Neem Leaf Extract Induces Radiosensitization in Human Neuroblastoma Xenograft Through Modulation of Apoptotic Pathway

JAMUNARANI VEERARAGHAVAN[1,2], SHEEJA ARAVINDAN[3], MOHAN NATARAJAN[4], VIBHUDUTTA AWASTHI[5], TERENCE S HERMAN[1] and NATARAJAN ARAVINDAN[1,2,6]

Departments of [1]Radiation Oncology, [2]Pathology, [3]Pediatrics, [5]Pharmaceutical Sciences and [6]Anesthesiology, University of Oklahoma Health Sciences Center, OUPB 1430, Oklahoma City, OK 73104, U.S.A.; [4]Department of Otorhinolaryngology, Head and Neck Surgery, University of Texas Health Sciences Center at San Antonio, San Antonio TX, 78229, U.S.A.

Abstract. *Induction of apoptosis is directly correlated with the biological effectiveness of ionizing radiation (IR). Accordingly, we investigated the efficacy of neem leaf extract (NLE) on IR-associated apoptotic transcriptional modulation and cell death in neuroblastoma (NB). Materials and Methods: NB xenografts exposed to single dose (SDR, 10 Gy) or fractionated (FIR, 2 Gy/d×5d) with or without NLE were examined for transcriptional activation of 84 apoptotic pathway genes using quantitative polymerase chain reaction. Apoptosis was measured using TdT nick-end labeling. Results: FIR induced 55 and suppressed 10 genes, while SDR induced 49 and suppressed 5 genes. Of these, 46 and 4 genes were commonly up/down-regulated after FIR and SDR. NLE inhibited IR-induced NAIP, BIRC6, BIRC8, NOL3 and enhanced BAK1, BAX, BCL10, CASP1, CASP10 CARD8 and CRADD. Furthermore, NLE conferred FIR- and SDR-induced cell death. Conclusion: These data imply that NLE may exert radiosensitization by activating pro-apoptotic signaling and negating survival signaling and may thus potentiate radiotherapy in NB.*

Neuroblastoma (NB), the most frequent extra cranial solid tumors in children (>90% are seen in patients aged ≤5 years), accounts for 8-10% of all childhood cancers (1) and 15% of childhood cancer fatalities (2). Although a dramatic increase in the survival rate has been achieved during the last two decades (53% to 71%), the risk of relapse and propensity to metastasize pose major challenges in the cure of NB. Recurring tumor may arise from remnant cells of the original neoplasm that have evaded therapeutic intervention and later become visible at the original site. To that end, radiotherapy (RT) is now widely used for high-risk NB patients after chemotherapy and the irradiation dosage depends on the age of the child. Traditionally, RT is delivered in multiple 2 Gy fractions (FIR) for 5 days a week to total ionizing radiation (IR) dose of 50-75 Gy in around 5-7 weeks. IR induces genomic instability (3, 4), adaptive radioresistance (5), and apoptotic response (6). Induced radioresistance in cancer cells might be associated with enhanced survival advantage resulting in tumor regrowth at the treatment site (7). Dose rates ranging from 1-2.5 Gy are enough to increase significantly the risk of a second malignant neoplasm (8). Thus, it is imperative to identify radiosensitizing agents that spare normal cells and induce cell death preferentially in tumor cells. To that end, neem (*Azadirachta indica*), a versatile medicinal plant, possess a wide spectrum of biological activities (9). The antioxidative properties of neem leaf extract (NLE) have been documented both *in vitro* and *in vivo* (9-11). Studies have revealed that NLE inhibits the development of experimental carcinogenesis by modulating multiple molecular targets in key signaling pathways (10-14). Azadirachtin and nimbolide, present in leaves and flowers, are recognized as the most potent neem limonoids that exhibit apoptosis-inducing effects against cancer cell lines *in vitro* (15, 16). Furthermore, studies have demonstrated the modulatory effects of azadirachtin and nimbolide on xenobiotic metabolizing enzymes, oxidative DNA damage, invasion, and angiogenesis *in vivo* (17). More importantly, azadirachtin and nimbolide, both of which are antiproliferative, target PCNA, p21, cyclin D1, GST-P, NFκB, p53, FAS, BCL-2, BAX, APAF-1, cytochrome *c*, Survivin,

Correspondence to: Natarajan Aravindan, Radiation Oncology, OUPB 1430, University of Oklahoma Health Sciences Center, 825 North East 10th Street, Oklahoma City, OK 73104. U.S.A. Tel: +1 4052713825, Fax: +1 4052713820, e-mail: naravind@ouhsc.edu

Key Words: Neuroblastoma, radiotherapy, neem leaf extract, apoptosis, transcriptional response.

caspases 3, 6, 8 and 9, and PARP. Therefore, this study aimed to delineate the radiosensitizing potential of NLE in the NB setting.

Many factors determine tumor resistance in RT, including tumor size, hypoxia, and intrinsic radiosensitivity. The fate of irradiated cells is believed to be controlled by the network of signaling elements that lead to different modes of cell death or survival. Many studies have correlated gene expression and response to RT (18-21) and *in vitro* radiosensitivity (22). Though many stress-responsive genes are inducible by IR (23), only a fraction of these are believed to play a key role in the stress-tolerance phenotype, including cell cycle checkpoints, apoptosis, and DNA repair (24, 25). More importantly, induction of apoptosis is directly correlated with the biological effectiveness of the IR (6, 26). To that end, the alteration of apoptotic transcriptional response after single-dose radiation (SDR) (27) was recently demonstrated as a function of time (28) and the response after chronic FIR (29) was further delineated in NB cells. Accordingly, herein, using an *in vivo* human NB xenograft model, this study investigated the apoptosis-specific transcriptional response after SDR (10 Gy) or FIR (2 Gy ×5) and further elucidated the effect of NLE in modulating IR-induced apoptotic transcriptional alterations in this setting.

Materials and Methods

NB Xenograft development and irradiation experiments. All experiments conformed to American Physiological Society standards for animal care and were carried out in accordance with guidelines laid down by the National Research Council and were approved by the host Institutional Animal Care and Use Committee. Seven-week-old athymic NCr-*nu*/*nu* nude mice (NCI, Frederick, MD, USA) received subcutaneous injections of SK-N-MC (5×10^6) cells suspended in 100 μl of culture media into their right flank. Tumor growth was periodically monitored, tumor volume was calculated using the formula volume=$[(\pi/6) \times \text{length} \times \text{width}^2]$ (30) and tumors were allowed to grow to a volume of approximately 500 mm^3. Xenografts were exposed to either SDR (10 Gy) or FIR (2 Gy/day for 5 days). A specially designed cerrobend shield was used to encase the body of the mice and the exposed flank tumor was irradiated using a Gamma Cell 40 Exactor (Nordion International Inc, Ontario, Canada) at a dose rate of 0.81 Gy/min. Mock-irradiated animals (controls) were treated identically except that xenografts were not subjected to IR. Irradiated animals were allowed to settle for an additional 24 h.

Collection of plant material and preparation of extract. Fresh mature leaves of neem were used for the preparation of the ethanolic extract, slightly modifying the procedure described by Chattopadhyay (31). In brief, oven-dried powder of neem leaves was mixed in 70% ethanol and incubated for 36 h followed by further extraction at 54˚C for 2 h with continuous agitation. The blend was cooled, filtered and completely dried in pre-weighed tubes using a speed vacuum. Dried NLE was weighed and dissolved in ethanol. For chemical analysis, high-performance liquid chromatography was carried out using a mobile phase consisting of isocratic solvent system consisting of 35% water and 65% acetonitrile and containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The stationary phase consisted of a Phenomenex (Torrance, CA, USA) Luna C-18 column (250×4.6 mm, 5 μm). The ultraviolet signal was recorded at 227 nm. Standard azadirachtin (Sigma, St. Louis, MO, USA), A7430) was found to have a retention time of 5.1 min in this solvent system. NLE showed two major peaks eluting within 6 min of chromatography. When the NLE was spiked with standard azadirachtin, the second peak was found to be enhanced, suggesting that the extract has significant amounts of this known active substance. For NLE treatment alone, animals were intraperitoneally injected with 10, 20, 50 mg/kg of NLE and the xenografts were harvested after 24 h. To determine the effect of NLE on IR-induced modulations, animals were injected with NLE and allowed to settle for 3 h before radiation.

QPCR profiling. Total RNA was extracted from treated xenografts with RNA-Stat60 reagent (Tel-Test Inc, Friendswood, TX, USA) following the manufacturer's instructions. Real-time QPCR profiling was performed as described previously (29, 32) using human apoptosis profiler (SA Biosciences Corporation, Frederick, MD, USA). In brief, cDNA mixed with SYBR Green based super mix were equally loaded in each well of the array plates equipped with 96 primer sets for a thoroughly researched set of 84 pathway focused genes. This highly selected RT-profiling was used instead of an all-encompassing gene arrays because the selected genes entail a well-characterized profile governing apoptosis, hence facilitating interpretation of data, simplifying data acquisition and analysis, and avoiding genes not functionally characterized. Each profiling plate was also equipped with RT controls, positive controls, genomic DNA control and house keeping genes. The $\Delta\Delta^{ct}$ values were calculated by normalizing the gene expression to that of the housekeeping genes. Normalized data were then compared between the groups, and the relative expression level of each gene was expressed as a fold change. When comparing each gene's signal intensity between groups, an increase/decrease of >1-fold was used to represent up-regulation and down-regulation respectively. Furthermore, intervention associated (group-wise comparisons) gene alterations were analyzed using ANOVA with Tukey's *post-hoc* correction (GraphPad, La Jolla, CA, USA). A p-value of <0.05 was considered to be statistically significant.

DNA fragmentation. The Fluorescein-FragEL kit (Oncogene Research Products, Boston, MA, USA) was used to detect DNA fragmentation in NB xenograft from mice treated with NLE (10, 20, 50 mg/kg), exposed to 10 Gy, 2 Gy ×5 days with or without NLE as described earlier (33). Relative fluorescence intensity levels were quantified and the groups were compared using ANOVA with Tukey's *post-hoc* correction. A p-value less than 0.05 was considered to be statistically significant.

Results

IR modulated apoptosis-related genes in human NB xenograft. Overall, compared to the mock-IR group, FIR induced 60 genes and down-regulated another 23 genes. One gene, *RIPK2*, did not show any modulations after FIR. On the other hand, SDR induced 65 genes and suppressed 19 genes. Interestingly, in this setting, FIR and SDR commonly

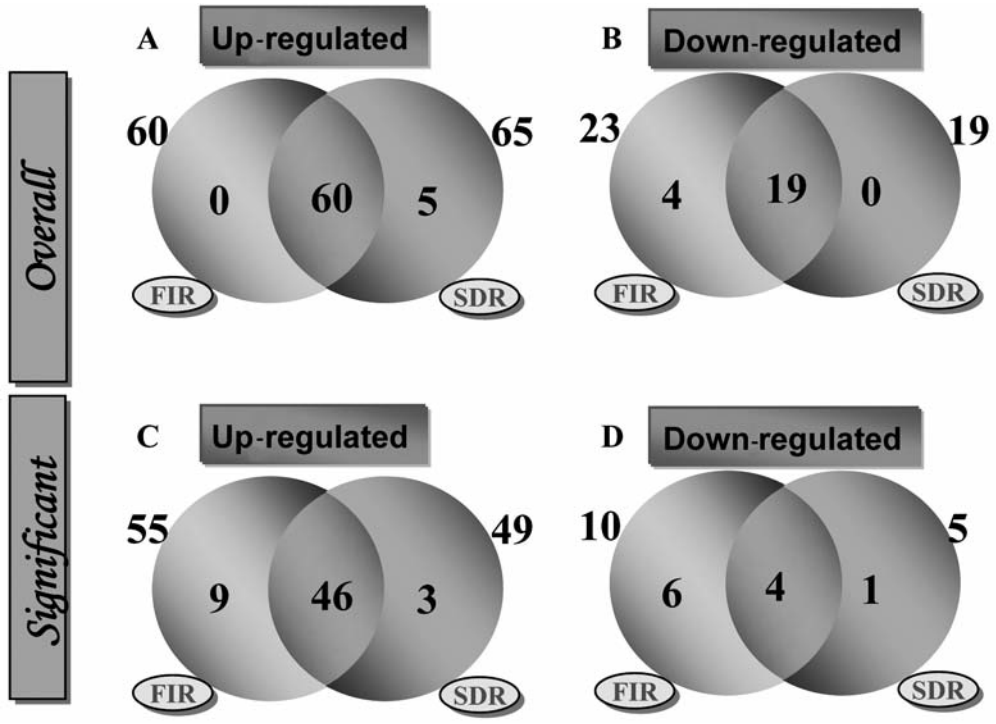

Figure 1. *Venn diagrams showing the overall number of (A) up-regulated or (B) down-regulated apoptosis-related genes in NB xenograft exposed to FIR and SDR. Venn diagrams showing the number of significantly (C) up-regulated or (D) down-regulated apoptosis-related genes in NB xenograft exposed to FIR and SDR. Numbers outside each circle represent the total number of genes up/down-regulated in response to the specific dose regimen. Numbers inside the greater part of the circles represent the genes that were selectively up-regulated at that particular dose. Numbers in two overlapping circles represent the number of genes up/down-regulated commonly under both conditions.*

up-regulated 60 genes (Figure 1A) and down-regulated another 19 genes (Figure 1B). *BCL2A1, BNIP2, CASP4, RIPK2* and *TRAF4* were selectively induced after SDR, while *BCL2A1, BNIP2, CASP4, TRAF4* were selectively suppressed after FIR. ANOVA with Tukey's *post-hoc* correction revealed that FIR significantly induced 55 out of 60 genes including TNF ligands (*CD40LG, FASLG, LTA, TNF, CD70, TNFSF8*) (Figure 2A), TNF receptors (*CD40, FAS, TNFRSF 10A, 10B, 1A, 21, 25*) (Figure 2B), BCL-2 family (*BAG1, BAG3, BAG4, BAK1, BAX, BCL10, BCL2L1, BCL2L10, BCL2L11, BCL2l2, BIK, BNIP1, BNIP3, BNIP3L, MCL1*) (Figure 2C and 2D), caspases (*CASP1*, 10, 2, 3, 5, 6) (Figure 2E), anti-apoptotic molecules (*BFAR, NAIP, BIRC6, BIRC8, BRAF, CFLAR, IGF1R*) (Figure 2F), TRAF and CARD family (*NOD1, CARD6, 8, CARDD, NOL3, PYCARD, TRAF3*) (Figure 2G) and death/CIDE domain, p53 and DNA damage-response molecules (*CIDEB, DAPK1, FADD, GADD45, TP53BP2, TP73, TRADD*) (Figure 2H). Conversely, of the 23 FIR down-regulated genes, expression of 10 *BIRC3* (*BCL2A1, CASP14, BIRC2, 3, XIAP, TRAF2, 4, ABL1, AKT1, DFFA*) was completely suppressed (Figures 2C and 2E-H). Like-wise, 49 out of 65 SDR-induced genes including *CD40LG, FASLG, LTA, TNF, CD70, TNFSF8* (Figure 2A), *CD40, FAS, TNFRSF 10A, 10B, 1A, 21, 25* (Figure 2B), *BAG1, BAG4, BAK1, BAX, BCL10, BCL2L11, BIK, BNIP1, BNIP3, BNIP3L, MCL1* (Figures 2C and 2D), *CASP1, CASP10, CASP5, CASP6* (Figure 2E), *BFAR, NAIP, BIRC8, CFLAR, IGF1R* (Figure 2F), *APAF1, NOD1, CARD6, 8, CARDD, NOL3, PYCARD, TRAF3, 4* (Figure 2G), *CIDEA, CIDEB, DAPK1, FADD, GADD45, TP53BP2 and TRADD* (Figure 2H) were significantly induced. Conversely, *BCL2, BIRC2, ABL1, AKT1, DFFA* of 19 SDR down-regulated genes were completely suppressed (Figures 2C, 2F and 2H). Interestingly, 46 genes were significantly up-regulated and another four genes were completely suppressed commonly after FIR or SDR exposure (Figure 1C and 1D).

NLE modulates cell death associated gene transcription. Altogether, NLE activated 65 genes and suppressed another 18 genes in NB xenografts. Of the 65, activeated genes, 49 including TNF ligands (*CD40LG, FASLG, LTA, TNF, CD70, TNFSF8*) (Figure 2A), TNF receptors (*CD40, FAS, TNFRSF 10A, 10B, 1A, 21, 25*) (Figure 2B), *BCL-2* family (*BAG1, BAG4, BAK1, BAX, BCL10, BCL2L11, BIK, BNIP3, MCL1*) (Figures 2C and 2D), caspases (*CASP1, 10, 2, 3, 4, 5, 6, 7*)

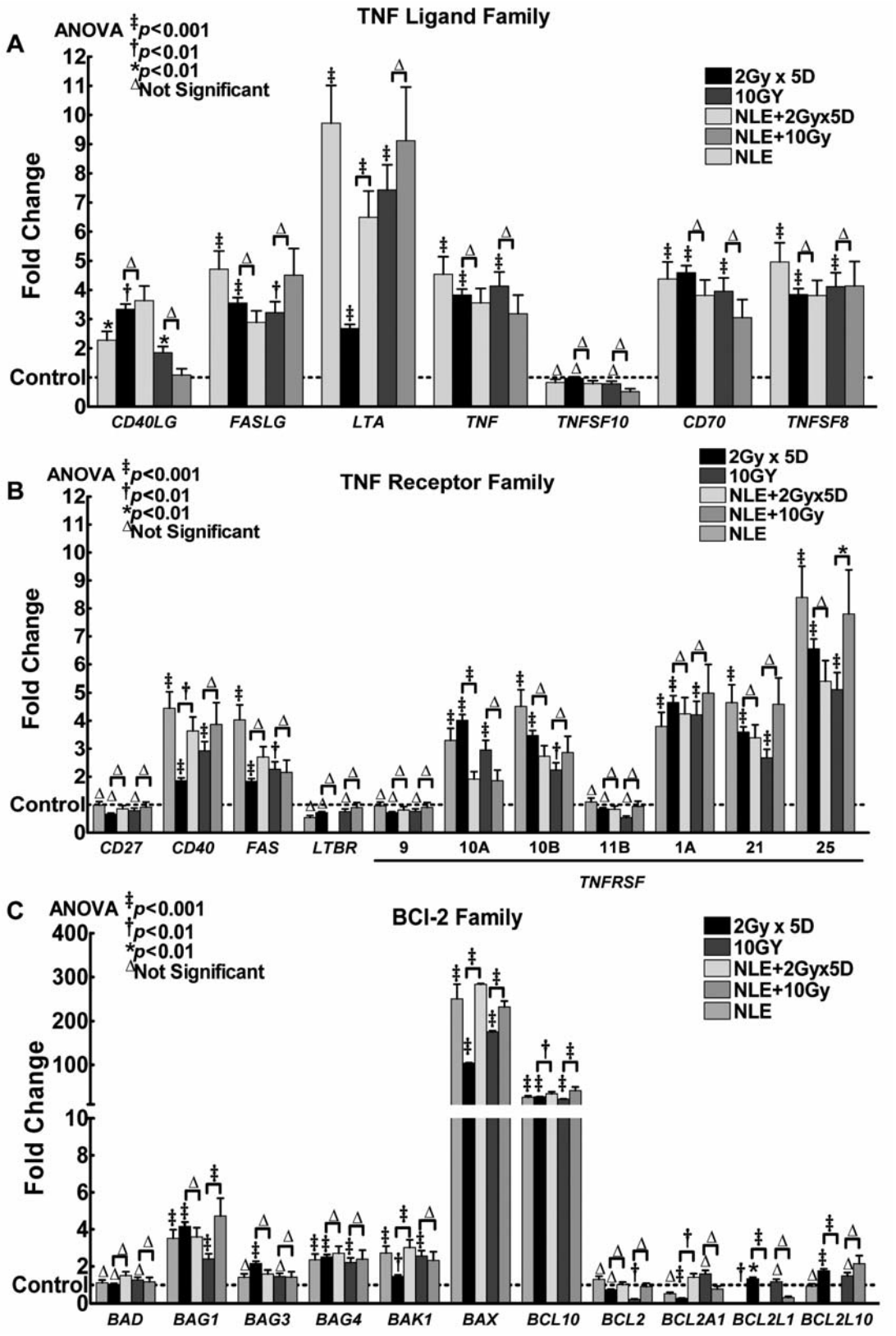

Figure 2. *continued*

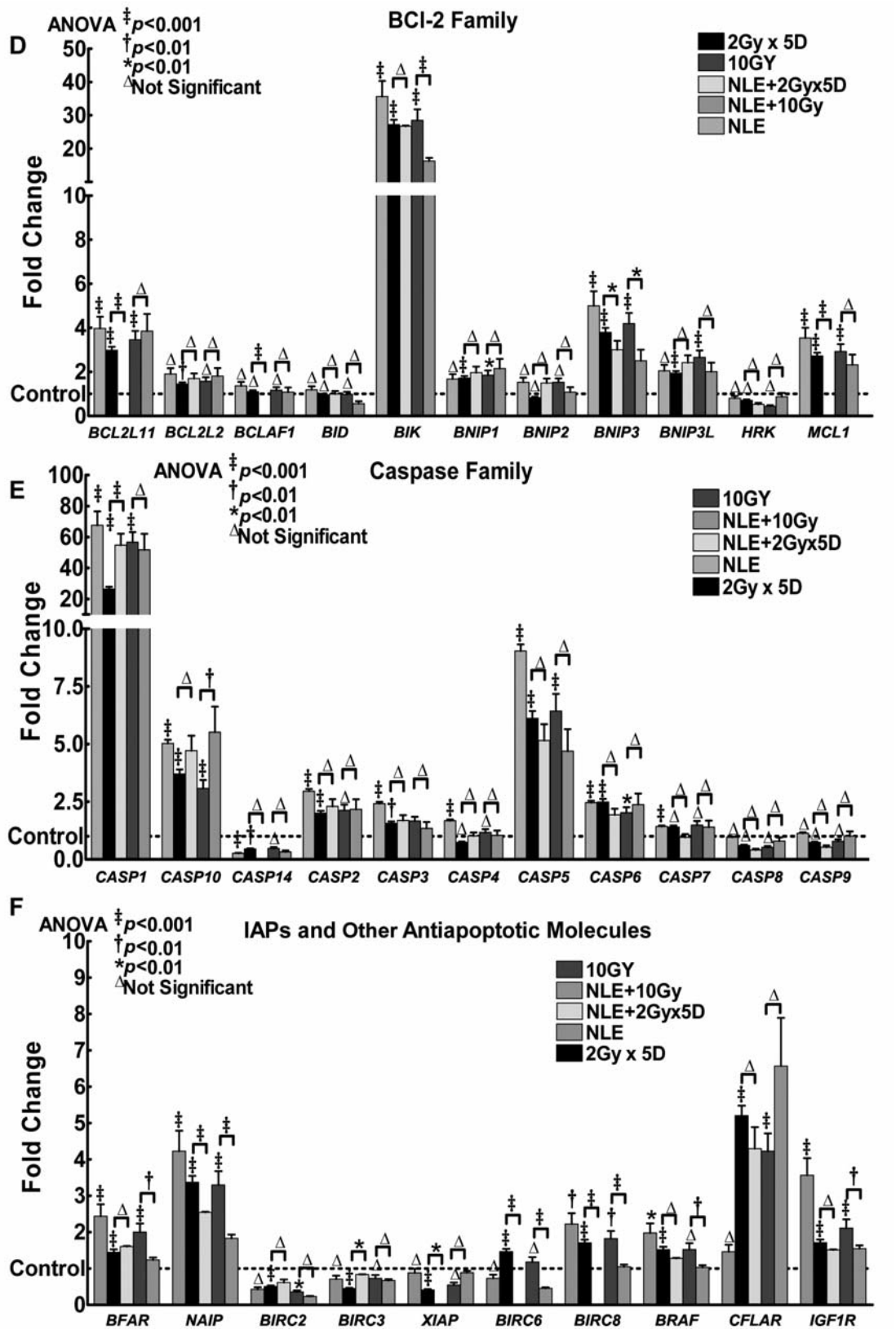

Figure 2. *continued*

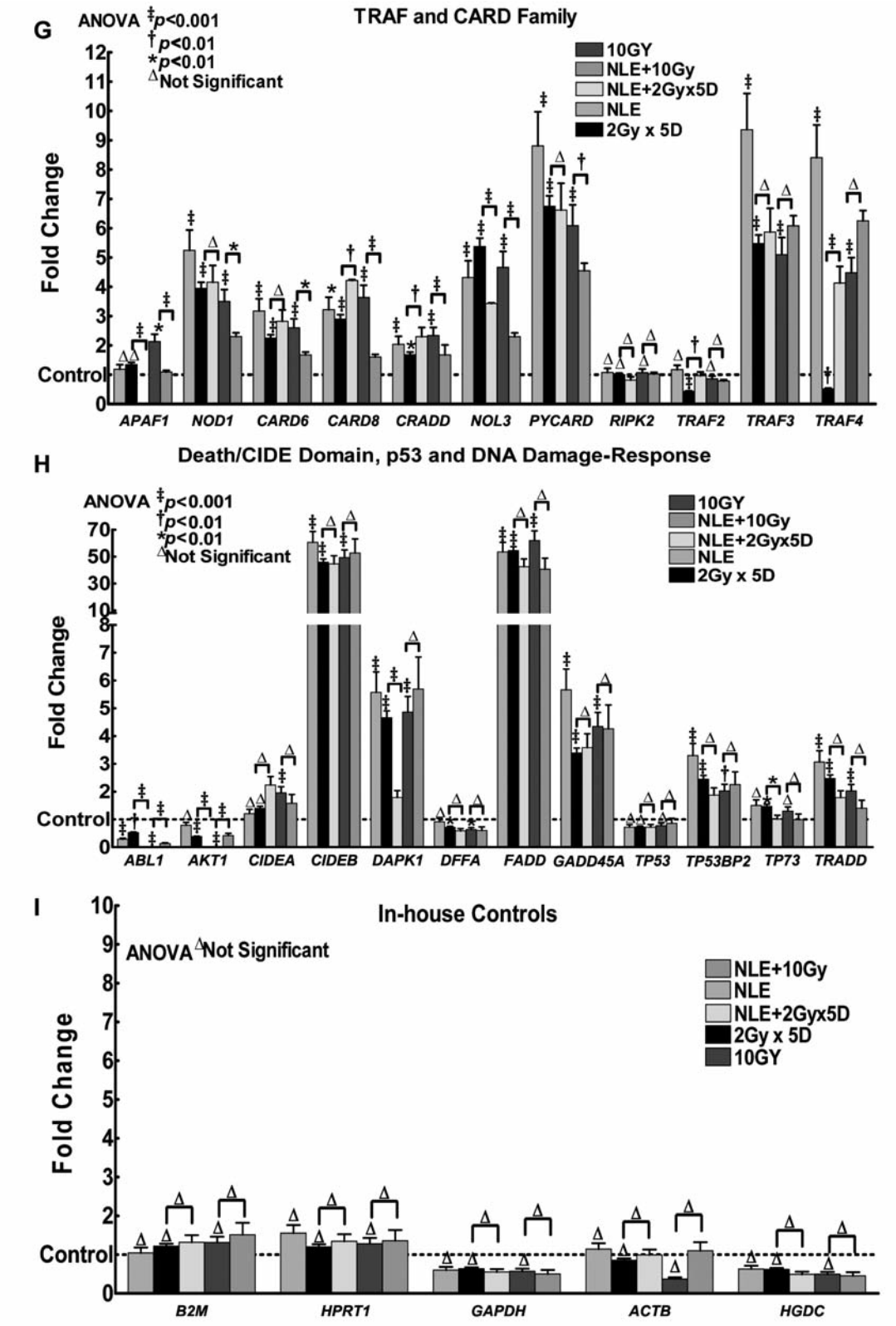

Figure 2. *Histograms showing the levels of (A) TNF ligands, (B) TNF receptors, (C&D) BCL2 family, (E) caspases, (F) IAPs and other anti-apoptotic molecules, (G) TRAF and CARD family, (H) death/CIDE domain, p53 and DNA damage-response molecules and (I) in-house controls in NB xenograft exposed to either mock-IR, SDR, FIR, NLE or treated with NLE and exposed to SDR/FIR.*

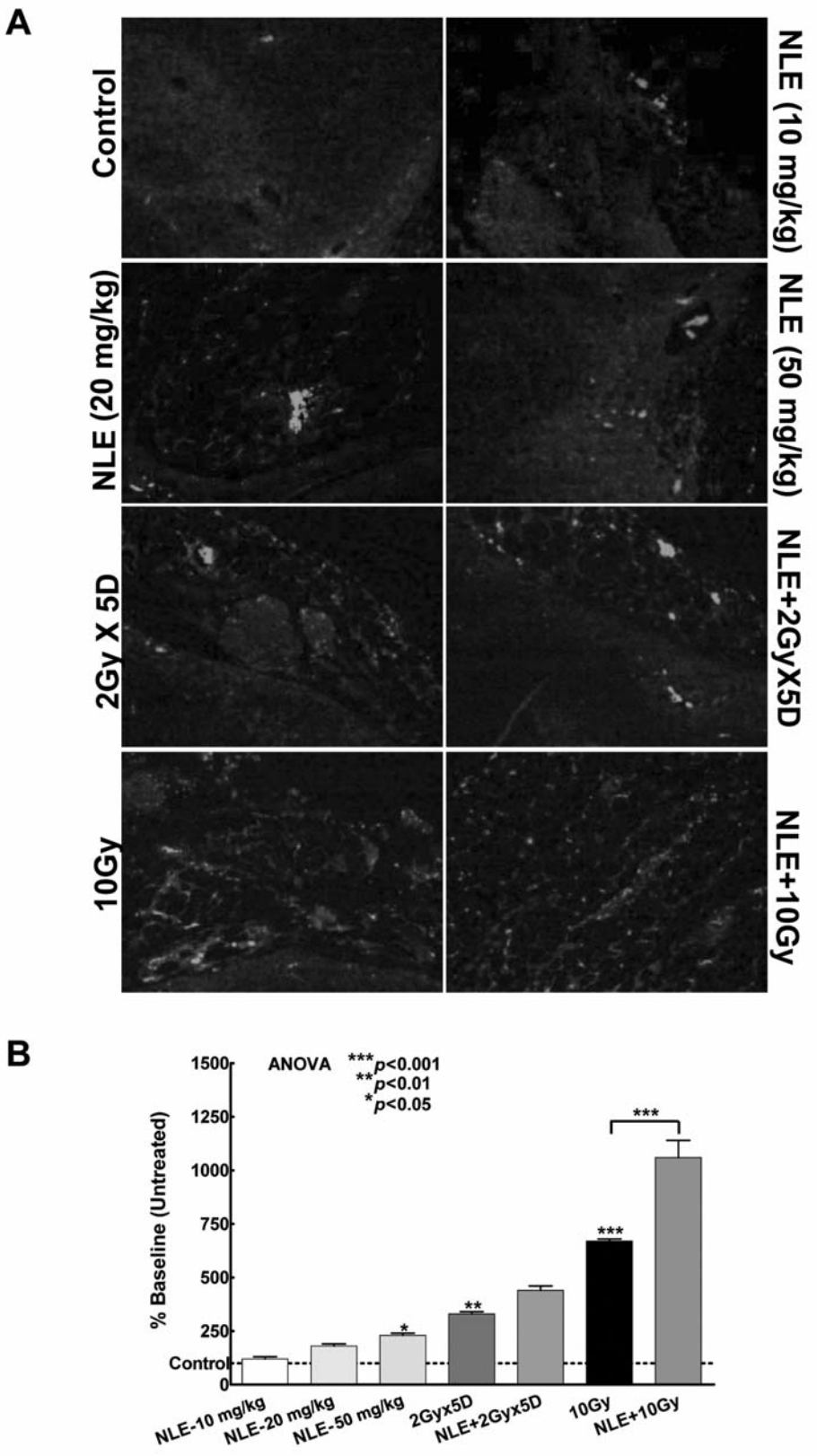

Figure 3. *Photomicrographs showing DNA fragmentation in NB xenograft exposed to SDR/FIR, treated with NLE (10, 20 or 50 mg/kg) with or without SDR/FIR. (B) Histogram showing significant and profound conferring effect of NLE on SDR- and FIR-induced DNA fragmentation in NB xenograft.*

(Figure 2E), anti-apoptotic molecules (*BFAR, NAIP, BIRC8, BRAF, IGF1R*) (Figure 2F), TRAF and CARD family (*NOD1, CARD6, CARD8, CARDD, NOL3, PYCARD, TRAF3, TRAF4*) (Figure 2G) and death/CIDE domain, p53 and DNA damage-response molecules (*CIDEB, DAPK1, FADD, GADD45, TP53BP2, TRADD*) were significantly enhanced (Figure 2H). Interestingly, all these molecules were significantly up-regulated after FIR/SDR in this setting, demonstrating the potential of NLE in modulating apoptosis in NB. Furthermore, of the 18 NLE down-regulated genes, *BCL2L1*, *CASP14* and *ABL1* were completely suppressed.

NLE regulates IR-induced apoptosis related transcriptional response. As opposed to FIR, an induced expression of 38 genes was observed in NLE pre-treated and FIR exposed xenografts. Conversely, NLE treatment reverted the induction of 47 FIR-modulated genes. However, ANOVA revealed that NLE significantly enhanced FIR-induced *LTA, CD40, BAK1, BAX, BCL10, CASP1, CARD8* and *CRADD* (Figure 2). Furthermore, NLE significantly induced FIR-inhibited *BCL2A1, BIRC3, TRAF2* and *TRAF4*. To that note, except for *TRAF4*, these IR-inhibited molecules were only brought back to the basal level with NLE. Conversely, NLE significantly inhibited FIR-induced *TNFRSF10A, BCL2L1, BCL2L10, BCL2L11, BCLAF1, BNIP3, MCL1, NAIP, BIRC6, BIRC8, APAF1, NOL3, DAPK1* and *TP73*. Moreover, NLE completely suppressed FIR-inhibited *XIAP, ABL1* and *AKT1* in the xenografts (Figure 2). Like wise, compared to SDR exposed xenografts, NLE up-regulated 40 and suppressed another 44 genes. ANOVA revealed that NLE significantly suppressed SDR-induced *BIK, BNIP3, BFAR, NAIP, BIRC6, BIRC8, BRAF, IGF1R, APAF1, NOD1, CARD6, CARD8, CRADD, NOL3* and *PYCARD* and further inhibited SDR-inhibited *ABL-1* (Figure 2). Conversely, NLE significantly enhanced SDR-induced *TNFRSF25, BAG1, BAX, BCL1* and *CASP10* (Figure 2). The functional importance of these NLE regulated molecules in SDR and/or FIR exposed NB xenografts is discussed below.

NLE regulates IR-induced DNA fragmentation. Compared to mock-IR, FIR ($p<0.01$) and SDR ($p<0.001$) significantly increased DNA fragmentation in the NB xenografts. Like-wise, NLE (50 mg/kg)-treated xenograft demonstrated a significant ($p<0.05$) induction of DNA fragmentation as opposed to mock-IR. Furthermore, it was observed that NLE markedly and significantly ($p<0.001$) induced DNA fragmentation after FIR and SDR exposure of xenograft respectively (Figures 3A and 3B).

Discussion

Drug development from natural products is currently emerging as a highly promising strategy to identify novel anticancer agents. Recently, limonoids, modified triterpenes formed as secondary metabolites by plants, have attracted considerable attention as promising anticancer candidates (34). Neem contains a vast array of bioactive phytochemicals, one-third of which are limonoids. Azadirachtin and nimbolide, present in neem leaves, are recognized as the most potent limonoids that exhibit cytotoxic-effects against various cancer cell lines (15, 16). Also, studies have shown their modulatory effect on xenobiotic metabolizing enzymes, oxidative DNA damage, invasion, and angiogenesis (17). Thus, azadirachtin and nimbolide have been shown to exert apoptosis by selectively targeting PCNA, p21, cyclin D1, GST-P, NFκB, IκB, p53, FAS, BCL2, BAX, APAF-1, cytochrome *c*, survivin, caspase 3, 6, 8, 9, and PARP (35). In the present study, for the first time, it was shown that NLE significantly inhibits clinically relevant radiation-induced *BNIP3, NAIP, BIRC6, BIRC8, APAF1, NOL3* in human NB xenograft.

Cancer cells are able to acquire resistance to apoptosis by up-regulating multiple survival factors. Inhibitors of apoptosis (IAPs) are a pivotal class of intrinsic cellular inhibitors of apoptosis (36). IAPs widely and potently suppress apoptosis against a large variety of apoptotic stimuli, including radiation, in cancer cells. Induced expression of IAPs (*NAIP, BIRC6, BIRC8*) observed in the current study, both after SDR or FIR in NB xenograft, confirms earlier findings and also serves as the positive control for the current study. Since IAPs function at the convergence of mitochondrial and death-receptor pathway, they can be described as an apoptosis 'brake' and IAP antagonists/inhibitors function to release this 'brake' (37). Inside a live cell, upon irradiation, multiple apoptosis pathway proteins are involved in shifting the balance of life and death signals. In the context of SDR/FIR, induction of *NAIP, BIRC6* and *BIRC8* observed in this study throws light on their roles in regulating IR-induced apoptosis and further dictates the outcome of the cell's response to therapy. Furthermore, NLE significantly inhibiting these molecules demonstrates IAP-inhibition-mediated radiosensitization and provides critical information as to how NLE works in the context of IR, and how NB cells respond better to the therapy. The latter has clear clinical relevance in that the information will be useful to predict or select the patients who will benefit the most from the molecular therapy targeting IAPs (37). Similarly, *NOL3* (ARC), an endogenous inhibitor of apoptosis antagonizes both central death (extrinsic death receptor or intrinsic mitochondrial/ER) cascades (38). While ARC binds to FAS, FADD, and procaspase-8 precluding the formation of death-inducing signaling complex, disabling the extrinsic pathway, it interacts with BAX preventing conformational activation and translocation to the mitochondria, thereby antagonizing the intrinsic pathway (38). To the Authors' knowledge, this study is the first report of clinically relevant doses of IR (SDR/FIR) robustly inducing the transcription of *NOL3*, at

least in NB cells. More importantly, the ARC inhibitory effect of NLE delineates its potential in conferring radiosensitization by inducing cell death. However, it is interesting to note that NLE also inhibits IR-induced pro-apoptotic *BNIP3* and *APAF1* in this setting. Although NLE inhibition of these molecules has been realized in earlier studies in other settings (17), the mechanism underlying this response and its functional significance, if any, in NLE-induced cell death in NB needs further investigation.

Many *in vitro* studies have demonstrated that the antiproliferative activities of NLE are mediated through induction of apoptosis. Here, it was shown that NLE radiosensitizes NB xenografts by blocking anti-apoptotic machinery and inducing apoptosis. Comprehensively, NLE either inhibits IR-induced anti-apoptotic molecules, completely confers IR-inhibited *XIAP, AKT1* or profoundly enhances proapoptotic genes such as *BAX*. Mitochondria, which play a pivotal role in apoptosis, are a major site of reactive oxygen species (ROS) generation. Excessive ROS generation can lead to the opening of the mitochondrial permeability transition pore with consequent release of cytochrome *c* from the inter membrane space into the cytosol, culminating in activation of the caspase cascade and apoptotic cell death. BCL-2 inhibits ROS production, cytochrome *c* release and caspase-3 activation, whereas BAX facilitates cytochrome *c* release, triggering caspase-mediated apoptotic cell death. BCL-2 and BAX have become attractive targets for designing new anticancer drugs, and agents that lower the BCL-2/BAX ratio are regarded as promising chemopreventive and chemotherapeutic agents. The decrease in the BCL-2/BAX ratio seen after exposure of NB xenograft to NLE that was observed in this study, together with decreased IAPs, ARC and others throw light on the initial molecular events that directs NLE associated radiosensitization in NB cells. The present study provides compelling evidence showing that NLE transduces apoptosis by both the mitochondrial and death receptor pathways.

In summary, the results demonstrate for the first time that NLE exerts radiosentization in NB cells by inducing apoptosis. The data indicate that NLE potentiates IR-induced cell death by targeting both extrinsic and intrinsic pathways, in particular, by inhibiting the anti-apoptotic signaling cascade. Furthermore, owing to the limitations of *in vitro* studies in delineating molecular orchestration in response to a stimuli or selective targeting, a more relevant pre-clinical human NB xenograft coupled with quantitative PCR profiling of more direct, yet comprehensive and functionally (apoptosis) characterized molecules were utilized to elucidate the molecular blueprint that underlies NLE associated radiosensitization. This study allowed the identification of a potential 'deliverable' that targets apoptosis transcriptional response. However, further studies are warranted to delineate its efficacy in mitigating NB progression and radiotherapy-associated NB relapse and metastasis.

Acknowledgements

This work was supported by the Presbyterian Health Foundation, Oklahoma City, OK, USA, ACS-IRG-05-066-0 and OUHSC-Radiation Oncology Research Development Funds to Natarajan Aravindan.

References

1 Bernstein ML, Leclerc JM, Bunin G, Brisson L, Robison L, Shuster J, Byrne T, Gregory D, Hill G and Dougherty G: A population-based study of neuroblastoma incidence, survival, and mortality in North America. J Clin Oncol *10*: 323-329, 1992

2 Kushner BH: Neuroblastoma: a disease requiring a multitude of imaging studies. J Nucl Med *45*: 1172-1188, 2004.

3 Morgan WF: Is there a common mechanism underlying genomic instability, bystander effects and other nontargeted effects of exposure to ionizing radiation? Oncogene *22*: 7094-7099, 2003.

4 Morgan WF and Murnane JP: A role for genomic instability in cellular radioresistance? Cancer Metastasis Rev 14: 49-58, 1995

5 Ch'ang HJ, Maj JG, Paris F, Xing HR, Zhang JP, Truman C, Cardon-Cardo C, Haimovitz-Friedman A, Kolesnick R and Fuks Z: ATM regulates target switching to escalating doses of radiation in the intestines. Nat Med *11*: 484-490, 2005.

6 Dewey WC, Ling CC and Meyn RE: Radiation-induced apoptosis: relevance to radiotherapy. Int J Radiat Oncol Biol Phys *33*: 781-796, 1995.

7 Ahmed KM, Dong S, Fan M and Li JJ: Nuclear factor-kappaB p65 inhibits mitogen-activated protein kinase signaling pathway in radioresistant breast cancer cells. Mol Cancer Res *4*: 945-955, 2006.

8 Paulino AC and Fowler BZ: Secondary neoplasms after radiotherapy for a childhood solid tumor. Pediatr Hematol Oncol *22*: 89-101, 2005.

9 Subapriya R and Nagini S: Medicinal properties of neem leaves: a review. Curr Med Chem Anticancer Agents *5*: 149-146, 2005.

10 Subapriya R, Kumaraguruparan R, Abraham SK and Nagini S: Protective effects of ethanolic neem leaf extract on DMBA-induced genotoxicity and oxidative stress in mice. J Herb Pharmacother *5*: 39-50, 2005.

11 Subapriya R, Bhuvaneswari V, Ramesh V and Nagini S: Ethanolic leaf extract of neem (*Azadirachta indica*) inhibits buccal pouch carcinogenesis in hamsters. Cell Biochem Funct *23*: 229-238, 2005.

12 Subapriya R, Kumaraguruparan R and Nagini S: Expression of PCNA, cytokeratin, Bcl-2 and p53 during chemoprevention of hamster buccal pouch carcinogenesis by ethanolic neem (*Azadirachta indica*) leaf extract. Clin Biochem *39*: 1080-1087, 2006.

13 Subapriya R, Velmurugan B and Nagini S: Modulation of xenobiotic-metabolizing enzymes by ethanolic neem leaf extract during hamster buccal pouch carcinogenesis. J Exp Clin Cancer Res *24*: 223-230, 2005.

14 Subapriya R, Kumaraguruparan R, Abraham SK and Nagini S: Protective effects of ethanolic neem leaf extract on *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine-induced genotoxicity and oxidative stress in mice. Drug Chem Toxicol *27*: 15-26, 2004.

15 Akudugu J, Gade G and Bohm L: Cytotoxicity of azadirachtin A in human glioblastoma cell lines. Life Sci *68*: 1153-1160, 2001.

16 Roy MK, Kobori M, Takenaka M, Nakahara K, Shinmoto H, Isobe S and Tsushida T: Antiproliferative effect on human cancer cell lines after treatment with nimbolide extracted from an edible part of the neem tree (*Azadirachta indica*). Phytother Res *21*: 245-250, 2007.
17 Priyadarsini RV, Manikandan P, Kumar GH and Nagini S: The neem limonoids azadirachtin and nimbolide inhibit hamster cheek pouch carcinogenesis by modulating xenobiotic-metabolizing enzymes, DNA damage, antioxidants, invasion and angiogenesis. Free Radic Res *43*: 492-504, 2009.
18 Joki T, Carroll RS, Dunn IF, Zhang J, Abe T and Black PM: Assessment of alterations in gene expression in recurrent malignant glioma after radiotherapy using complementary deoxyribonucleic acid microarrays. Neurosurgery *48*: 195-201; discussion 201-192, 2001.
19 Lehnert S: Prediction of tumor response to therapy: molecular markers and the microenvironment. Apoptosis and chips: an overview of the proceedings. Radiat Res *154*: 121-124, 2000.
20 Ishigami T, Uzawa K, Higo M, Nomura H, Saito K, Kato Y, Nakashima D, Shiiba M, Bukawa H, Yokoe H, Kawata T, Ito H and Tanzawa H: Genes and molecular pathways related to radioresistance of oral squamous cell carcinoma cells. Int J Cancer *120*: 2262-2270, 2007.
21 Fukuda K, Sakakura C, Miyagawa K, Kuriu Y, Kin S, Nakase Y, Hagiwara A, Mitsufuji S, Okazaki Y, Hayashizaki Y and Yamagishi H: Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation. Br J Cancer *91*: 1543-1550, 2004.
22 Rosen EM, Fan S, Rockwell S and Goldberg ID: The molecular and cellular basis of radiosensitivity: implications for understanding how normal tissues and tumors respond to therapeutic radiation. Cancer Invest *17*: 56-72, 1999.
23 Fornace AJ, Amundson SA, Bittner M, Myers TG, Meltzer P, Weinsten JN and Trent J: The complexity of radiation stress responses: analysis by informatics and functional genomics approaches. Gene Expr *7*: 387-400, 1999.
24 Maity A, McKenna WG and Muschel RJ: The molecular basis for cell cycle delays following ionizing radiation: a review. Radiother Oncol *31*: 1-13, 1994.
25 Schmidt-Ullrich RK, Contessa JN, Dent P, Mikkelsen RB, Valerie K, Reardon DB, Bowers G and Lin PS: Molecular mechanisms of radiation-induced accelerated repopulation. Radiat Oncol Investig *7*: 321-330, 1999.
26 Meyn RE, Stephens LC and Milas L: Programmed cell death and radioresistance. Cancer Metastasis Rev *15*: 119-131, 1996.
27 Aravindan N, Madhusoodhanan R, Ahmad S, Johnson D and Herman TS: Curcumin inhibits NFκB mediated radioprotection and modulate apoptosis related genes in human neuroblastoma cells. Cancer Biol Ther *7*: 569-576, 2008.
28 Aravindan N, Madhusoodhanan R, Natarajan M and Herman TS: Alteration of apoptotic signaling molecules as a function of time after radiation in human neuroblastoma cells. Mol Cell Biochem *310*: 167-179, 2008.
29 Madhusoodhanan R, Natarajan M, Veeraraghavan J, Herman TS, Jamgade A, Singh N and Aravindan N: NFκB signaling-related molecular alterations in human neuroblastoma cells after fractionated irradiation. J Radiat Res (Tokyo) *50*: 311-324, 2009.
30 Staab HJ and Anderer FA: Growth of human colonic adenocarcinoma and development of serum CEA in in athymic mice. I: Strict correlation of tumour size and mass with serum CEA concentration during logarithmic growth. Br J Cancer *46*: 841-847, 1982.
31 Chattopadhyay RR: Possible biochemical mode of anti-inflammatory action of *Azadirachta indica* A. Juss. in rats. Indian J Exp Biol *36*: 418-420, 1998.
32 Madhusoodhanan R, Natarajan M, Veeraraghavan J, Herman TS and Aravindan N: NFκB activity and transcriptional responses in human breast adenocarcinoma cells after single and fractionated irradiation. Cancer Biol Ther *8*: 765-773, 2009.
33 Aravindan N, Cata JP, Hoffman L, Dougherty PM, Riedel BJ, Price KJ, Shaw AD: Effects of isoflurane, pentobarbital, and urethane on apoptosis and apoptotic signal transduction in rat kidney. Acta Anaesthesiol Scand *50*: 1229-1237, 2006.
34 Setzer WN and Setzer MC: Plant-derived triterpenoids as potential antineoplastic agents. Mini Rev Med Chem *3*: 540-556, 2003.
35 Priyadarsini RV, Murugan RS, Sripriya P, Karunagaran D and Nagini S: The neem limonoids azadirachtin and nimbolide induce cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells. Free Radic Res *44*: 624-634
36 Srinivasula SM and Ashwell JD: IAPs: What's in a name? Mol Cell *30*: 123-135, 2008.
37 Devi GR: XIAP as target for therapeutic apoptosis in prostate cancer. Drug News Perspect *17*: 127-134, 2004.
38 Mercier I, Vuolo M, Madan R, Xue X, Levalley AJ, Ashton AW, Jasmin JF, Czaja MT, Lin EY, Armstrong RC, Pollard JW and Kitsis RN: ARC, an apoptosis suppressor limited to terminally differentiated cells, is induced in human breast cancer and confers chemo- and radiation-resistance. Cell Death Differ *12*: 682-686, 2005.

Received November 19, 2010
Revised December 14, 2010
Accepted December 15, 2010