Curcumin-altered p53-Response Genes Regulate Radiosensitivity in *p53*-Mutant Ewing's Sarcoma Cells

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Abstract. *Aim: Curcumin has been demonstrated to have antitumor effects including radiosensitization by modulating many molecular targets including p53. Herein, we investigated the radiosensitizing effect of curcumin in p53 mutant Ewing's sarcoma (ES) cells. Materials and Methods: Cells exposed to radiation with or without curcumin were examined for transcriptional and translational levels of p53 downstream targets and its influence in regulated apoptosis, DNA fragmentation, cell survival and clonal expansion. Results: Curcumin significantly caused radiation induced expression of p21 and Bax, and reduced BclXl, Mcl1 with only marginal Bcl2 modulation. As a positive control to the study, both transcriptional and translational levels of p53 remained unchanged after radiation with/without curcumin. Conversely, curcumin caused radiation-induced apoptosis and DNA fragmentation. Consistently, curcumin enhanced radiation-induced cytotoxicity and clonal expansion. Conclusion: These results suggest that curcumin potentially radiosensitizes p53-mutant ES cells by regulating IR-modulated p53-response genes. However, the curcumin-associated p53-independent regulation of downstream targets remains to be explored.*

Ewing's sarcoma (ES), the third most frequent primary sarcoma of bone (1) and the second most common bone tumor (after osteosarcoma) occurring in children and adolescents, accounts for ~3% of all pediatric and ~10% of all primary bone malignancies (1). ES has a strong potential to metastasize (28%) to the lungs, bone, bone marrow, lymph nodes, liver and/or brain (1). Despite advances in the treatment of localized ES, the prognosis for detectable metastatic ES remains poor with <10% 5-year event-free and overall survival (1). Clinical and laboratory evidence suggests that several common types of human cancer contain populations of rapidly proliferating clonogens that can have substantial impact on local control following chemoradiation or conventional radiotherapy (RT) (2). To that end, RT has well-recognized applications in the control of both primary and metastatic ES (3, 4). The RT dose for local control is 55.8-60.0 Gy, with a fractionation of 180 to 200 cGy/d, 5 days a week (4). However, tumor radioresistance remains a critical obstacle in clinical radiotherapy that results in local recurrence and RT-induced sarcomas (5). The risk of developing solid tumors, including secondary ES, appears to be greatest in patients treated with RT and is most pronounced in skeletally immature patients (5). Over the years, particularly in the last decade, molecular signaling and transcriptional regulations involved in the induced radioresistance of cancer cells has been extensively studied. Recently, studies have demonstrated that despite *p53* mutations, its downstream pathway and the DNA damage signaling pathway are functionally intact in ES (6). Thus, it is imperative to identify anticancer/radiosensitizing agents that are highly effective in inducing cell death, preferably in a p53-independent targeting of pro-apoptotic molecules in ES cells.

Curcumin (diferuloylmethane), a dietary polyphenol derived from turmeric, *Curcuma longa*, is a pharmacologically safe and effective agent that has been demonstrated to have anti-inflammatory, anti-proliferative, and anti-tumor effects by modulating many potential molecular targets (7). Curcumin is widely used at low doses as a food dye and at higher doses in Ayurvedic medicine. Because of its use as a food additive and its potential for cancer chemoprevention, curcumin has undergone through extensive toxicological screening and pre-clinical investigations in rats, mice, dogs, and monkeys (8, 9). In clinical trials, cancer patients have not shown adverse effects with doses from 2000 to 8000 mg/day (10). More

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recently, it has been demonstrated that curcumin injected peripherally crosses the blood-brain barrier (11). In the cancer literature, voluminous data have demonstrated that curcumin has a potent role in inhibiting cellular migration (12), anti-proliferation (13), inhibiting cancer growth (14), preventing the progression of cancer to a hormone-refractory state, (15) and cell killing (16, 17). Recently, a number of studies in various cancer cells demonstrated the involvement of *p53* in curcumin-induced apoptosis (18-22). Conversely, curcumin has also been demonstrated to induce *p53*-independent cell death by inhibiting ubiquitin-dependent proteasome pathways. Accordingly, we investigated whether curcumin confers a radiosensitizing effect in *p53*-mutant ES (SK-N-MC) cells. Our results demonstrate that curcumin conferred IR-induced apoptosis, DNA fragmentation, cytotoxicity and clonal expansion in ES cells. Furthermore, the results suggest that curcumin targets *p53* response molecules involved in cell cycle, apoptosis (*Bcl2:Bax* ratio) and DNA damage signaling pathways in these p53 mutant ES cells.

Materials and Methods

Cell culture, curcumin treatment and irradiation experiment. Human ES (SK-N-MC) cells were maintained as monolayer cultures as previously described (23, 24). Cells plated in 100 mm tissue culture plates (70-80% confluence) were exposed to single radiation (IR, 2 Gy) dose using Gamma Cell 40 Exactor (Nordion International Inc., Ontario, Canada) at a dose rate of 0.81 Gy/min. Mock irradiated cells were treated identically except that the cells were not subjected to IR. The experiments were repeated at least three times in each group. Curcumin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMSO (36.8 mg/ml) to a stock concentration of 100 mM was further serially diluted in plain medium to a working concentration of 10 μM. For experiments with curcumin alone, ES cells were treated with 10, 50 or 100 nM curcumin and allowed to incubate at 37˚C for an additional period of 3 hours. For IR experiments, cells incubated with 10, 50 or 100 nM of curcumin for 3 hours were then exposed to IR and collected at 24 hours post IR incubation, unless otherwise indicated.

Transcriptional response of p53 and p53 target genes. Curcumin-associated alterations of IR-modulated transcriptional responses of p53 and its downstream molecules Bcl2, Bax, p21, Mcl1, Bclxl and Gadd45 were analyzed using an RNAse protection assay and validated using real-time QPCR. RPA was carried out using the RiboQuant™ Multi-Probe RNAse Protection Assay System (PharMingen, Mississauga, ON, Canada) as described earlier (25). Similarly, total RNA extraction and QPCR were performed as described in our earlier studies (23, 24) using *Bax* (sense, 5'- GAG CGG CGG TGA TGG A-3'; anti-sense, 5'- TGG ATG AAA CCC TGA AGC AAA-3'), *Bcl2* (sense, 5'-TCG CCC TGT GGA TGA CTG A-3'; anti-sense, 5'-CAG AGA CAG CCA GGA GAA ATC A-3'), *BclXl* (sense, 5'-CCA CTT ACC TGA ATG ACC ACC TAG A-3'; anti-sense, 5'-CAG CGG TTG AAG CGT TCC-3'), *MCl1* (sense, 5'- TTC CAA GGC ATG CTT CGG AAA C-3'; anti-sense, 5'- TCT GCT AAT GGT TCG ATG CAG C-3'), *p21* (sense, 5'- CAG AAC CGG CTG GGG AT-3'; anti-sense, 5'- CGG CGT TTG GAG TGG TAG-3') and *p53* (sense, 5'- ATG GAG GAG CCG CAG-3'; anti-sense, 5'- AAA TCA TCC ATT GCT T-3') gene specific primers. We used *β-actin* as a positive control (sense, 5'- ATG ACC CAG ATC ATG TTT GA- 3'; anti-sense, 5'- TAC GAC CAG AGG CAT ACA G- 3') and a negative control without template RNA was also included. Each experiment was carried out four times and the $\Delta\Delta^{ct}$ values were calculated by normalizing the gene expression levels to *β-actin* and the relative expression level was expressed as a fold change. Group-wise comparisons were made using ANOVA with Tukey's post-hoc correction (Graph Pad, La Jolla, CA, USA). A *P*-value of <0.05 is considered statistically significant.

Translational modifications of p53 and p53 target genes. Total protein extraction and western blot analysis was performed as described in our earlier studies (26, 27). For this study, the protein transferred membranes were incubated with either anti-mouse p53 (Santa Cruz Biotech, Santa Cruz, CA, USA), *Bcl2* (Invitrogen, Carlsbad, CA, USA) or anti-rabbit Mcl1, p21 (Santa Cruz Biotech), Bax (BD biosciences, Mississauga, ON, Canada) antibodies in 1% BSA-PBST. Blots were stripped and reblotted with mouse monoclonal anti-α-tubulin antibody (Santa Cruz Biotech) to determine equal loading of samples. Stripping was accomplished using 0.3 M NaOH supplemented with 0.005% sodium azide for 10 min.

Radiosensitization. In order to assess curcumin-induced radiosensitization in ES cells, we examined the induced apoptosis, cytotoxicity and clonal expansion. Because induced apoptotic cell death could not be precisely validated with a single assay, we examined the curcumin-induced effect on cell killing by both DNA fragmentation detection (Fluorescein-FragEL, Oncogene Research Products, Boston, MA, USA) and annexin V-FITC staining as described earlier (23, 24). Cell survival was analyzed using MTT and clonal expansion using clonogenic assay as described in our previous studies (23). For the colony-forming assay, ES cells were seeded at a density of 2500 cells (Countess) per 30 mm plate in 3 ml complete media and exposed to 2 Gy with or without curcumin pre-treatment, and were allowed to incubate at 37˚C for 14 days. Plates for untreated (unirradiated) controls were also included. Plates from all groups including untreated controls were transported to the irradiation facility and handled similarly to normalize any variations between groups. Digitalized images were subjected to computed colony counting using Image Quant (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) implying the standard sensitivity, noise factor and background. For MTT assay, cells (1000 cells/300 μl in a 24-well plate) were either mock-irradiated, or exposed to IR and treated with Curcumin, or exposed to IR and treated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (30 μl/well from 5 mg/ml stock) for 4 hours. Solubilization of the converted purple formazan dye was accomplished by adding 200 μl/well of acid-isopropanol with continuous shaking for 20 min at 37˚C. The reaction product was quantified by measuring the absorbance at 570 nm using a Synergy II microplate reader (Biotek, Winooski, VT, USA). Cell survival response was compared using ANOVA with Tukey's post-hoc correction (Graph Pad, La Jolla, CA, USA).

Results

Curcumin significantly modulated p53 downstream apoptosis-related molecules in a p53-independent manner. Compared to the mock-irradiated controls, IR exposure with or without curcumin did not reveal any significant variation in *p53* mRNA

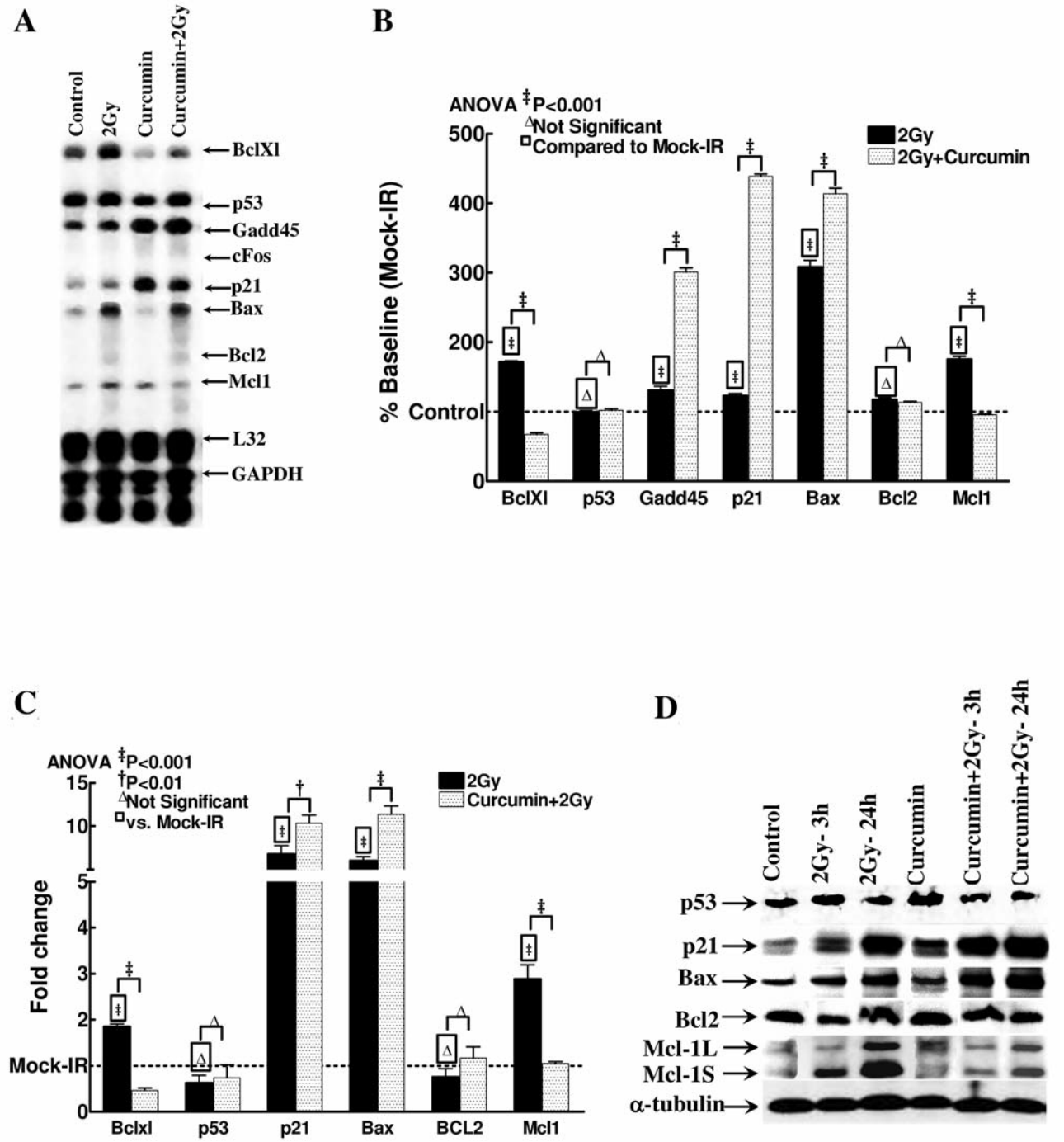

Figure 1. *Effect of curcumin on p53 and its downstream molecular targets in p53-mutant human ES cells. A: Representative autoradiogram of RNase protection assay showing the expression levels of BclXl, p53, Gadd45, cFos, p21, Bax, Bcl2 and Mcl1 mRNA. B: Densitometric analysis of RPA showing the curcumin-dependent profound alterations of IR-modulated BclXl, p53, Gadd45, cFos, p21, Bax, Bcl2 and Mcl1 mRNA. C: Real time Q-PCR analysis showing Bcl2, p53, Mcl1, p21, BclXl and Bax mRNA expression in human ES (SK-N-MC) cells exposed to IR (2 Gy), curcumin (100 nM) or pretreated with curcumin and exposed to IR. β-Actin was used as the positive control. Negative controls without template RNA were also included. D: Western blot analysis showing the expression of p53, p21, Bax, Bcl2 and Mcl1 proteins in mock-IR; IR (2 Gy) and harvested after 3 h and 24 h; treated with curcumin (100 nm); and pretreated with curcumin (100 nM), exposed to IR and harvested after 3 h and 24 h. The expression was digitally captured using Kodak image Station 4000R.*

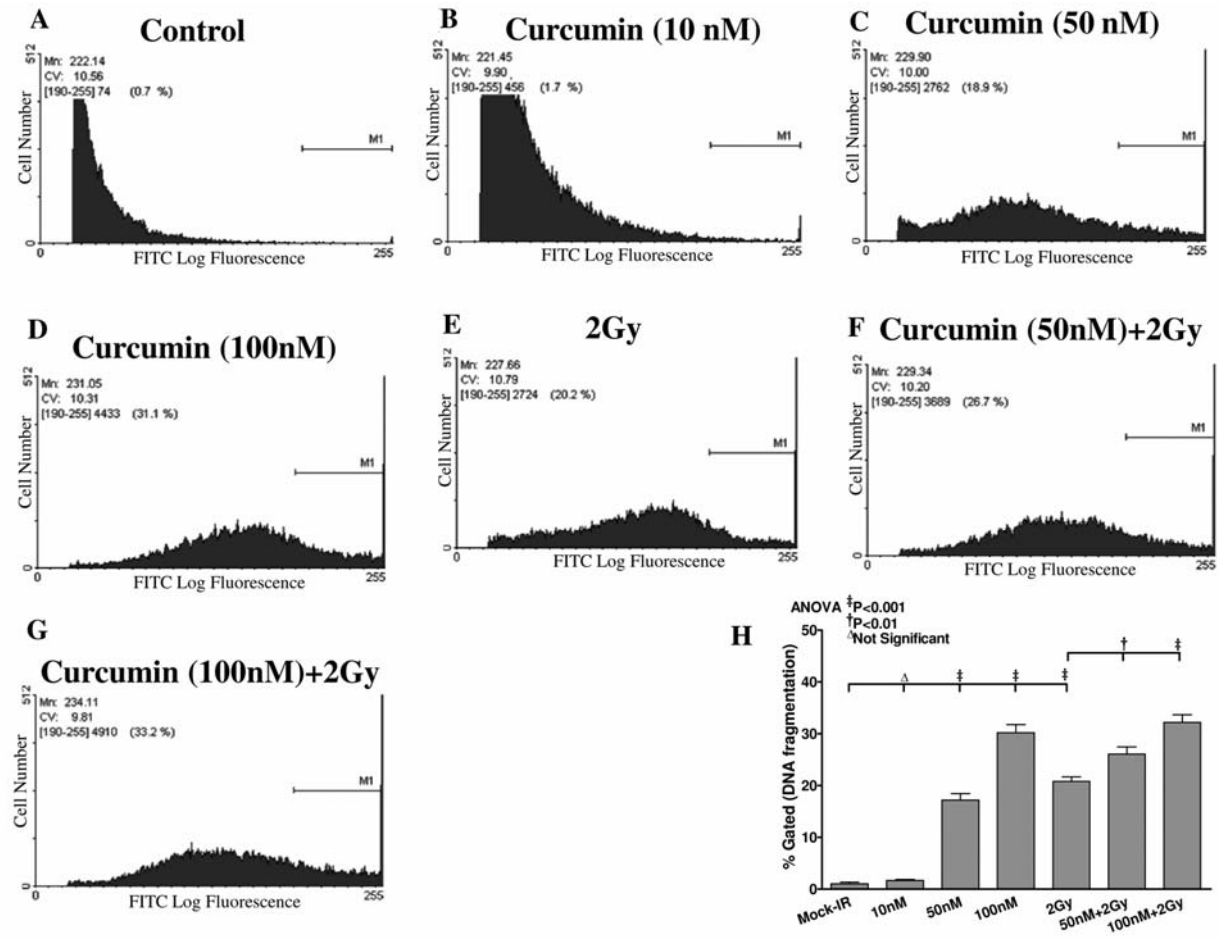

Figure 2. *Fluorescein Fragel DNA fragmentation analysis showing DNA fragmentation in ES cells. A: Exposed to mock-IR, B: treated with 10 nM C: 50 nM and D: 100 nM curcumin, E: exposed to IR (2 Gy), or exposed to IR with F: 50 nM and H: 100 nM curcumin pre-treatment, H: histogram showing the significant and dose-dependent effect of curcumin on IR-induced DNA fragmentation in human ES cells.*

levels as evidenced with RPA analysis (Figures 1A and 1B). Consistent with the RPA observation, real-time QPCR analysis precisely demonstrated similar transcriptional levels of *p53* in these cells (Figure 1C). Consistently, immunoblot analysis showed no variation in *p53* expression after exposing the cells to 2 Gy and harvested after 3 hours or 24 hours. Moreover, compared to the mock irradiated controls, curcumin treatment alone or with IR exposure showed minimal effect on the cellular localization of p53 (Figure 1D). No alterations both in p53 transcriptional and translational levels after IR exposure with or without curcumin treatment in p53 mutant ES cells served as positive controls for the study.

RPA analysis revealed a marked increase in *p21* transactivation as opposed to mock IR controls. This IR-induced *p21* mRNA levels were significantly enhanced in cells pre-treated with curcumin (Figure 1A and B). Consistent with RPA results, QPCR analysis revealed, IR-induced *p21* mRNA levels and to that end, significantly ($P<0.001$) enhanced levels with curcumin treatment (Figure 1C). Furthermore, Western blot analysis revealed that IR induced p21 3h post-IR. However, we observed a robust p21 cellular localization after 24 hours in these cells. More importantly, curcumin treatment resulted in significant enhancement of IR-induced p21 expression both after 3 and 24 hours (Figure 1D).

Compared to Mock IR cells, IR exposure with or without curcumin failed to modulate *Bcl2* transcriptional levels at least in these cells as evident from both RPA (Figures 1A and B) and QPCR (Figure 1C) analysis. Furthermore, immunoblot analysis revealed consistent Bcl2 protein levels in cells exposed to mock-IR, IR or treated with curcumin and exposed to IR (Figure 1D). Conversely, RPA analysis revealed that IR significantly induced *Bax* mRNA levels in SK-N-MC cells (Figures 1A and 1B). Consistently, QPCR analysis validates the induced *Bax* mRNA levels in cells exposed to 2 Gy (Figure 1C). IR-induced *Bax* transcriptional levels were significantly enhanced in cells pretreated with

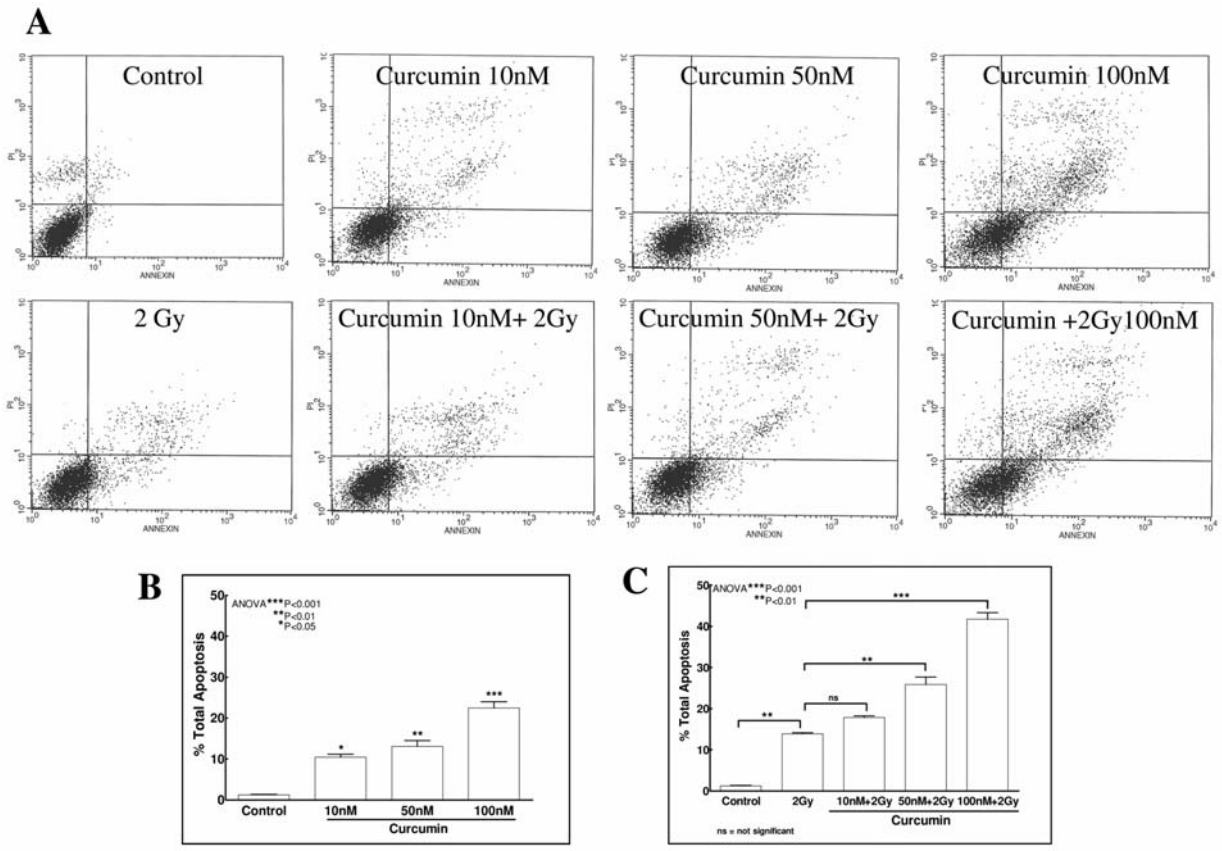

Figure 3. *A: Annexin V-FITC staining showing apoptosis in ES cells exposed to mock-IR, treated with curcumin (10, 50, 100 nM), exposed to IR (2 Gy) with or without curcumin pre-treatment. B: Histogram showing curcumin-induced significant and dose-dependent induction of apoptosis in ES cells. C: Histogram showing the profound effect of curcumin on IR-induced apoptosis in human ES cells.*

curcumin. As opposed to Bcl2 expression, Bax immunoblot analysis revealed a time-dependent induction after IR exposure. More importantly, this IR-induced Bax expression was significantly increased with curcumin treatment (Figure 1D). Up-regulation of Bax and no changes in Bcl2 levels in curcumin plus IR-treated cells altered the Bcl2:Bax ratio.

IR profoundly (Figures 1A and B) and significantly (P<0.001) induced *Mcl1* mRNA expression (Figure 1C) in ES cells as opposed to mock IR exposed cells. This IR-induced *Mcl1* mRNA expression was profoundly suppressed in the presence of curcumin (Figures 1A-C). Similarly, we observed an induced Mcl1 protein expression in cells exposed to 2 Gy after 3 hours and this induced expression was robust after 24 hours. However, curcumin pre-treatment profoundly inhibited IR-induced Mcl1 expression both after 3 hours and 24 hours (Figure 1D).

Compared to mock-IR cells, 2 Gy significantly up-regulated *BclXl* mRNA levels as evident by both RPA (Figures 1A and 1B) and QPCR (Figure 1C) analysis. However, the expression of *BclXl* mRNA was significantly (P<0.001) suppressed in cells pretreated with curcumin and exposed to IR.

Curcumin confers radiosensitization. Compared to mock-IR cells, Fluorescein fragel DNA fragmentation analysis revealed that curcumin at low (10 nM) concentration did not induce any significant (1.7±0.3%) DNA fragmentation in human SK-N-MC cells (Figures 2B and H). However, higher concentrations (17.2±2.5% with 50 nM and 30.2±3.1% with 100 nM) of curcumin markedly induced DNA fragmentation (Figures 2C, 2D and 2H). Conversely, compared to the untreated controls (1±0.7%), IR significantly (20.8±1.8%) induced DNA fragmentation (Figure 2E). Pretreatment with either 50 nM (26.1±2.7%) or 100nM (32.2±2.9%) of curcumin enhanced the IR-induced apoptosis (Figures 2F-H).

Similarly, annexin V-FITC staining revealed a significant and dose dependent induction of apoptosis in cells exposed to curcumin, with a maximum cell death at 100 nM concentration (Figures 3A and B). Likewise we observed significant induction of apoptosis in cells exposed to IR as opposed to mock-IR exposed cells (Figures 3A and C). Pre-treating the cells with 10 nM of curcumin marginally increased the IR-induced apoptosis (Figure 3A). However, cells pre-treated with higher concentrations of curcumin

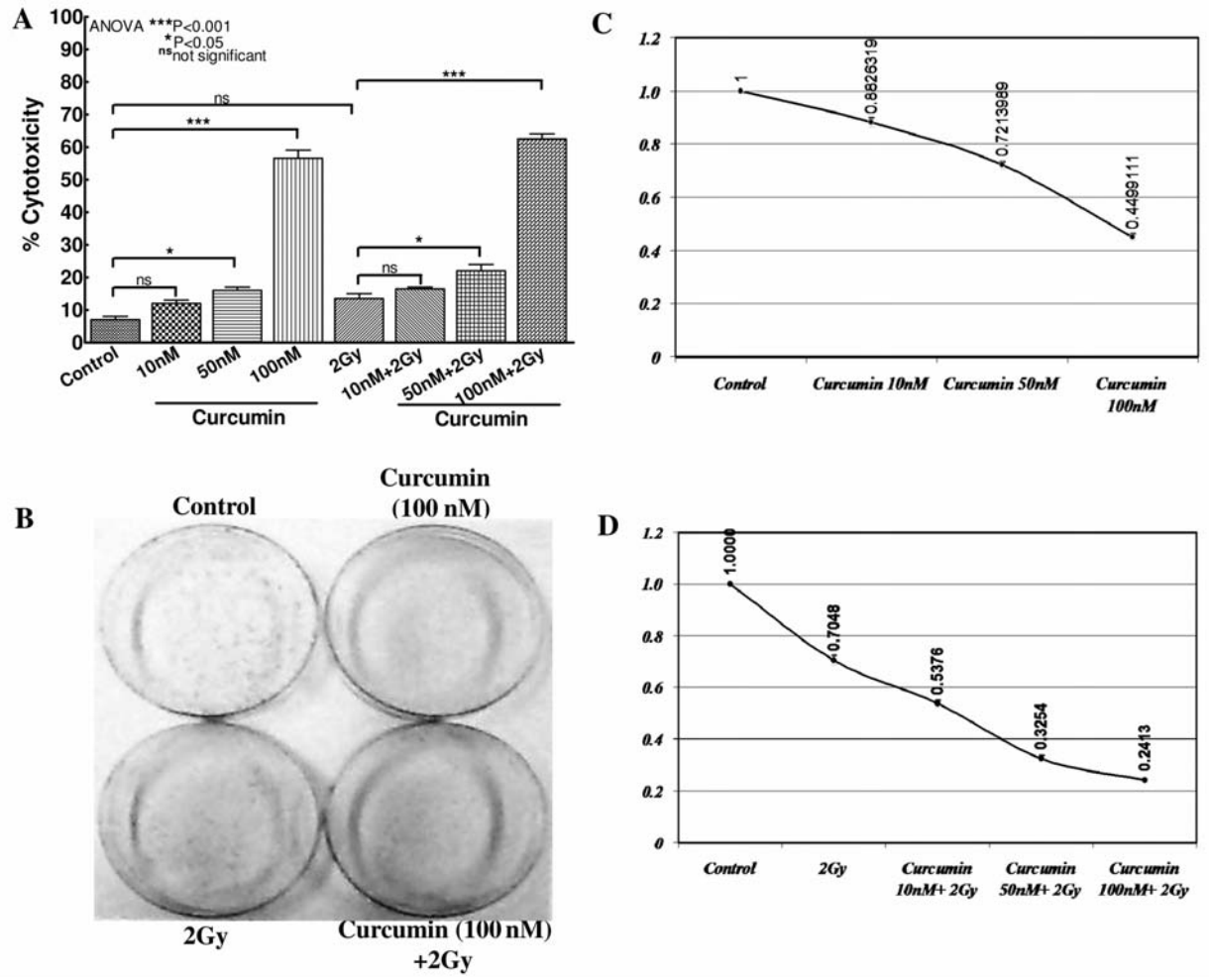

Figure 4. *A: Effect of curcumin on IR-induced cytotoxicity in human ES cells exposed to mock-IR, treated with curcumin (10, 50, 100 nM), exposed to IR (2 Gy) with or without curcumin pre-treatment. MTT assay was performed in triplicate. The reaction product was quantified by measuring the absorbance at 570 nm. B: Colony-forming assay showing stained colonies in control, IR, curcumin and curcumin+IR treated plates. Cells (1000 cells/plate) seeded in 100 mm culture plates were exposed to IR with or without curcumin pre-treatement and incubated for 14 days. The colonies were stained with crystal violet and counted manually under low magnification, C: Dose-dependent inhibition of colony-forming units in cells pretreated with curcumin. D: Curcumin IR-suppression of colony-forming units in SK-N-MC cells. Group-wise comparisons were made using ANOVA with Tukey's post-hoc correction.*

showed in increased and dose-dependent induction of IR-induced cell death (Figures 3A and C).

In order to assess the effect of curcumin on radiosensitization, we examined the induced modulations both in the metabolic and clonogenic activities of the cells. MTT analysis showed that compared to the mock-IR cells, IR significantly reduced cell survival (Figure 4A). Similarly, curcumin treatment showed a dose-dependent inhibition of cell survival in these ES cells. More importantly, IR-induced inhibition of cell survival was significantly increased with curcumin. To that end, we observed a dose-dependent effect on IR-induced inhibition of cell survival (Figure 4A). To delineate the curcumin effect in IR-inhibited clonal expansion in ES cells, human SK-N-MC cells either mock-irradiated or exposed to 2 Gy with or without different concentrations of curcumin were assessed for colony-forming capacity (Figure 4B). Compared to the mock-IR cells, IR significantly (P<0.001) induced clonal expansion (Figure 4D). Consistent with these findings, curcumin treatment exhibited significant (P<0.001) dose-dependent inhibition of clonal expansion (Figure 4C). The maximum inhibition was observed at a concentration of 100 nM. More importantly, as opposed to IR-exposed cells, cells pretreated with curcumin profoundly (P<0.001) augmented the IR-suppressed clonal expansion in a dose-dependent manner (Figure 4D).

Discussion

The tumor suppressor p53 is mutated in more than 50% of all tumors. Human SK-N-MC cells express mutant *p53* with a deletion in exons 2 and 3 at the *N*-terminus of *p53* (28-30). Importantly, most clinically useful antineoplastic and radiosensitizing agents are less potent and efficacious in the context of mutant *p53*. Similarly, loss of *p53* function plays a key role in sustained high level resistance to chemoradiotherapy in childhood tumors (31). In the present study we determined the effect of curcumin, a dietary polyphenol in conferring radiosensitization in SK-N-MC cells *via* molecular mechanisms independent of *p53*. Our results showed that IR with or without curcumin failed to modulate the expression of *p53* in these cells. However, curcumin significantly enhanced *p53*-responsive *p21* that plays a key role in G_2/M arrest of the cell cycle, which is sensitive to IR and therefore this leads to enhanced radiosensitization. A growing body of evidence indicates that expression of the tumor suppressor *p21*, a potent cyclin-dependent kinase (cdk) inhibitor (32), was implicated in growth arrest in response to a variety of conditions, including DNA damage and terminal differentiation (33). Moreover, up-regulation of *p21* was required for growth inhibition of human cancer cells. In contrast, in *p21*-deficient mouse embryo fibroblasts, γ-IR induced arrest of the cell cycle was compromised (34). Induction of *p21* in response to DNA damage requires the function of the *p53* tumor suppressor protein (35). However, *p21* expression was shown to be induced in several cell lines by agents that cause terminal differentiation through a *p53*-independent mechanism (35). Consistently, in the present study, we observed induced expression of *p21* and *Bax* in curcumin-pretreated cells with or without IR and no or little modulations in *p53* expression. The induction of *p21* and *Bax* and inhibition of *BclXl* and *Mcl1* by curcumin was associated with the induction of apoptosis and radiosensitization. Similarly, numerous studies reported that curcumin can cause cell cycle arrest and apoptosis in several tumor cell lines (18, 20, 36). Recently, Chendil and colleagues (37) reported that in *p53*-mutant PC3 cells, curcumin altered *Tnfα* induced *NFκB* mediated *Bcl2:Bax* ratio and enhanced radiosensitization. Likewise, Jaiswal and colleagues (38) demonstrated that curcumin induced *p53* independent growth arrest (G_2/M) and apoptosis in colon cancer cells. More recently, Li and colleagues confirmed that curcumin exerts chemo- and radiosensitization by modulating *Mdm2*, *p21* and *Bax* in a *p53*-independent manner (39). It is believed that cell cycle arrest is an irreversible process leading to apoptosis if cells are unable to repair their damaged DNA. Further studies need to be performed to verify whether curcumin affects the activity of *cdks* and their regulatory subunits important for cell cycle transitions, that are controlled by *p21*. Moreover, the molecular mechanism(s) involved in *p21* induction by curcumin (*i.e.* transcriptional up-regulation or posttranscriptional stabilization) remains to be established.

Curcumin has been reported to be a potent anti-proliferative agent for many tumor types and it acts as a pro-apoptotic agent in a variety of cancer cell lines (40-44). Our results demonstrate that curcumin enhanced IR-induced apoptosis and increased IR-suppression of cell survival in human SK-N-MC cells. Induction of *p21* induces cell cycle arrest in response to DNA damage, allowing repair enzymes to function. Apoptosis of such arrested cells could well be mediated by a combination of up-regulation of the pro-apoptotic *Bax* and down-regulation of the anti-apoptotic protein *Bcl-2*. We found that curcumin profoundly induced the expression of Bax and had no effect on Bcl2 expression in SK-N-MC cells, which lead to the modulation of the *Bcl2:Bax* ratio. To that end, curcumin also significantly inhibited the IR-induced anti-apoptotic *BclXl* and *Mcl1* expression-thereby facilitating apoptosis. In recent clinical trails, curcumin was given at a dosage of 8000 mg/day, and the peak serum concentration of 1.77 to 2 μM after 2 hours of intake of curcumin has been reported (10). In our results, a significant enhancement of radiosensitizing effect was observed at 100 nM of curcumin. Hence, it is possible to achieve curcumin concentrations of 100 nM in the serum by consumption of much less than 8000 mg/day and this dose of curcumin in the serum will enhance the IR effect in ES patients.

In summary, our studies demonstrate that curcumin pretreatment in human ES (SK-N-MC) cells enhances IR-induced *p21* expression, modulates the *Bcl2:Bax* ratio, inhibits anti-apoptotic *BclXl* and *Mcl1* expression, which potentiates DNA fragmentation, cytotoxicity and cell death. In view of the accumulating evidence that curcumin may serve as a potent radiosensitizer, in *p53* mutant cells in particular, further efforts to explore this therapeutic strategy are necessary.

Acknowledgements

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