

# Synergistic Effect Between Curcumin (diferuloylmethane) and Radiation on Clonogenic Cell Death Independent of p53 in Prostate Cancer Cells

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## Abstract

Radiotherapy is a common treatment for prostate cancer, but failure is observed 30 to 40% of the time. It is more common in patients with abnormal p53. The phytochemical diferuloylmethane (curcumin) a naturally occurring flavinoid derived from the rhizome of *Curcuma longa*, shows potential radiosensitizing effects. In the present study, the effect of curcumin and radiation on cell viability, apoptosis and clonogenic cell death was examined in LNCaP (wild type p53) and PC3 (mutant p53) prostate cancer cells.

The expression of p53 and p53 target genes was examined using RNase protection assay and Western blot analysis. Cell cycle and apoptosis was evaluated by flow cytometry. Cell viability and colony formation was examined using MTT assay and clonogenic assay respectively.

The expression of p53, p21 and gadd45 increased in LNCaP cells after radiation exposure. In PC3 cells, there was no change in expression in mutant p53 after radiation or curcumin treatment. However, the expression of p21 and gadd45 increased after curcumin treatment in PC3 cells independent of p53. Curcumin induced cell growth arrest and apoptosis in both the cell lines. The cell viability and cell proliferation decreased in presence of both curcumin and radiation as compared to curcumin or radiation alone in both the cell lines. Regardless of p53 status, combination treatment with curcumin (2.5 to 10  $\mu$ M) and radiation (2 Gy) had a synergistic effect on clonogenic cell death in both LNCaP and PC3 cells.

**Conclusion:** Curcumin appears to radiosensitize prostate cancer cells and may be a possible adjuvant to radiotherapy in the treatment of prostate cancers.

**Keywords:** *Curcuma longa*; Curcumin; Radiation; Clonogenic cell death; Synergy; p53; Prostate cancer

## Introduction

Cancer is the second leading cause of death in the United States and prostate cancer is the most prevalent male cancer. Approximately 217,730 new cases of prostate cancer and 32,050 deaths are projected to occur in USA in 2010 (Jemal et al., 2010). Most of the prostate cancer patients are treated during the course of their illness by radiotherapy alone or as an adjuvant treatment for surgery (Swanson, 2006; Swanson et al., 2006; Thompson et al., 2006). However, failure of radiotherapy is observed in about 30-40% of prostate cancer patients (Kalkner et al., 2007; Thompson et al., 2007). The radio resistance of prostate cancer cells is a limitation of radiotherapy. The effectiveness of radiotherapy mainly depends on the molecular context of the tumor. The molecular mechanisms leading to radiosensitivity and radioresistance development remain elusive (Rosen et al., 1999; Skvortsova et al., 2008). Therefore, there is immense interest in understanding the critical determinants in radiation-induced cell killing and developing new agents for radiosensitization.

The p53 tumor suppressor protein plays an important role in radiation response and radiotherapy failure is more common in prostate cancer patients with abnormal p53 (Razoky et al., 1999; Ritter et al., 2002). p53 is activated in response to different anti-cancer agents including ionizing radiation (Levine et al., 1991; Haupt et al., 2003). Active p53 functions as a transcriptional regulator and in turn regulates its downstream genes like p21, gadd45, and bax, -resulting in cell growth arrest, apoptosis and cellular senescence (Nayak and Das, 2002; Fridman and Lowe, 2003). p53 is mutated or inactivated in early as well as in advanced-stage prostate cancers (Bookstein et

al., 1993; Chi et al., 1994; Gumerlock et al., 1997; Stapleton et al., 1997; Downing et al., 2003). Mutant or inactive p53 may limit the effectiveness of radiotherapy in prostate cancers (Razoky et al., 1999; Ritter et al., 2002; Lehman et al., 2007). Wild-type p53 significantly increases clonogenic cell death through p53-dependent cellular senescence and apoptotic pathways in prostate cancer (Lehman et al., 2007). Therefore, if treatment could be tailored as to abrogate the need for an intact p53, the results of radiotherapy in prostate cancer could be improved. There are agents that act on the molecular level that may make this possible.

Curcumin (diferuloylmethane) (Figure 1) is a polyphenol derived from the rhizome of the turmeric plant (*Curcuma longa*, family Zingiberaceae). It is used as a flavoring and coloring agent, as a food preservative and in Ayurvedic medicine (Corson and Crews, 2007; Shishodia et al., 2007). It has been consumed for centuries as a dietary spice at the rate of up to 100 mg/day by people in

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Asian countries (Ammon and Wahl, 1991). It is believed that lower incidence of prostate cancer in Asian countries than in Western countries may be due to differences in diet (Hebert et al., 1998; Denis et al., 1999; Hsing et al., 2000). Curcumin possesses diverse anti-inflammatory and anti-cancer properties (Kawamori et al., 1999; Aggarwal et al., 2005; Khor et al., 2006; Herman et al., 2009; Khan et al., 2010). Studies have shown that curcumin can induce apoptosis by different mechanisms, such as, by inhibiting the expression of the anti-apoptotic genes bcl-2 and bcl-x<sub>l</sub>, by inhibiting AP-1 and NF-κB transcription factors (Mukhopadhyay et al., 2001; Anto et al., 2007; Shankar and Srivastava, 2007; Li et al., 2007; Jutooru et al., 2010). Preclinical, phase I and phase II clinical studies suggest that curcumin is pharmacologically safe and it is well tolerated without any toxic effects (Cheng et al., 2001, Sharma et al., 2001; Sharma et al., 2004; Dhillon et al., 2008). In the present study, we tested the hypothesis that combination of curcumin with radiation would enhance apoptosis and clonogenic cell death of prostate cancer cells independent of the p53 status.

## Materials and Methods

### Cell lines

The PC3 and LNCaP prostate cancer cells were purchased from ATCC. The cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) and 1% Penicillin/Streptomycin. The cells were maintained in T-75 tissue culture flasks with filter caps in a humidified 5% CO<sub>2</sub>/95% air atmosphere at 37°C.

### Ionizing radiation

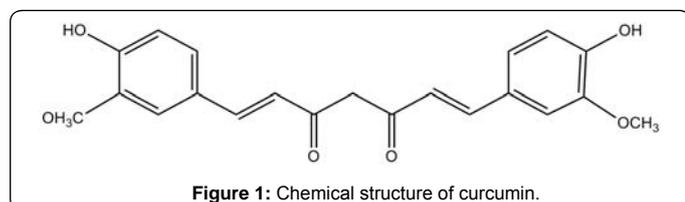
Cells were exposed to a 2 to 20 Gy dose of <sup>137</sup>Cs gamma rays (Atomic Energy of Canada/Nordion). The dose rate was ~1.1 Gy/minute. The cells were exposed at room temperature in 100 mm culture dishes or in T-25 culture flasks or in 96-well plates immediately after removal from the 37°C incubator. The flasks were returned to the incubator immediately after the exposure.

### Curcumin

Curcumin ([HOC<sub>6</sub>H<sub>3</sub>(OCH<sub>3</sub>)CH=CHCO]<sub>2</sub>CH<sub>2</sub>) (Figure 1) was purchased from Sigma-Aldrich (product number C7727; St. Louis, MO). The source and country of manufacture was India. On high pressure liquid chromatograph, purity was 99% total curcuminoids and 93% curcumin. Cells were exposed to radiation (2 to 20 Gy) alone, curcumin (2.5 to 20 μM) alone, or the combination of the two. For combination treatment, cells were treated with curcumin (2.5 to 10 μM) for 2 hr prior to radiation exposure (2 Gy). The control for the effects of curcumin was radiation without the compound.

### RNAse protection assay

Expression of p53 and p53 target genes was examined using the RNase protection assay (RPA) as previously described (Nayak and Das, 2002). The human riboprobe set containing bcl-x<sub>l</sub>, p53, c-fos, p21, bax, bcl-2, Mcl-1, L32, and GAPDH was used (BDBiosciences, San Jose, CA). RNA expression of L32 and GAPDH were used as



internal loading controls. The riboprobe set was labeled with T7 RNA polymerase in the presence of [ $\alpha^{32}$ P] UTP, and was used for RPA following the manufacturer's protocol (BDBiosciences). The gels were scanned using a Typhoon 8600 Variable Mode Imager. Quantitative assessment was carried out using Image Quant (Molecular Dynamics, Sunnyvale, CA).

### Western blot analysis

In addition, expression of p53 and p21 was examined using Western blot analysis (Nayak and Das, 2002). A 50-μg sample of the cell lysate was electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman Inc., Florham Park, NJ). The membrane was incubated with primary antibody [p53 (DO-1, Santa Cruz Biotechnology), p-p53-Ser15 (9284, Cell Signaling Technology, Inc., Danvers, MA), p21 (C-19, Santa Cruz Biotechnology), bax (sc-493, Santa Cruz Biotechnology), bcl2 (sc-492, Santa Cruz Biotechnology), actin (A1978, Sigma-Aldrich), GAPDH, sc-25778, Santa Cruz Biotechnology)] overnight at 4°C. Actin and GAPDH were used as internal loading controls. The membrane was developed using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences). Quantitation of Western blot was carried out using the Scion Image 4.03 software program.

### Caspase 9 assay

Apoptosis was examined by quantifying active caspase 9 using the carboxyfluorescein caspase detection kit (Biocarta, San Diego, CA). With this system, active caspases are detected in live cells through the use of a carboxyfluorescein (FAM) labeled peptide fluoromethyl ketone (FMK) caspase inhibitor (FAM-Peptide-FMK). Caspase positive cells were distinguished from caspase negative cells in a Becton Dickinson FACSCalibur Flow Cytometer.

### Annexin V binding assay

Apoptosis was also assayed using annexin V-FITC apoptosis detection kit (Calbiochem, La Jolla, CA). The cells were then stained with annexin and annexin V-positive cells were analyzed using flow cytometry.

### Cell cycle analysis

Cell cycle analysis was carried out at 48h post-exposure. The cells were harvested, and resuspended in PBS containing propidium iodide (50 μg/mL), RNase A (500 μg/mL), and Triton X-100 (0.5%), and incubated in the dark for 1 hour. The cell cycle analysis was performed by flow cytometry in a Becton Dickinson FACSCalibur Flow Cytometer. The results were determined using ModFit software and reported as percentage G1, percentage S, and percentage G2/M cells.

### MTT assay

Cell viability was examined by using MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich). Cells were treated with 2.5 to 10 μM curcumin. After 2 hours of curcumin treatment, cells were exposed to 2 Gy dose of ionizing radiation. The MTT assay was performed after 7 days of seeding. The cell precipitate was dissolved in 100 μL of dimethyl sulfoxide and the optical density was measured at 570 nm using a Spectrafluor plate reader.

### Clonogenic assay

PC3 (~2,000) and LNCaP (~9,000) cells were seeded in 100 mm tissue culture dishes with 10 mL complete media. After incubation, the cells are treated with curcumin (2.5 to 20 μM) alone, radiation

(2 to 20 Gy) alone or a combination of curcumin (2 to 10 μM) and radiation (2 Gy). After 12 days of incubation period, the colonies were stained with 10 mL of Crystal Violet (0.625% in methanol). Colonies of more than 50 cells were counted. Plating efficiency was used to calculate percent survival for all experimental groups. Plating efficiency was calculated from the number of colonies formed divided by the number of cells seeded. The percent survival of the control group was considered 100%. The percent survival of the experimental groups was calculated as 100/plating efficiency of control x plating efficiency of the experimental group.

**Combination index**

The combination index (CI) was calculated according to the method of Chou and Talalay (1984). The CI value for different combination groups of curcumin and radiation was calculated using the equation  $CI = D1/Dx1 + D2/Dx2$ , where D1 and D2 are the concentrations of curcumin and radiation respectively used in the combination to achieve a specific (x%) effect. Dx1 and Dx2 are the concentrations of curcumin and radiation respectively required to achieve the same effect as single agents. Dx1 and Dx2 are calculated from the individual dose response curve for curcumin (2.5 to 20 μM) and radiation (2 to 20 Gy). A CI value of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism respectively.

**Statistical analysis**

The number of colonies from three replicate clonogenic assay in each experimental group was analyzed using the student's

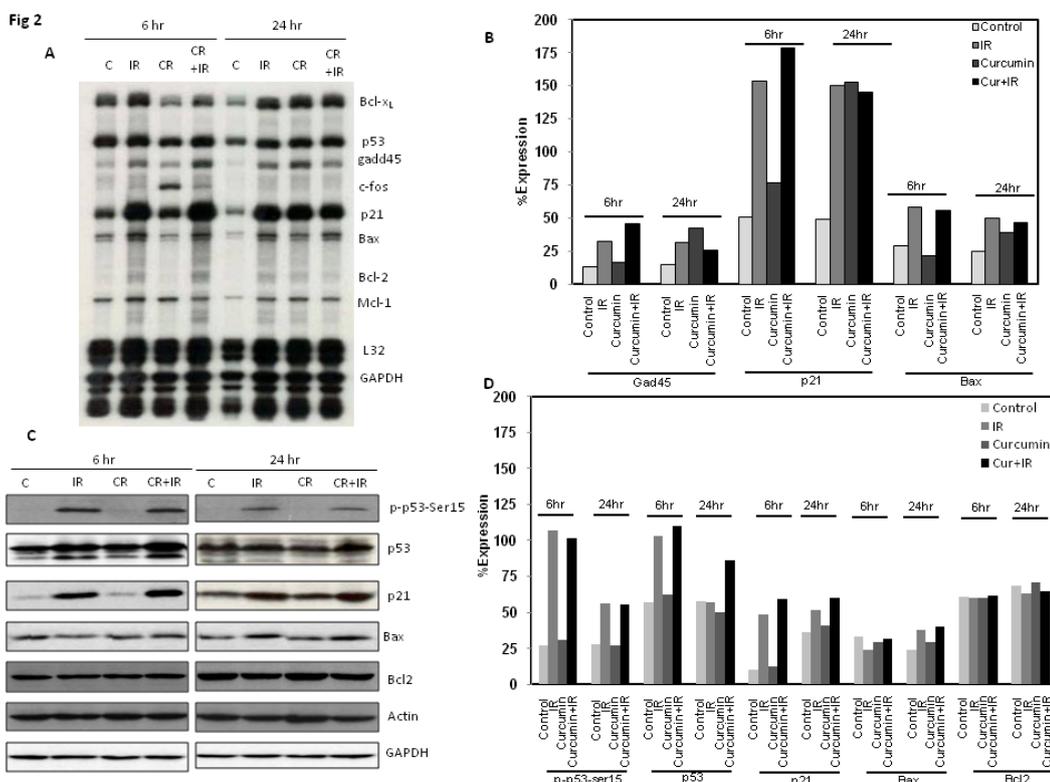
paired t-test (p<0.05). The t-test was performed between the following experimental groups: control vs. radiation, control vs. curcumin, curcumin vs. curcumin+radiation, and radiation vs. radiation+curcumin.

**Results**

The study was undertaken to examine the effect of curcumin and radiation on cell viability, apoptosis and clonogenic cell death in LNCaP (wild-type p53) and PC3 (mutant p53) prostate cancer cells.

The expression of p53 and p53 target genes (p21, gadd45, and bax) was examined in LNCaP cells using RNase protection assay (Figure 2A and 2B) and Western blot analysis (Figure 2C and 2D). The level of phospho-p53 (p-p53 at serine 15) increased ~4 fold after radiation exposure (Figure 2C and 2D), but not after curcumin treatment (Figure 2C and 2D) when compared with control. Similarly, the level of p53 protein expression increased ~1.8 fold after radiation exposure (Figure 2C, at 6 hr) and not after curcumin treatment (Figure 2C), indicating that p53 is activated by radiation exposure.

The expression of p21 increased at the RNA level (Figure 2A and 2B) after radiation exposure (~3 fold at 6 hr and 24 hr), after curcumin treatment (~1.5 fold at 6 hr and 3.1 fold at 24 hr), and in the presence of both curcumin and radiation (~3.6 fold at 6 hr and 3 fold at 24 hr) when compared with control. The p21 expression at the protein level (Figure 2C and 2D) increased after radiation exposure (~5 fold at 6 hr and 1.5 fold at 24 hr), and in the presence of both curcumin and radiation (~6 fold at 6 hr and 1.7 fold at 24 hr) when compared with



**Figure 2:** Expression of p53 and p53 target genes by curcumin and radiation in LNCaP cells. **A:** RNase protection assay (RPA) showing expression of p21, gadd45, and bax at 6 and 24 hr post radiation exposure. L32 and GAPDH are loading controls. C-control, IR- 2 Gy radiation, CR-10 μM curcumin, CR+IR-10 μM curcumin+2 Gy. **B:** Quantitation of RPA showing gadd45, p21, and bax expression level normalized to GAPDH **C:** Western blot analysis showing expression of p-p53-Ser 15, p53, p21, bax, bcl2 at 6 and 24 hr post radiation exposure. Actin and GAPDH are loading controls. C-control, IR- 2 Gy radiation, CR-10 μM curcumin, CR+IR-10 μM curcumin+2 Gy. **D:** Quantitation of Western blot showing p53, p21, bax, and bcl2 expression level normalized to GAPDH.

control. The gadd45 expression increased after radiation exposure (~2.5 fold at 6 hr and 2.3 fold at 24 hr), after curcumin treatment (~1.3 fold at 6 hr and 3.2 fold at 24 hr), and in the presence of both curcumin and radiation (~3.5 fold at 6 hr and 1.9 fold at 24 hr) when compared with the control. Bax expression increased at RNA level (Figure 2A) after radiation exposure (~2 fold at 6 hr and 2.1 fold at 24 hr), after curcumin treatment (~1.6 fold at 24 hr) and in the presence of both curcumin and radiation (~2 fold at 6 hr and 2 fold at 24 hr) when compared with control. At the protein level (Figure 2C, and 2D), Bax expression increased after radiation exposure (~1.6 fold at 24 hr), and in the presence of both curcumin and radiation (~1.7 fold at 24 hr). There was no change in bcl2 expression after radiation or curcumin treatment (Figure 2C and 2D).

In PC3 cells (Figure 3): the p-p53-Ser15 was not detected and there was no change in expression in mutant p53 protein after radiation or curcumin treatment (Figure 3C), indicating that the mutant p53 was not activated by either treatment. The expression of p21 did not change after radiation exposure, but increased after curcumin treatment, and increased in the presence of both curcumin and radiation (Figure 3A and 3B; Figure 3C and 3D) when compared with the control. The expression of p21 increased at the RNA level (Figure 3A and 3B) after curcumin treatment (~2.5 fold at 6 hr and 1.3 fold at 24 hr), and in the presence of both curcumin and radiation (~2.5 fold at 6 hr and 4 fold at 24 hr). The p21 expression at the protein level (Figure 3C and 3D) increased after curcumin treatment (~1.5 fold at 6 hr and 2.1 fold at 24 hr), and in the presence of both curcumin and radiation (~1.2 fold at 6 hr and 2.3 fold at 24

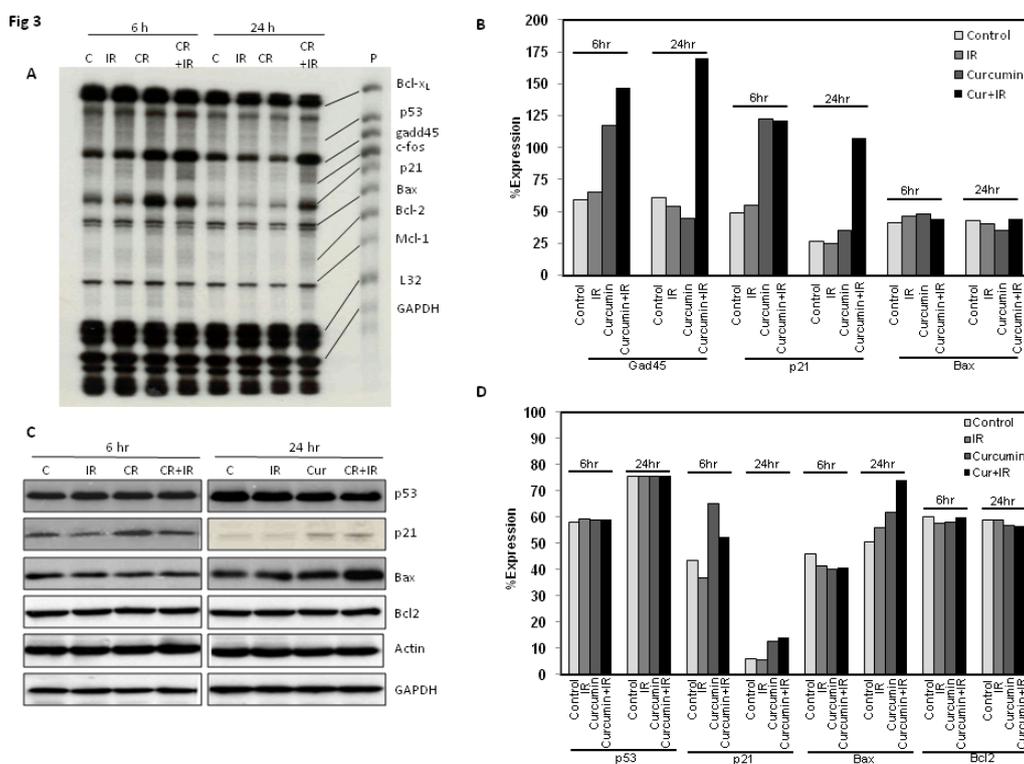
hr). The gadd45 expression did not change after radiation exposure (Figure 3A), increased after curcumin treatment (~2 fold at 6 hr), and increased in the presence of both curcumin and radiation (~2.5 fold at 6 hr and 2.8 fold at 24 hr). The bax expression (Figure 3A and 3C) did not change after radiation or curcumin exposure, but increased in the presence of both curcumin and radiation (~1.5 fold at 24 hr, Figure 3C) as compared with the control. There was no change in bcl2 expression after radiation or curcumin treatment when compared with the control (Figure 3C and 3D). The results showed that curcumin increased the expression of p21 and gadd45 in both the cell lines independent of p53.

The effect of curcumin and radiation on cell cycle arrest was examined by flow cytometry. The cells were treated with curcumin (10 μM) alone, radiation (2 Gy) alone or in combination of curcumin (10 μM) with radiation (2 Gy). For combination treatment, cells were treated with curcumin for 2 hr prior to radiation exposure. The cell cycle distribution was examined after 48 hr post radiation exposure (Figure 4).

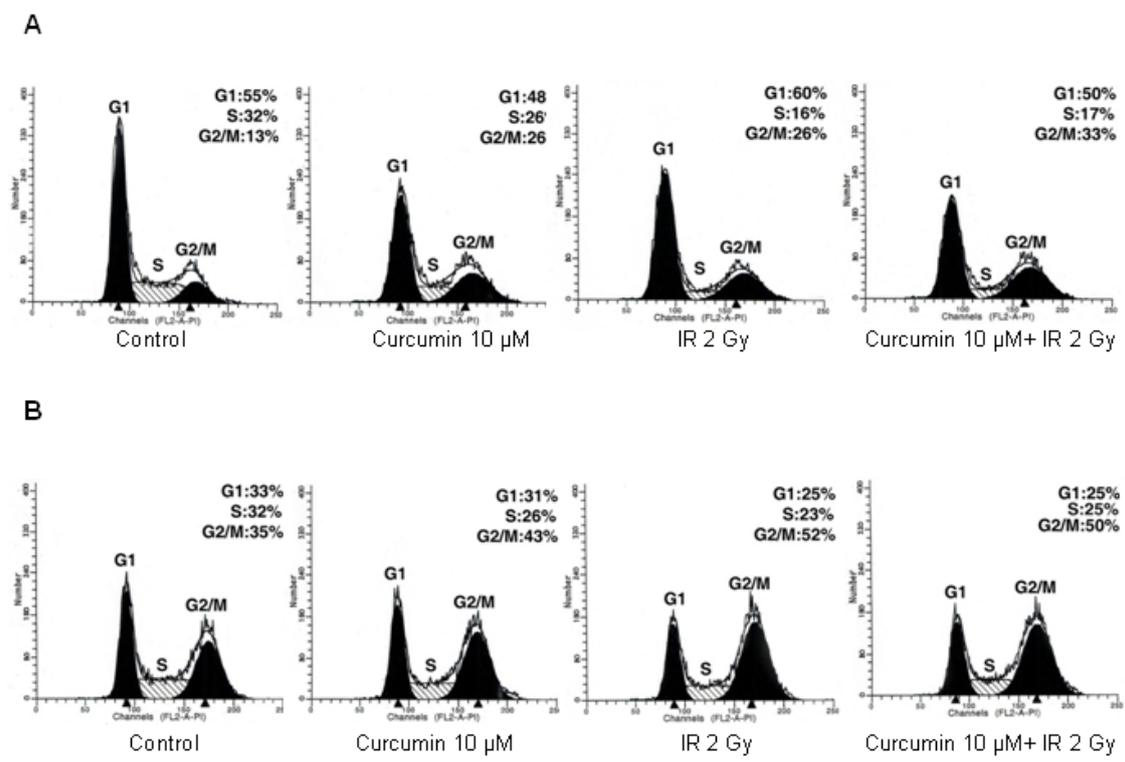
The percentage of cells in each phase of the cell cycle for both the LNCaP and PC3 cells is shown in Table 1 and Figure 4. As can be seen,

Experimental Groups	LNCaP			PC3		
	G1	S	G2/M	G1	S	G2/M
Control	55%	32%	13%	33%	32%	35%
Curcumin 10 μM	48%	26%	26%	31%	26%	43%
IR 2 Gy	60%	16%	26%	25%	23%	52%
Curcumin 10 μM + IR 2 Gy	50%	17%	33%	25%	25%	50%

**Table 1:** Cell cycle analysis in LNCaP and PC3 cells.



**Figure 3:** Expression of p53 and p53 target genes by curcumin and radiation in PC3 cells. **A:** RNase protection assay showing expression of p21, gadd45, and bax at 6 and 24 hr post radiation exposure. L32 and GAPDH are loading controls. P-probe, C-control, IR- 2 Gy radiation, CR-10 μM curcumin, CR+IR-10 μM curcumin+2 Gy. **B:** Quantitation of RPA showing gadd45, p21, and bax expression level normalized to GAPDH. **C:** Western blot analysis showing expression of p53, p21, bax, bcl2 at 6 and 24 hr post radiation exposure. Actin and GAPDH are loading controls. C-control, IR- 2 Gy radiation, CR-10 μM curcumin, CR+IR-10 μM curcumin+2 Gy. **D:** Quantitation of Western blot showing p53, p21, bax, and bcl2 expression level normalized to GAPDH.



**Figure 4:** Cell cycle distribution after curcumin and radiation treatment in LNCaP and PC3 cells. Cells were treated with curcumin (10 μM) alone or radiation (2 Gy) alone or in combination of curcumin (10 μM) with radiation (2 Gy). Cell cycle distribution was examined after 48 hr of radiation exposure and presented as percent G1, S, and G2/M. A: LNCaP B: PC3.

there was cell cycle arrest in response to curcumin and radiation exposure in both the cell lines with an increase in cells at G2/M.

The effect of curcumin and ionizing radiation (IR) on apoptosis induction was examined either by measuring the level of active caspase 9 or by assessing the level of annexin V binding. The cells were exposed with radiation (2 Gy) alone or treated with curcumin (10 to 50 μM) alone, or in combination of curcumin (10 to 50 μM) with radiation (2 Gy). For combination treatment, cells were treated with curcumin for 2 hr prior to radiation exposure. The apoptosis was measured after 48 hr post radiation exposure. The change in apoptosis is shown in Table 2a and Figure 5. As can be seen, at lower

Experimental Groups	LNCaP		PC3	
	% Apoptosis	% survival (MTT) Mean±SE	% Apoptosis	% survival (MTT) Mean±SE
Control	8	100	7	100
Curcumin 2.5 μM		76±1.8		76±1.5
Curcumin 5 μM		62±0.9		62±2
Curcumin 10 μM	11	49±1.8	8	50±1.2
Curcumin 20 μM	15		12	
Curcumin 50 μM	76		87	
IR 2 Gy	15	78±1.2	11	80±0.9
Curcumin 2.5 μM + IR 2 Gy		56±1.2		63±1.5
Curcumin 5 μM + IR 2 Gy		41±1.8		51±1.5
Curcumin 10 μM + IR 2 Gy	17	20±1.8	12	42±0.9
Curcumin 20 μM + IR 2 Gy	20		15	
Curcumin 50 μM + IR 2 Gy	79		86	

**Table 2a:** Apoptosis and cell viability (MTT assay) in LNCaP and PC3 cells. Apoptosis data is from a representative experiment and MTT data is from triplicate experiment..

Experimental Groups	LNCaP		PC3	
	% Apoptosis	% survival (MTT)	% Apoptosis	% survival (MTT)
Control	8	100	7	100
Curcumin 2.5 μM		76		76
Curcumin 5 μM		62		62
Curcumin 10 μM	11	49	8	50
Curcumin 20 μM	15		12	
Curcumin 50 μM	76		87	
IR 2 Gy	15	78	11	80
Curcumin 2.5 μM + IR 2 Gy		56		63
Curcumin 5 μM + IR 2 Gy		41		51
Curcumin 10 μM + IR 2 Gy	17	20	12	42
Curcumin 20 μM + IR 2 Gy	20		15	
Curcumin 50 μM + IR 2 Gy	79		86	

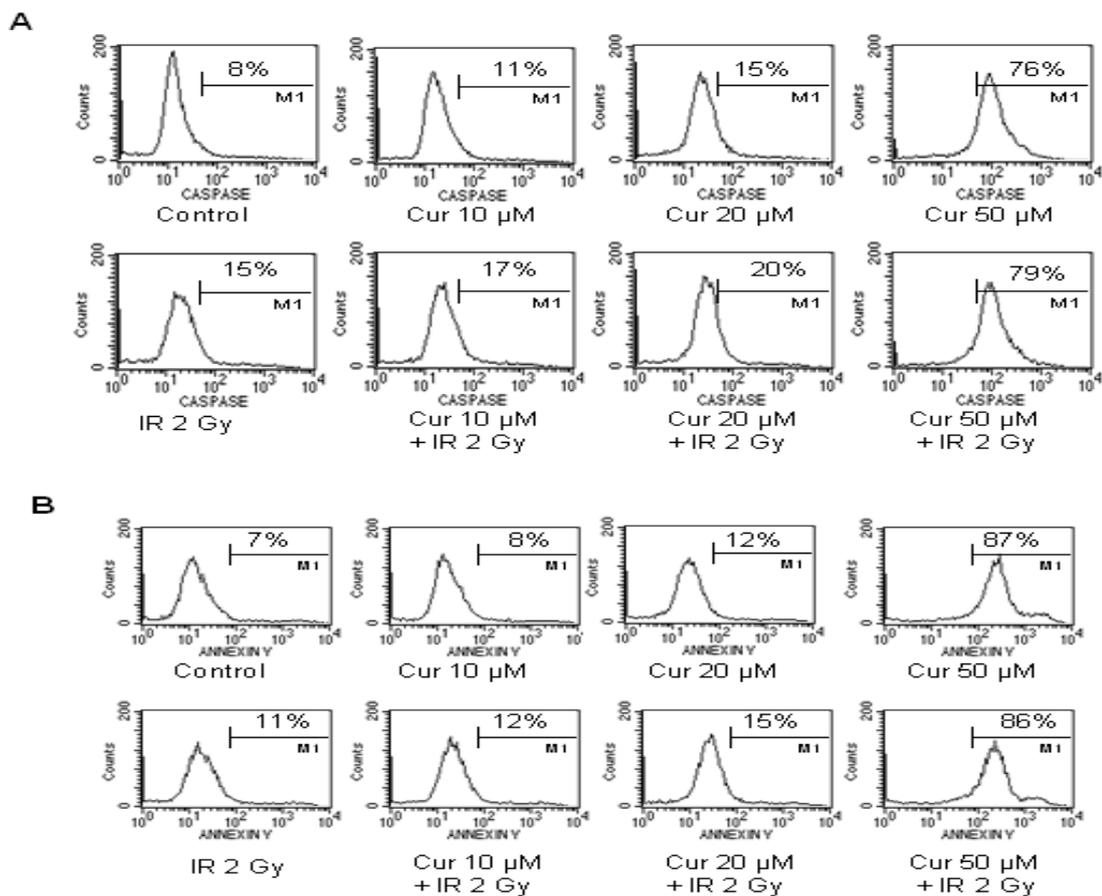
**Table 2b:** Apoptosis and cell viability (MTT assay).

levels, both curcumin and radiation induced apoptosis in both the cell lines, which was only modestly additive with both modalities.

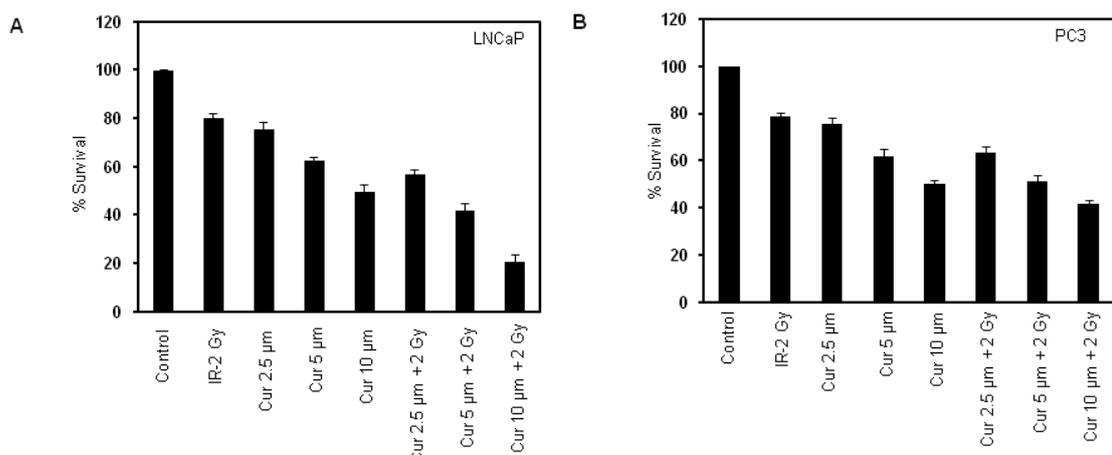
The effect of curcumin and radiation on cell viability was examined using the MTT assay. The cells were exposed with radiation (2 Gy) alone or treated with curcumin (2.5 to 10 μM) alone, or in combination of curcumin (2.5 to 10 μM) with radiation (2 Gy). The MTT assay was performed after 7 days of radiation exposure. The results are shown in Table 2b and Figure 6.

The results showed that the cell viability decreased in presence of both curcumin and radiation as compared to control and radiation exposed cells. There was clearly an additive effect, especially as the curcumin dose increased.

We examined the effect of both curcumin and radiation on



**Figure 5:** Curcumin induces apoptosis in LNCaP and PC3 cells. Cells were treated with curcumin (10, 20, and 50  $\mu$ M) alone, radiation (2 Gy) alone or in combination of curcumin with radiation. **A:** Caspase 9 assay in LNCaP cells **B:** Annexin V binding assay in PC3 cells.



**Figure 6:** Decrease in cell viability in LNCaP and PC3 cells examined using MTT assay. Cells were treated with curcumin (2.5, 5, and 10  $\mu$ M) alone, radiation (2 Gy) alone or in combination of curcumin with radiation. **A:** LNCaP **B:** PC3 cells.

clonogenic cell death by performing colony forming assay. Two independent colony forming experiment were performed in both the cell lines; triplicate plates were included in each experiment. A dose-response curve for curcumin alone, radiation alone and combination of curcumin and radiation is presented in Figure 7. Combination index

(CI) value for combination of curcumin and radiation is shown in Table 3a. A representative set of data and figures from one experiment are presented in Figures 8 and 9. Clonogenic cell death is a predominant mechanism of cell death caused by radiation that includes late apoptotic and reproductive (senescence) cell death (Dewey et al.,

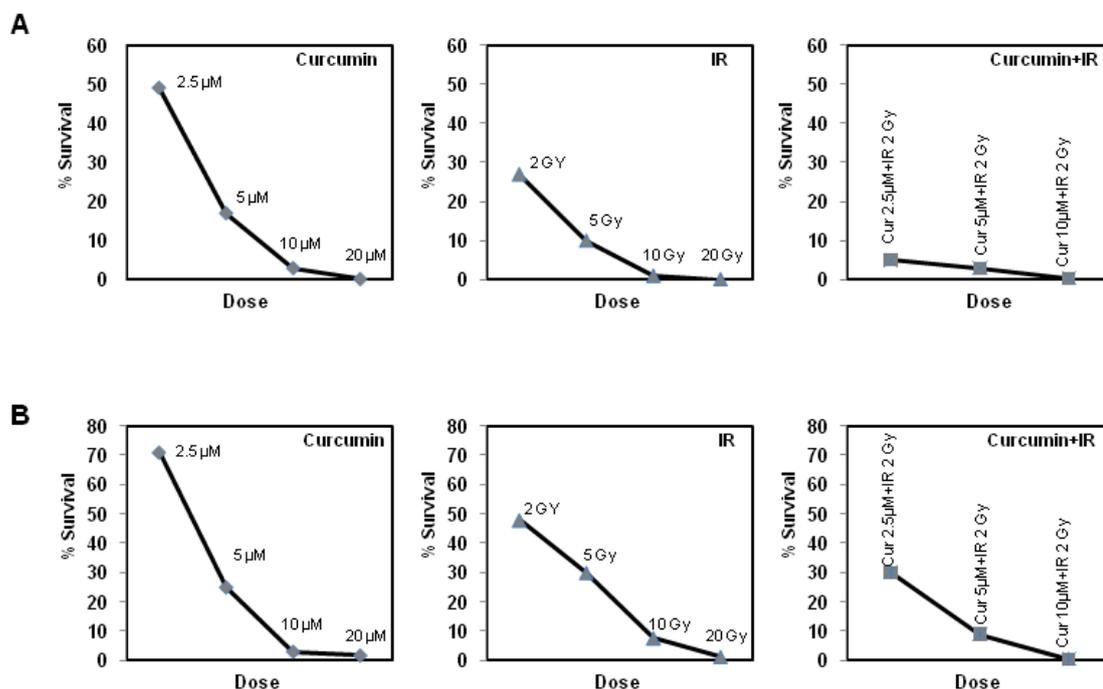


Figure 7: Dose-response curve for curcumin, radiation, and curcumin+radiation on colony formation. A: LNCaP B: PC3.

Experimental Groups	LNCaP				PC3			
	Average colonies from three plates Mean±SE	Plating efficiency (%)	Percent survival	CI	Average colonies from three plates Mean±SE	Plating efficiency (%)	Percent survival	CI
Control	2142±11.1	23.8	100		421±10.1	21	100	
IR 2 Gy	583±12	6.4	27		198±6.2	10	48	
IR 5 Gy	215±5.2	2.4	10		127±5.0	6.4	30	
IR 10 Gy	22±1.2	0.2	1		32±3.2	1.6	7.6	
IR 20 Gy	3±0.4	0.03	0.1		5±1.1	0.25	1.2	
Curcumin 2.5 μM	1066±8.2	11.8	49		305±10.3	15	71	
Curcumin 5 μM	354±7.5	4	17		105±3.5	5	25	
Curcumin 10 μM	60±5.5	0.7	3		14±3.5	0.7	3	
Curcumin 20 μM	5±3.0	0.05	0.2		7±1.3	0.35	1.7	
Curcumin 2.5 μM + IR 2 Gy	110±4.6	1.2	5	0.78	128±12.0	6	30	0.92
Curcumin 5 μM + IR 2 Gy	74±3.5	0.8	3	0.47	37±6.0	2	9	0.50
Curcumin 10 μM + IR 2 Gy	09±1.2	0.1	0.4	0.26	2±0.6	0.1	0.5	0.16

Table 3a: Clonogenic cell survival in LNCaP and PC3 cells. CI-combination index.

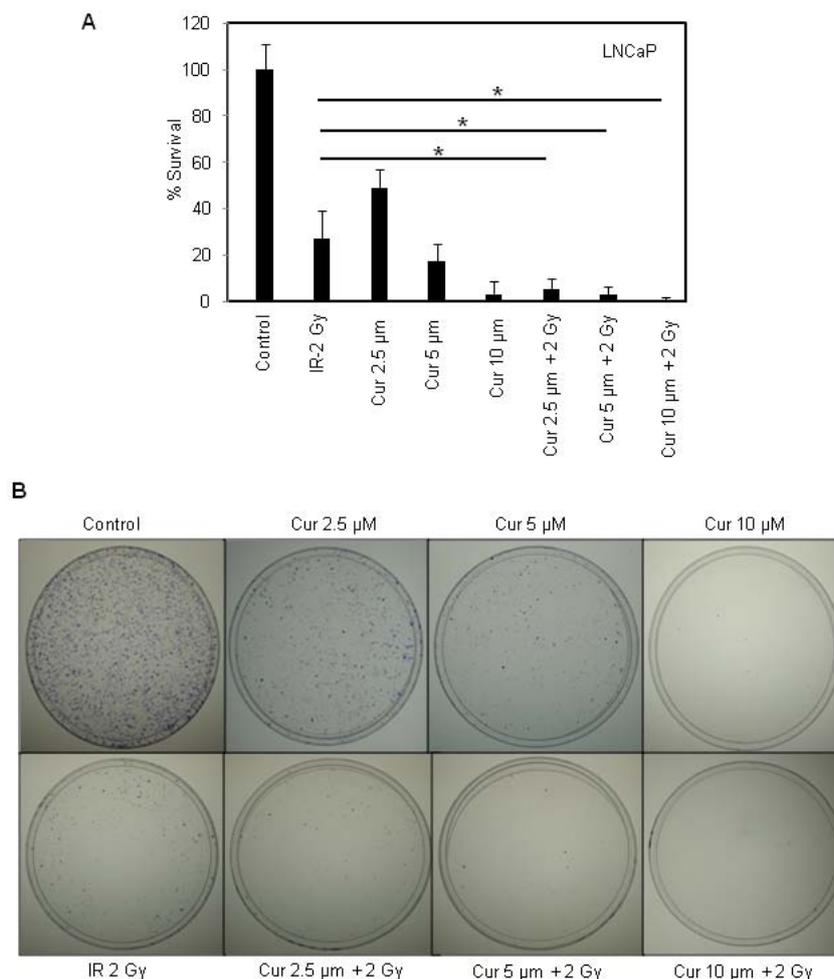
Experimental Groups	LNCaP			PC3		
	Average colonies from three plates	Plating efficiency (%)	Percent survival	Average colonies from three plates	Plating efficiency (%)	Percent survival
Control	2142	23.8	100	421	21	100
IR 2 Gy	583	6.4	27	198	10	48
Curcumin 2.5 μM	1066	11.8	49	305	15	71
Curcumin 5 μM	354	4	17	105	5	25
Curcumin 10 μM	60	0.7	3	14	0.7	3
Curcumin 2.5 μM + IR 2 Gy	110	1.2	5	128	6	30
Curcumin 5 μM + IR 2 Gy	74	0.8	3	37	2	9
Curcumin 10 μM + IR 2 Gy	09	0.1	0.4	2	0.1	0.5

Table 3b: Clonogenic Cell survival.

1971; Lehman et al., 2007). The cells were exposed with radiation (2 to 20 Gy) alone, or treated with curcumin (2.5 to 20 μM) alone, or in combination of curcumin (2.5 to 10 μM) with radiation (2 Gy). The clonogenic assay was performed after 12 days of radiation exposure. Table 3b shows the percent survival and combination index (CI) values. The combination of curcumin with radiation had synergistic effect (CI value less than 1 in all combinations) on clonogenic cell

death in both the cell lines regardless of p53.

The effect of combination of curcumin and radiation compared with curcumin or radiation alone was statistically significant in both the cell types. In LNCaP cells, the p values were: IR 2 Gy vs. curcumin 2.5 μM+IR 2 Gy (p=0.001), IR 2 Gy vs. curcumin 5 μM+IR 2 Gy (p=0.0009), IR 2 Gy vs. curcumin 10 μM+IR 2 Gy (p=0.0003),



**Figure 8:** Synergistic effect of curcumin and radiation on clonogenic cell death in LNCaP cells. Cells were treated with curcumin (2.5, 5, and 10 μM) alone, radiation (2 Gy) alone or in combination of curcumin with radiation. **A:** Bar chart showing percent survival in different experimental groups (\*p<0.05). **B:** A representative clonogenic assay showing different experimental groups.

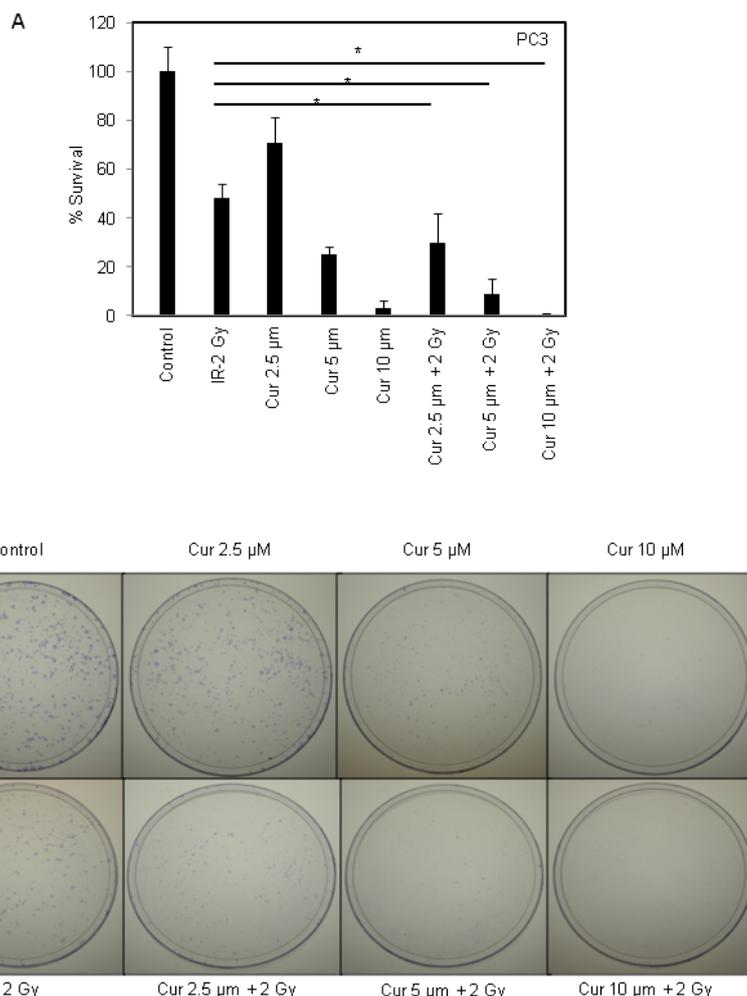
curcumin 2.5 μM vs. curcumin 2.5 μM+IR 2 Gy (p=0.0005), curcumin 5 μM vs. curcumin 5 μM+IR 2 Gy (p=0.0008), and curcumin 10 μM vs. curcumin 10 μM+IR 2 Gy (p=0.009). In PC3 cells, the p values were: IR 2 Gy vs. curcumin 2.5 μM+IR 2 Gy (p=0.027), IR 2 Gy vs. curcumin 5 μM+IR 2 Gy (p=0.001), IR 2 Gy vs. curcumin 10 μM+IR 2 Gy (p=0.001), curcumin 2.5 μM vs. curcumin 2.5 μM+IR 2 Gy (p=0.0004), curcumin 5 μM vs. curcumin 5 μM+IR 2 Gy (p=0.01), and curcumin 10 μM vs. curcumin 10 μM+IR 2 Gy (p=0.05).

## Discussion

Radiotherapy is one important treatment choice for prostate cancer (Swanson, 2006; Thompson et al., 2007). A conventional radiation dose of 72 Gy is preferred as the treatment modality for management of prostate cancers. Radiation dose escalation to improve effectiveness of radiotherapy is achieved mainly through the use of intensity modulated radiation therapy (IMRT) or brachytherapy. However, radioresistance of prostate cancer cells remains a limitation of radiotherapy. Research efforts are needed to identify and develop new agents that can be used as radiosensitizers. Epidemiologic studies indicate that lower incidence of prostate cancer in Asian populations than Western populations may be due to differences in their food habits (Hebert et al., 1998; Denis et al., 1999; Hsing

et al., 2000). The dietary spice curcumin is one such phytochemical. Curcumin has been shown to have diverse anti-cancer properties and could be developed as a potent therapeutic agent for prostate cancer treatment (Aggarwal, 2008; Khan et al., 2010). The aim of this study was to examine the effect of curcumin and radiation on cell viability, apoptosis, and clonogenic cell death in prostate cancer cells with wild-type and mutant p53 status. We clearly show that combination treatment of curcumin and radiation had a synergistic effect on clonogenic cell death in prostate cancer cells independent of the p53 status.

The radiosensitivity of prostate cancer cells has been shown to be associated with different molecular pathways, such as, bcl-2 (Chendil et al., 2004; Scott et al., 2002; An et al., 2007), mdm-2 (Mu et al., 2004; Lehman et al., 2007), AP-1 and NF-κB (Mukhopadhyay et al., 2001; Chendil et al., 2004), androgen receptor (Nakamura et al., 2002; Choi et al., 2010) and PI3 kinase/Akt (Li et al., 2007; Yu et al., 2008). Most of these above mentioned pathways have been shown to be modulated by curcumin (Aggarwal, 2008; Khan et al., 2010). Curcumin induces apoptosis by downregulating bcl-2 (Mukhopadhyay et al., 2001; Chendil et al., 2004), cyclin D1 (Mukhopadhyay et al., 2002), AP-1 and NF-κB transcription factors (Mukhopadhyay et al., 2001), EGFR signaling pathway (Kim et al., 2006), and by activating p53



**Figure 9:** Synergistic effect of curcumin and radiation on clonogenic cell death in PC3 cells. Cells were treated with curcumin (2.5, 5, and 10  $\mu$ M) alone, radiation (2 Gy) alone or in combination of curcumin with radiation. **A:** Bar chart showing percent survival in different experimental groups (\* $p < 0.05$ ). **B:** A representative clonogenic assay showing different experimental groups.

and bax (Shankar and Srivastava, 2007) in different prostate cancer cells. It is reported that curcumin can inhibit cell proliferation and induce apoptosis in androgen-dependent, androgen-independent and metastatic prostate cancer cells (Dorai et al., 2000, Deeb et al., 2003; Dorai et al., 2004). Recently it has been shown that curcumin augmented the inhibitory effect of radiation on growth of head and neck (Khafif et al., 2009; Yallapu et al., 2010) and ovarian cancer (Javadi et al., 2010). Chendil et al. (2004) have shown that curcumin can enhance radiation-induced apoptosis in prostate cancer cells by altering bcl2: bax ratio. In the present study we show that wild type p53 was activated by radiation and not by curcumin (Figure 2C). However, curcumin induced p21 and gadd45 expression independent of p53 in PC3 cells (Figure 3) and there was induction of cell growth arrest (Figure 4) and apoptosis (Figure 5) by curcumin in both the LNCaP and PC3 cell lines. The role of p21 and gadd45 in cell growth arrest and apoptosis independent of p53 is well documented (Sheikh et al., 2000; Don et al., 2001; Hirose et al., 2003; Zhang et al., 2007). Though curcumin induced apoptosis, combination of curcumin and radiation did not show enhancement of apoptosis examined using caspase 9 and annexin V assays (Figure 5). This could be due to the fact that a 2 Gy dose of radiation resulted only in cell growth arrest and there was no induction of apoptosis observed in our study examined

within 48 hours of radiation exposure. However, clonogenic assay (performed after 12 days of exposure) showed a synergistic effect between curcumin and radiation in both the cell lines (Figure 8, 9 and Table 3a). This may be due to the fact that clonogenic cell death takes into account the late apoptotic cell death and the reproductive (senescence) cell death (Dewey et al., 1971; Lehman et al., 2007). Recently, Lehman et al. (2007) have reported that p53 protein is essential for irradiation-induced senescence and clonogenic death of prostate cancer cells. Our results show radiation-induced clonogenic cell death in PC3 cells having mutant p53 (Figure 9 and Table 3b). However, the clonogenic survival in LNCaP cells with normal p53 was lower (27%) than the PC3 cells with mutant p53 (48%). Further, combination of curcumin and radiation had a synergistic effect on clonogenic cell death in LNCaP and PC3 cells regardless of the p53 status.

To improve therapeutic outcomes, radiotherapy is often combined with chemotherapeutic drugs (Belka et al. 2004; Garzotto et al., 1999; Swanson et al., 2006). Curcumin and curcumin analogues are being developed as potential anti-prostate cancer agents (Lin et al., 2006). It has been shown that curcumin sensitizes colorectal cancer xenografts in nude mice to radiation (Kunnumakkara et al., 2008). Phase I and phase II clinical trials using curcumin alone has

been undertaken in colorectal and pancreatic cancers (Sharma et al., 2001; Dhillon et al., 2008). Phase I clinical trial using combination of curcumin and docetaxel has been initiated in patients with advanced and metastatic breast cancer (Bayet-Robert et al., 2010). However, there is no report of any preclinical or clinical trial study using combination of curcumin and radiation in prostate cancer. In this report, we clearly show a synergistic effect between curcumin and radiation on apoptosis induction and clonogenic cell death in prostate cancer cells. These findings highlight the potential of curcumin in radiosensitizing prostate cancer cells and merits future clinical trials using combination of curcumin and radiation in prostate cancers.

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