. 6*d*). However, at selected concentrations of EGCG, no significant changes were observed in the protein levels of cdk4, cdk6, cyclin D2 and cyclin E (data not shown).

Discussion

Our study is the first systematic effort to assess the chemopreventive effects of green tea polyphenol EGCG on melanoma. Melanoma is the deadliest form of skin cancer and the standard biochemotherapy of melanoma with interferons has failed to offer any clear benefit in terms of survival.[1,10] Furthermore, other than sun-protecting efforts such as sunscreens and clothing, no known method is available to prevent new primary melanomas in individuals at increased risk for these tumors. The success of systemic therapy of metastatic melanoma has been minimal and associated

with high toxicities that often result in discontinuation of the treatment regimen.[10] Chemoprevention *via* natural or synthetic agents may be helpful in the management of melanoma.[11] This is largely an unexplored strategy in melanoma research.[2] The epicatechin derivatives or polyphenols, present in green tea, have been shown to be effective chemopreventive agents for several cancer types including nonmelanoma skin cancers.[3,4]

Our study was designed to assess the chemopreventive effects of EGCG against melanoma. The data presented in our study demonstrated that EGCG exerts direct and selective anti-proliferative and pro-apoptotic effects on melanoma cells, without affecting the normal melanocytes at similar concentrations. The issue of pharmacologically attainable dose and bioavailability of EGCG following green tea consumption is complex and depends on various factors. However, based on published data, it appears that such a dose is attainable following 4–6 cups of green tea consumption per day by an adult human being. For our initial experiments, we employed a wide concentration-range of EGCG (0, 1, 5, 10, 20, 40 and 80 μg/ml). However, the mechanistic studies were conducted with lower EGCG concentrations (0, 1, 2.5, 5, 10 μg/ml) which are closer to the physiologically attainable concentrations of EGCG. Although some studies have reported the effects of EGCG at submicromolar levels, most experiments require concentrations of $>$ 10 or 20 mmol/l to demonstrate an effect.[12]

Our data demonstrated that EGCG treatment resulted in a significant antiproliferative effects against the melanoma cells without affecting normal melanocytes. EGCG treatment resulted in S-phase arrest in Hs-294T cells, G1 phase arrest in A-375 cells and induction of apoptosis in both cell types. These observations are important because dysregulation of normal cell cycle- and apoptotic-machinery plays a critical role in the development of neoplasms.[13,14] In addition, cell cycle and apoptotic pathways are being increasingly appreciated as targets for intervention against cancer.[13,14] The observed induction of apoptosis and cell cycle arrest by EGCG in melanoma cells is important because these are physiological processes, which function as essential mechanisms of tissue homeostasis and are regarded as preferable ways for eliminating unwanted cells.[11]

Furthermore, to investigate the mechanism of EGCG-mediated cell cycle arrest and induction of apoptosis in melanoma cells, we tested the hypothesis that EGCG will impart antiproliferative effects *via* modulation(s) in Bcl-2 family proteins and/or cyclin kinase inhibitor (cki)-cyclin dependent kinase (cdk) machinery. Melanocytes and melanoma cells are known to express relatively high levels of anti-apoptotic Bcl-2. In fact, the link between apoptosis and cancer was discovered when Bcl-2 (B-cell lymphoma 2), which is the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death.[15,16] This discovery gave birth to the concept, now widely embraced, that impaired apoptosis is a crucial step in the process of development of cancer including melanoma.[17,18] In our study, we have shown that EGCG treatment of human melanoma cells results in a significant decrease in the levels of anti-apoptotic Bcl-2 protein and an increase in the pro-apoptotic Bax protein, thus shifting the Bax/Bcl-2 ration in favor of apoptosis (Fig. 6*a*). Studies have shown that Bcl-2 forms a heterodimer with Bax and might thereby neutralize its pro-apoptotic effects.[16,19,20] Synthetic small interfering RNA (siRNA) compounds targeting Bcl-2 have caused a significant down regulation of Bcl-2 expression in melanoma cells, an increase in apoptotic cell death and inhibition of cell growth.[21] In addition, Bcl-2 is also known to prevent the release of caspases.[22]

Furthermore, EGCG treatment of melanoma cells resulted in increased levels of caspase -3, -7 and 9. This is an important observation because the execution phase of apoptosis is believed to be mediated by caspases, which carry out the apoptotic program through a sequential activation cascade of initiator and executioner caspases.[22,23] The activation of the caspase-cascade results in the cleavage of PARP and subsequent DNA degradation and apoptotic death.[23] Thus, our data suggest that EGCG-induced apoptosis of human melanoma cells is mediated *via* caspase activation triggered by modulations in Bcl-2 family proteins and the associated events (Fig. 6*b*).

Because many studies have shown the involvement of cell cycle regulation-mediated apoptosis as a mechanism of cell growth inhibition,[22–24] we investigated the involvement of cki-cyclin-cdk machinery during the induction of cell cycle arrest and apoptosis of melanoma cells by EGCG. It is well established that in eukaryotes, the passage through the cell cycle is controlled by a family of protein kinase complexes, which are composed minimally of a catalytic subunit, the cdk, and its essential activating partner, the cyclin.[25–28] These complexes are activated at specific intervals and through a series of events, result in a progression of cells through different phases of the cell cycle thereby ensuring normal cell proliferation.[25–28] A defect in this machinery causes altered cell cycle regulation resulting in unwanted cellular proliferation that may lead to the development of cancer.[25–29] Our data also demonstrate that EGCG treatment of the melanoma cells results in significant decreases in cyclin D1 and cdk-2 (Fig. 6*c*). Reports indicate that cyclin D1 may not only be a tumor cell growth promoter but may also function as a survival factor for neoplastic cells and down regulation of cyclin D1 may be suggestive of apoptosis.[30]

During the progression of the cell cycle, the cdk-cyclin complexes are inhibited *via* their binding to ckis such as the CIP/KIP and INK4 families of proteins.[25–29] Because our studies have demonstrated that EGCG causes either an S-phase or a G_1-phase arrest of the cell cycle in the experimental cell lines, we examined the effect of EGCG on cell cycle regulatory molecules operative in these 2 phases of the cell cycle. Our data demonstrated an upregulation of the ckis $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p16^{Ink4a}$ by EGCG (Fig. 6*d*), albeit at different levels. The cyclin dependent kinase inhibitor 2A (CDKN2A) locus, also known as inhibitor of (cyclin-dependent) kinase 4A (INK4a) or alternative reading frame (ARF) protein, is a major tumor suppressor locus and its inactivation leads to the development of human and mouse melanoma *via* abrogation of RB and p53 functions.[31,32] The capability of EGCG to generate p16 in melanoma is remarkable. Similarly, up regulation of universal cyclin kinase inhibitor $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ is quite impressive.

Taken together, our data demonstrate that EGCG may impart cancer chemopreventive/antiproliferative effects against melanoma by inducing apoptosis and affecting cell cycle regulation machinery that is mediated *via* modulations in Bcl-2 family proteins and the cki-cyclin-cdk network, respectively. The exact mechanism(s) by which EGCG imparts its effects on these elements, leading to cell cycle arrest and apoptosis, is not known. However, based on the available literature, we suggest that these effects may be a result of the antioxidant effect of EGCG. EGCG is an exceptionally strong antioxidant and studies have shown that the antioxidant potential of EGCG is several-fold stronger than the well-known physiological antioxidants, *viz*. vitamin E and vitamin C. We suggest that EGCG enforces different redox environments in normal versus tumor cells favoring either normal cell survival or tumor cell destruction. This may be a reason for the observed differential effect of EGCG in melanoma cells *vs*. normal melanocytes.[33]

To our knowledge, ours is the first systematic study to demonstrate the direct, selective anti-proliferative/pro-apoptotic effects of EGCG against melanoma cells. This raises the possibility that EGCG, at physiologically attainable concentrations, may have chemopreventive and even therapeutic potential for human melanoma. Based on these findings, we believe that EGCG, either alone or in combination with the standard therapy, could be a novel strategy for the management of melanoma. However, further studies are needed to validate our findings in appropriate animal models.

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