

Low-Dose Fractionated Radiation Potentiates the Effects of Paclitaxel in Wild-type and Mutant p53 Head and Neck Tumor Cell Lines

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ABSTRACT

This study was designed to: (a) evaluate the induction of hyper-radiation sensitivity (HRS), a phenomenon observed at low doses of radiation (<1 Gy); (b) compare the potentiating effects of single dose radiation (2 Gy) versus the effect of low-dose fractionated radiation (LDFRT; <1 Gy) on Paclitaxel; and (c) understand the molecular mechanism of LDFRT-mediated chemo-potentiating effects, in wild-type p53 SCC-61 and p53 mutant SQ-20B head and neck squamous cell carcinoma cell lines. Both cell lines exhibited the HRS phenomenon at low radiation doses. Compared with SCC-61 cells, SQ-20B cells were resistant to radiation and Paclitaxel alone. A significant enhancement of radiation sensitization by Paclitaxel (0.5 or 1 nM) was observed in both cell lines. Chemo-potential of Paclitaxel by single 2-Gy radiation was observed in SCC-61 cells but not in SQ-20B cells. However, LDFRT (0.5 Gy in four fractions) significantly chemo-potentialized the effect of Paclitaxel in both cell lines. The cell cycle regulator p53 and its target genes *p21^{waf1/cip1}* and *BAX* were induced in SCC-61 cells treated with 2 Gy, Paclitaxel, or in combination, but not in SQ-20B cells. These treatments elevated the antiapoptotic BCL-2 protein in SQ-20B cells but not in SCC-61 cells. Interestingly, LDFRT treatment in both cell lines with or without Paclitaxel down-regulated nuclear factor κ B activity and BCL-2 protein expression and simultaneously up-regulated BAX protein. These findings strongly suggest that LDFRT (at these doses, HRS phenomenon is observed) can be used

in combination with Paclitaxel to overcome the antiapoptotic effects of BCL-2 and nuclear factor κ B.

INTRODUCTION

Cancers of the head and neck represent ~6% of cancers diagnosed in the United States each year with ~28,900 cases of SCCHN² being diagnosed annually (1–3). Most advanced cancers are treated with chemo-radiation with or without surgery. In spite of these approaches, <30% of patients achieve long-term remission, and recurrence commonly occurs loco-regionally (3). To improve on these poor results, the use of neo-adjuvant chemotherapy and radiation has been investigated. These protocols have produced response rates ranging from 60 to 90% (4) but unfortunately have not had an impact on long-term patient survival.

Recent studies suggest that induction of apoptosis in tumor cells has an important role in the efficacy of radiation therapy and chemotherapy (5). Because of their complex genetic composition, many tumors tend to demonstrate resistance to therapy at the outset or during initial therapy. One of the functions of the putative tumor suppressor gene *p53* is the induction of apoptosis (6). Gene expression studies have revealed that there exists more than one pathway regulating growth inhibition and apoptotic processes (7). The pathway mediated through the tumor suppressor *p53* gene in cell cycle arrest and apoptosis form an important molecular determinant regulating the response to ionizing radiation. Wild-type p53 protein confers radiation responsiveness, which causes either G₁ cell cycle arrest and/or apoptotic death. This effect is mediated by activation of other downstream target genes, such as *p21^{waf1/cip1}*, *BAX*, and *BCL-2*, which act as cross-point regulators that can induce, enhance, delay, or inhibit apoptosis (7, 8).

There is growing evidence that p53 is an important determinant in apoptosis induction by radiation (8) and by a number of chemotherapeutic agents (9). Paclitaxel is a chemotherapeutic agent (member of taxane family) that has been postulated to act as a cell cycle-specific radiation sensitizer (10, 11) because it promotes and stabilizes premature microtubule assembly and consequently arrests cells in the radiosensitive G₂ and M phases of the cell cycle (12, 13). This ability of Paclitaxel to arrest cells in G₂-M makes it a potential radiosensitizer. Thus, G₂-M arrest

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² The abbreviations used are: SCCHN, squamous cell carcinoma of head and neck; HRS, hyper-radiation sensitivity; IRR, induced radiation resistance; LDFRT, low-dose fractionated radiation; NF κ B, nuclear factor κ B; ER, enhancement ratio; TUNEL, terminal transferase-mediated dUTP-digoxigenin nick end labeling; EMSA, electrophoretic mobility shift assay; SF, surviving fraction; D₀, dose required to reduce the fraction of cells to 37%, indicative of single-event killing.

is considered the underlying mechanism of Paclitaxel-induced radiosensitization (14).

Until recently in the field of radiation biology, the initial slope of the radiation cell survival curve (doses of <1 Gy) was presumed to be an ineffective dose range for human tumor therapy. However, Joiner *et al.* (15, 16) revolutionized the thinking regarding low doses of radiation (<1 Gy) by demonstrating an initial phase of hypersensitivity to radiation (using doses <1 Gy). Increased resistance to radiation was found from doses >1 Gy, a phenomenon termed IRR.

Low-dose radiation has been extensively studied *in vitro*. At doses <1 Gy, several cell lines from various cancer types, including SCCHN, have demonstrated the presence of HRS region in the initial slope of cell survival curve induced by low doses of radiation (17–19). Although this has been studied in murine models as well (20), it has not been adequately explored in humans. Interestingly, this phenomenon of HRS at low doses of radiation is most pronounced in radio-resistant cells, defined as those with mutant p53 expression (7, 21). The discovery that HRS does not stimulate cellular repair mechanisms, such as those seen at higher doses, provides a plausible explanation of why there is no induction of radio resistance with HRS, as measured *in vitro* (21). However, as Short *et al.* (21) have pointed out, to take advantage of the benefits of HRS radiation dose in the clinical setting, therapy would have to be extended over 7–12 weeks, allowing tumor proliferation that would abolish the gain attributable to enhanced cell killing. One logical alternative to exploit the enhanced cell killing at low doses of radiation (at which HRS is observed) is to combine it with systemic chemotherapy.

In light of the radio-sensitizing properties of Paclitaxel, as well as its documented activity in SCCHN, we designed this study to investigate the influence of wild-type and mutant p53 function on the radio-sensitizing effects of Paclitaxel in combination with single radiation dose and LDFRT (at which HRS is induced) and investigate LDFRT as a chemo-potentiator for Paclitaxel, as well as compare the chemo-potentiating effects of single standard dose radiation (2 Gy) *versus* LDFRT (1 Gy of two fractions or 0.5 Gy of four fractions). Furthermore, we studied the mechanism of chemo-potentialiation by single dose radiation at 2 Gy *versus* LDFRT by analyzing the kinetics of pro-survival factors, such as BCL-2 expression and NFκB activity, and pro-apoptotic factors, such as BAX gene expression.

MATERIALS AND METHODS

Cell Culture. Two established head and neck cancer cell lines from moderately differentiated SCCHN origin (SCC-61 and SQ-20B) were obtained from American Type Culture Collection (Rockville, MD). SCC-61, which contains wild-type p53 (22), and SQ-20B, which contains mutant p53 (23), were cultured in DMEM with high glucose, supplemented with 15% fetal bovine serum, 2 mM L-Glutamine, 1% penicillin streptomycin, and 0.4 μg/ml hydrocortisone, at 37°C and 5% CO₂.

Cell Treatments. Cells were treated with Paclitaxel (Taxol®; Bristol-Myers Squibb Co., Princeton, NJ) formulated in Cremophor EL (polyoxyethylated castor oil) and dehydrated alcohol, at a stock concentration of 6 mg/ml.

A 100-kV industrial X-ray machine (Phillips, Hamburg,

Germany) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm Al plus 1-mm Be filter was ~2.64 Gy/min at a focus surface distance of 10.5 cm.

Cell lines (SQ-20B and SCC-61) were left untreated or exposed to 1–6 Gy dose of radiation or to different concentrations of Paclitaxel. For combined experiments, the cells were treated with Paclitaxel (0.5 or 1 nM), and 24 h later, cells were exposed to radiation without changing the medium. For multi-fractionated experiments, cells were exposed to 0.5 nM Paclitaxel, and 24 h later, the cells were exposed to radiation without changing the medium at doses of 0.5 or 1 Gy fractions to a total dose of 2 Gy, with 8-h time intervals between each fraction.

Colony Forming Assay. Clonogenic survival assays were performed as described earlier (23, 24). The radiation ER by Paclitaxel was calculated as follows:

$$\text{Radiation ER} = \frac{[\text{survival fraction of radiation}^3 \text{ alone}]}{[\text{survival fraction of radiation}^3 + \text{Paclitaxel}^4]}$$

Paclitaxel ER by radiation was calculated using the following formula:

$$\text{Paclitaxel ER} = \frac{[\text{survival fraction of Paclitaxel alone}]}{[\text{survival fraction of radiation}^3 + \text{Paclitaxel}^4]}$$

Quantification of Apoptosis. Apoptosis was quantified by TUNEL assay. The ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD), which detects DNA strand breaks by terminal TUNEL, was used as described earlier (25). Briefly, cells were seeded in chamber slides and exposed to Paclitaxel alone (0.5 nM), single dose radiation alone (2 Gy), a combination of Paclitaxel plus single dose radiation (2 Gy), and Paclitaxel plus four fractions of 0.5 Gy radiation doses. Enhancement of radiation-induced apoptosis by Paclitaxel was calculated using the following formula:

$$\text{Radiation ER} = \frac{[\text{percentage of induction of apoptosis by radiation}^5 + \text{Paclitaxel}^6]}{[\text{percentage of induction of apoptosis by radiation}^5]}$$

Enhancement of Paclitaxel-induced apoptosis by radiation was calculated using the following formula:

$$\text{Paclitaxel ER} = \frac{[\text{percentage of induction of apoptosis by radiation}^5 + \text{Paclitaxel}^6]}{[\text{percentage of induction of apoptosis by Paclitaxel}^6]}$$

Definition of the terms “Radio-Sensitization” and “Chemo-Potentialiation.” Terms such as radio-sensitization and chemo-potentialiation are used throughout this manuscript to assess the combined effects of standard 2 Gy dose radiation or LDFRT with Paclitaxel. Radio-sensitization is defined as the term used when Paclitaxel increases the sensitivity of cells to radiation (as assessed by clonogenic inhibition or apoptosis). This is calculated as per the formula listed above and represented in form of radiation ERs. Thus, radiation ER is defined as the ratio of surviving cells with radiation alone (2 Gy or LDFRT) compared with combination of radiation (2 Gy or LDFRT) and Paclitaxel exposures.

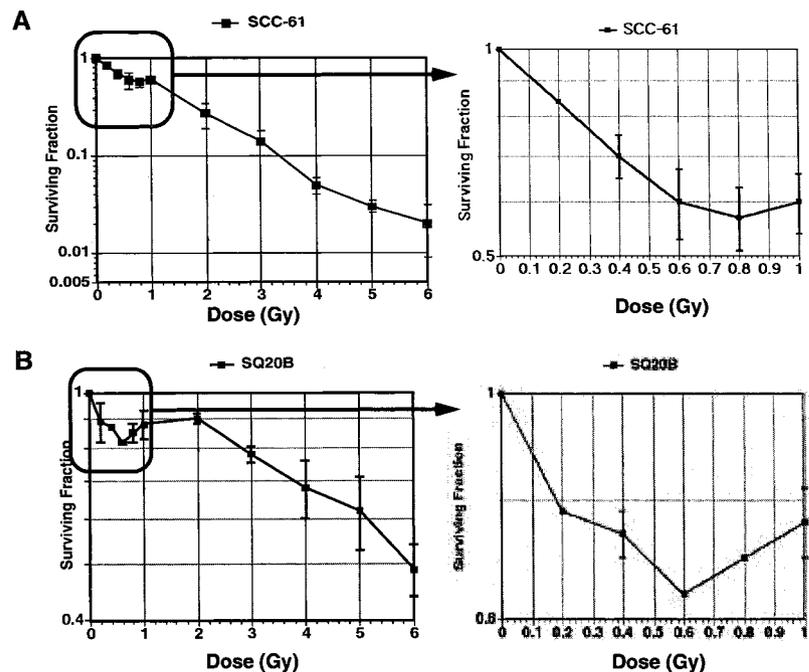
³ Can be single standard dose radiation (2 Gy) or LDFRT (1 Gy of two fractions or 0.5 Gy of four fractions).

⁴ Concentrations of 0.5 or 1 nM.

⁵ Can be single standard dose radiation (2 Gy) or LDFRT (1 Gy of two fractions or 0.5 Gy of four fractions).

⁶ Concentration of 0.5 nM.

Fig. 1 Presence of HRS phenomenon at low-dose radiation in SCC-61 cells (A) and SQ-20B cells (B). Cell survival curves for SCC-61 and SQ-20B cells after radiation as analyzed by Single Hit Multi-Target model curve fit. Error bars, SE from the mean of three separate experiments.



Chemo-potiation is defined as the term used when radiation increases the sensitivity of cells to Paclitaxel (as assessed by clonogenic inhibition or apoptosis). This is calculated as per the formula listed above and represented in form of Paclitaxel ERs. Thus, Paclitaxel ER is defined as the ratio of surviving cells with Paclitaxel alone compared with combination of radiation (2 Gy or LDFRT) and Paclitaxel exposures.

Western Blot Analysis. Total protein extracts from untreated and treated cells at various time intervals were subjected to Western blot analysis as described previously (25) using p53 antibody (sc-126; Santa Cruz Biotechnology, Santa Cruz, CA), p21^{waf1/cip1} antibody (sc-817; Santa Cruz Biotechnology), BAX antibody (sc-493; Santa Cruz Biotechnology), or BCL-2 monoclonal antibody (sc-509; Santa Cruz Biotechnology). Anti- β -actin antibody (Sigma Chemical Co., St. Louis, MO) was used as an internal loading control. These proteins were detected using the chemi-luminescent method.

EMSA. Preparation of nuclear extracts from untreated and treated cells was prepared, and EMSA was performed as described previously (24). Analysis of DNA binding by EMSAs was performed using 2 mg of poly (dI-dC; Sigma) as nonspecific competitor DNA. The binding reactions contained 10,000 cpm of ³²P-labeled, double-stranded oligonucleotide probe with a high affinity for NF κ B binding (Promega, Madison, WI). Binding reactions were electrophoresed on a 4% PAGE in 0.5 \times Tris-borate EDTA buffer to separate the bound and unbound probe.

Statistical Analysis. The Student *t* test was used to test the statistical significance using the means of radiation inactivation estimates (SF₂ and D₀) and percentage of apoptosis (TUNEL-positive cells) obtained from the data in three different treatment groups of two cell lines.

RESULTS

SQ-20B Cells Show High Levels of Endogenous Mutant p53 Protein with Absence of p21^{waf1/cip1}. To ascertain and characterize the basal and radiation-induced total p53 protein levels and its downstream target genes in SCC-61 and SQ-20B SCCHN, Western blot analysis was performed after various time intervals using p53, p21^{waf1/cip1}, BCL-2, and BAX antibodies. Our findings clearly demonstrate that SQ-20B cells contain nonfunctional p53 protein, because these cells lack elevation of BAX and p21^{waf1/cip1} protein expression. SCC-61 cells contain functional p53 protein because an up-regulation of p53 and its downstream effector genes, such as p21^{waf1/cip1} and BAX, were observed (figure not shown).

Low-Dose Radiation-induced HRS Phenomenon in Head and Neck Tumor Cell Lines. Having ascertained the functional status of p53 in these two cell lines, we analyzed the induction of the HRS phenomenon at low doses of radiation, and these observations were further correlated with p53 functional status. In both cell lines, low radiation dose (0–100 cGy) induced the HRS phenomenon. However, p53 mutant SQ-20B demonstrated a more pronounced HRS region when compared with the wild-type SCC-61 cells. In wild-type p53 SCC-61 cells, a low dose of 80 cGy produced the maximum HRS phenomenon, whereas a low dose of 60 cGy produced the maximum HRS phenomenon in p53 mutant SQ-20B cells. These observations indicate that induction of HRS phenomenon at low doses is observed irrespective of p53 functional status (Fig. 1).

SCC-61 Cells Were More Sensitive to Paclitaxel and Ionizing Radiation than SQ-20B Cells. Radiation caused clonogenic inhibition in both cell lines. The estimates of radiation inactivation for the two cell lines are presented in Table 1. The Paclitaxel alone D₀ values for SCC-61 and SQ-20B were

Table 1 Cell inactivation estimates by single dose radiation, Paclitaxel and single dose radiation plus Paclitaxel

Treatments	SCC-61				SQ-20B			
	SF ^c	D ₀	Enhancement ratios		SF	D ₀	Enhancement ratios	
			Radio-sensitization ^a				Chemo-potential ^b	
			SF	ER			SF	ER
RT	0.27	121 cGy			0.85	245 cGy		
P (0.5 nM)	0.38				0.64			
P (1 nM)	0.12				0.26			
RT + P (0.5 nM)	0.125	85.5 cGy	2.16	3.04	0.59	206 cGy	1.44	1.08
RT + P (1 nM)	0.082	68.5 cGy	3.29	1.46	0.22	134 cGy	3.86	1.18

^a Enhancement of radiation effects by Paclitaxel.

^b Enhancement of Paclitaxel effects by radiation.

^c SF, surviving fraction at 2 Gy for radiation or surviving fraction at indicated concentration for Paclitaxel; RT, radiation; P, Paclitaxel; D₀, calculated as per single hit multi-target model using surviving fractions obtained from doses 1–6 Gy, and defined as the dose required to reduce the surviving fraction of cells to 31%.

0.412 and 0.635 nM, respectively. On the basis of these findings, SCC-61 cells were more sensitive to Paclitaxel than SQ-20B cells. In addition, compared with the SQ-20B cells, the SCC-61 cells were significantly more sensitive to ionizing radiation (Table 1). When Paclitaxel and single radiation dose (2 Gy) were combined, an enhanced radio-sensitizing effect was observed in both the cell lines (Table 1). The radiation ER by Paclitaxel in SCC-61 was found to be 2.16 and 3.29 for 0.5 nM Paclitaxel with single 2 Gy radiation dose and 1 nM Paclitaxel with single 2 Gy radiation dose, respectively ($P < 0.0006$). The radiation ERs for SQ-20B were 1.44 and 3.86 for 0.5 nM Paclitaxel with single 2 Gy radiation dose and 1 nM Paclitaxel with single 2 Gy radiation dose, respectively ($P < 0.00008$; Table 1). These findings indicate that Paclitaxel conferred significant radio-sensitizing effect, irrespective of p53 status.

Next, we determined whether single radiation dose potentiated the effects of Paclitaxel (refer to Paclitaxel ER formula in "Materials and Methods"). The Paclitaxel ER (for Paclitaxel at 0.5 nM) by 2 Gy dose of radiation was 3.04 and 1.08 for SCC-61 and SQ-20B cells, respectively. Hence, single radiation dose at 2 Gy potentiated the effects of Paclitaxel in wild-type p53 SCC-61 cells but not in mutant p53 SQ-20B cells (Table 1). However, Paclitaxel at 1 nM dose when combined with single radiation 2 Gy dose did not show significant chemo-potential in either cell lines (SCC-61 = 1.46 and SQ-20B = 1.18). Together, these findings indicate that a single radiation dose of 2 Gy is ineffective in potentiating the effects of Paclitaxel in mutant p53 SQ-20B cells.

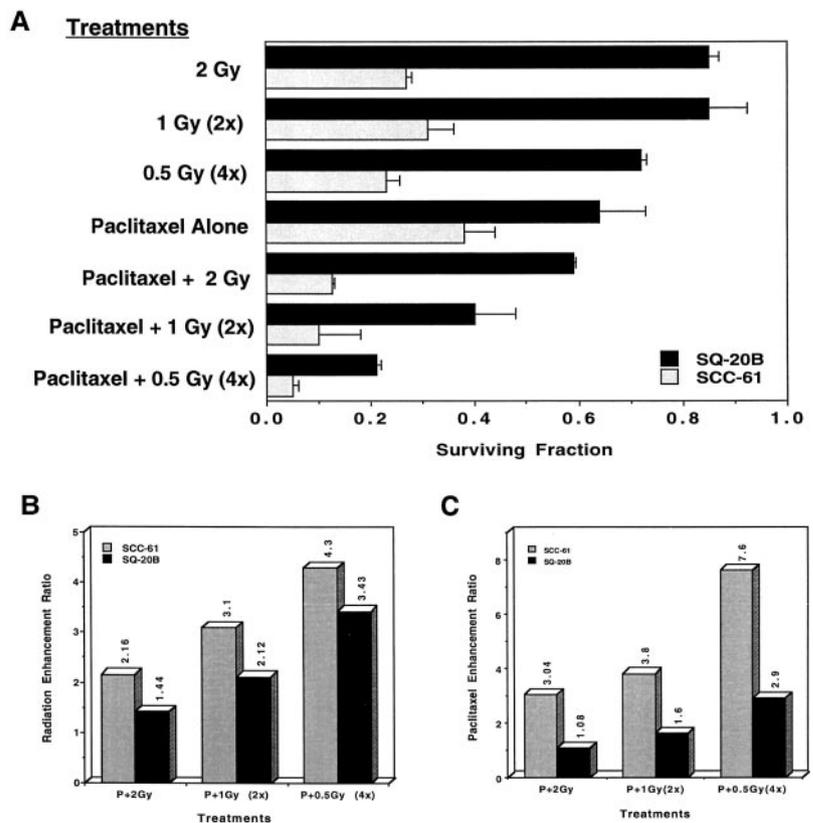
LDVRT Potentiated the Effects of Paclitaxel-induced Clonogenic Inhibition in Both Wild-type and Mutant p53 Cells. The data summarized in Table 1 indicate that the significant radio-sensitizing effect of Paclitaxel with single 2 Gy radiation dose in both cell lines is irrespective of p53 status. On the contrary, single 2 Gy radiation dose failed to produce the chemo-potentiating effects of Paclitaxel in mutant p53 SQ-20B cells (Table 1). Thus, we further investigated whether LDFRT will chemo-potentiate the effects of Paclitaxel in these cells. Experiments were designed to compare the effects of multifractionated low doses of radiation alone *versus* Paclitaxel (0.5 nM) combined with LDFRT (1 Gy of two fractions or 0.5 Gy of four fractions). In both the cell lines, two fractions of 1 Gy radiation did not alter the SF when compared with 2 Gy of single dose

fraction, whereas four fractions of 0.5 Gy reduced the SFs marginally (Fig. 2A). In combination with Paclitaxel, two fractions of 1 Gy reduced the SF significantly ($P < 0.0034$) in both cell lines (Fig. 2A). A further significant reduction ($P < 0.00023$) was observed when four fractions of 0.5 Gy (50 cGy) were given in combination with Paclitaxel (Fig. 2A). Thus, Paclitaxel in combination with single 2 Gy radiation dose showed a radiation ER of 2.16 and 1.44 for SCC-61 and SQ-20B cells, respectively (Table 1; Fig. 2B). However, when Paclitaxel was combined with two 1 Gy fractions or four 0.5 Gy fractions of radiation dose, the radiation ER increased significantly to 3.1 or 4.3, respectively, in SCC-61 cells ($P < 0.00008$; Fig. 2B). A similar significant increase in radiation ER was observed for SQ-20B cells, and this was 2.12 or 3.43 for the two 1 Gy fractions or four 0.5 Gy fractions of radiation dose, respectively ($P < 0.003$; Fig. 2B). Together, these findings strongly indicate that Paclitaxel significantly sensitized the effects of single 2 Gy radiation dose or LDFRT, irrespective of p53 status.

In terms of chemo-potential, as stated earlier, Paclitaxel in combination with single 2 Gy radiation dose showed a Paclitaxel ER of 3.04 and 1.08 for SCC-61 and SQ-20B cells, respectively (Table 1; Fig. 2C). Interestingly, Paclitaxel ER significantly increased when Paclitaxel was combined with two fractions of 1 Gy radiation dose (ER of 3.8 and 1.6 for SCC-61 and SQ-20B cells, respectively; Fig. 2C). An additional significant increase in Paclitaxel ER [SCC-61 = 7.6 ($P < 0.001$) and SQ-20B = 2.9 ($P < 0.003$)] was observed when Paclitaxel was combined with four fractions of 0.5 Gy radiation dose (Fig. 2C). Thus, these findings strongly indicate that LDFRT is a potent chemo-potentiator of Paclitaxel as opposed to single radiation dose-mediated chemo-potential.

LDVRT Significantly Potentiated the Effects of Paclitaxel-induced Apoptosis in Mutant p53 SQ-20B Cells when Compared with Wild-type p53 SCC-61 Cells. Radiation or Paclitaxel alone exposures caused apoptosis in both cell lines; however, SCC-61 cells showed a marginal increase in cell death when compared with SQ-20B cells (Fig. 3A). A proportionate increase in cell death was observed in both cell lines when Paclitaxel and single 2 Gy radiation dose were combined (Fig. 3A). Thus, in terms of apoptotic radiosensitization by Paclitaxel, the radiation ER for cell death was 1.64 and 1.77 in SCC-61 and

Fig. 2 Bar graph showing SFs for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and Paclitaxel (0.5 nM) plus LDFRT, respectively (A). Bar graph showing radiation ERs for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and Paclitaxel (0.5 nM) plus LDFRT, respectively (B). Bar graph of Paclitaxel ERs for SCC-61 and SQ-20B cells treated with combination of Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and combination of Paclitaxel (0.5 nM) plus LDFRT, respectively (C).



SQ-20B cells, respectively (Fig. 3B). These findings indicate that Paclitaxel conferred significant radio-sensitizing apoptotic effects, irrespective of p53 status. In terms of apoptotic chemo-potential by single 2 Gy radiation dose, the Paclitaxel ER was 3.1 and 4.86 for SCC-61 and SQ-20B cells, respectively (Fig. 3C). These findings indicate that single radiation dose at 2 Gy potentiated the effects of Paclitaxel in both wild-type p53 SCC-61 and mutant p53 SQ-20B cells. However, for LDFRT, the radiation ER by Paclitaxel was 2.63 and 2.37 for SCC-61 and SQ-20B cells, respectively (Fig. 3B). These findings suggest that Paclitaxel significantly sensitized the effects of LDFRT, irrespective of p53 status. Interestingly, LDFRT (four fractions of 0.5 Gy radiation dose) chemo-potentiated the effect of Paclitaxel, resulting in a significant increase in Paclitaxel ER to 6.93 ($P < 0.64 \times 10^{-6}$) and 10.36 ($P < 0.23 \times 10^{-11}$) for SCC-61 and SQ-20B, respectively (Fig. 3C). These findings indicate that LDFRT is a significant chemo-potentiator of Paclitaxel-induced apoptosis in mutant p53 SQ-20B cells as compared with wild-type p53 SCC-61 cells ($P < 0.0001$).

Loss of Induction of NF κ B Activity and Significant Up-Regulation of BAX Protein by LDFRT: A Possible Mechanism of Chemo-Potentiating Effect of LDFRT. A single dose of 2 Gy radiation or Paclitaxel or in combination caused induction of BCL-2 in SQ-20B cells. Because BCL-2 is an antiapoptotic protein, induction of this protein in response to single dose radiation might have played a role in the loss of radiation (2 Gy)-mediated chemo-potentiating effect. However, LDFRT (0.5 Gy of four fractions) significantly chemo-potentiated the effects of Paclitaxel in SQ-20B cells. This prompted us

to analyze the kinetics of antiapoptotic factors, such as BCL-2 and NF κ B, and pro-apoptotic proteins, such as BAX, in response to LDFRT alone or in combination with Paclitaxel. In wild-type p53 SCC-61 cells, LDFRT caused significant induction of pro-apoptotic protein BAX, at 3 and 6 h of third and fourth fractions, with significant down-regulation of BCL-2 protein. Paclitaxel alone did not change BCL-2 and BAX levels (Fig. 4A). LDFRT in combination with Paclitaxel caused significant induction of BAX with significant down-regulation of BCL-2 (Fig. 4A). NF κ B, a pro-survival transcription factor and transactivator of BCL-2 (26), was up-regulated by single 2 Gy dose radiation, whereas LDFRT alone failed to induce NF κ B activity in SCC-61 cells (Fig. 4B). Paclitaxel alone and Paclitaxel in combination with LDFRT did not show any presence of NF κ B activity (Fig. 4B). In SQ-20B cells, LDFRT alone caused significant induction of BAX protein with marginal increase in BCL-2 of 0.5 Gy LDFRT treatment fractions (Fig. 5A). Induction of NF κ B activity was observed by single 2 Gy radiation dose but not with LDFRT. Paclitaxel alone caused a marginal induction of NF κ B activity with no significant changes in BCL-2 or BAX protein (Fig. 5B). When Paclitaxel was combined with LDFRT, significant induction of BAX was observed in all 0.5 Gy fractions (Fig. 5A). A weak induction of NF κ B activity was observed in response to Paclitaxel plus LDFRT. Together, these findings strongly indicate that the molecular mechanisms signaling the chemo-potentiating effects of LDFRT are mediated through the mitigation of the induction of anti-apoptotic factors, such as BCL-2 and NF κ B.

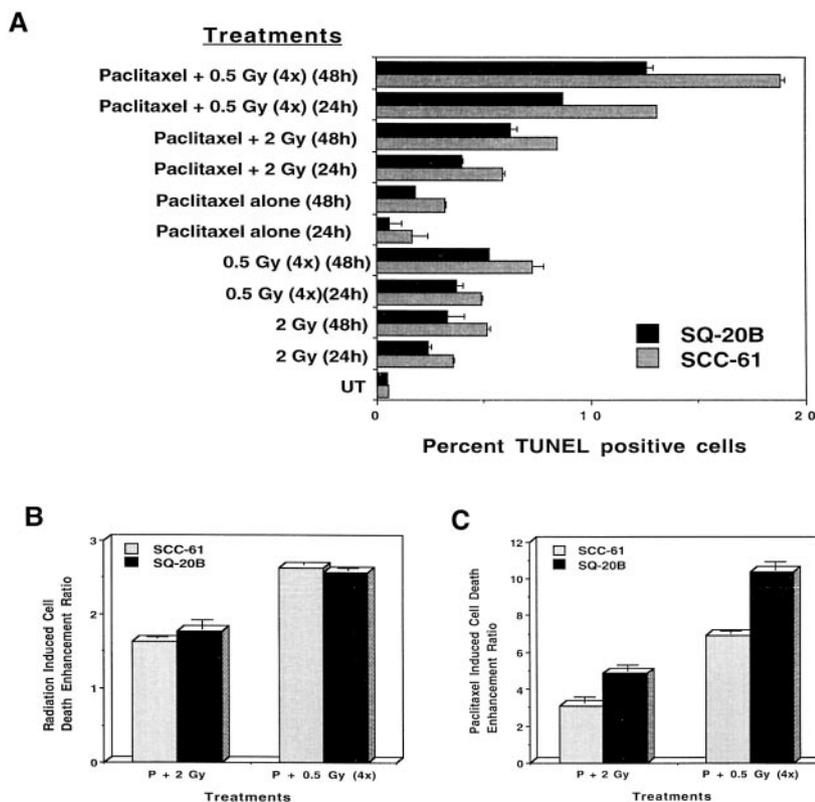


Fig. 3 Bar graph showing radiation-induced cell death (as a percentage of TUNEL-positive cells) for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and Paclitaxel (0.5 nM) plus LDFRT, respectively (A). Bar graph showing radiation-induced cell death ERs for SCC-61 and SQ-20B cells treated with Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and Paclitaxel (0.5 nM) plus LDFRT, respectively (B). Bar graph of Paclitaxel-induced cell death ERs for SCC-61 and SQ-20B cells treated with combination of Paclitaxel (0.5 nM) plus single 2 Gy of radiation dose and combination of Paclitaxel (0.5 nM) plus LDFRT, respectively (C).

DISCUSSION

Mutations and deletions of p53 have been identified in many head and neck carcinomas (23). The p53 gene is an essential component of the pathway leading to apoptosis caused by DNA damage. Wild-type p53 protein confers radiation responsiveness, which causes either G₁ cell cycle arrest and/or apoptotic death resulting from activation of other downstream target genes, such as p21^{waf1/cip1}, BAX, and mdm-2 (8, 9). Induction of nuclear p21^{waf1/cip1} protein leads to inhibition of cyclin-dependent kinase complex (27, 28), which results in accumulation of unphosphorylated retinoblastoma gene product (29). Hypo-phosphorylated retinoblastoma abrogates the activation of the E2F transcription factors that would otherwise signal for entry into S phase (30). Together, these mechanisms lead to G₁ arrest, which allows the cell to repair DNA damage (31).

We used the head and neck tumor cell lines SCC-61 and SQ-20B, which have been extensively characterized by both *in vitro* and *in vivo* experiments as radio-resistant and radiosensitive cells, respectively (32–34). In this study, 2 Gy radiation caused an increase in p53, p21^{waf1/cip1}, and BAX proteins in SCC-61 cells, suggesting that elevation of p53 involved the induction of its downstream effector genes p21^{waf1/cip1} and BAX. This is supported by a previous report that SCC-61 cells contain wild-type p53 (22). However, in SQ-20B cells, which harbor mutant p53 (23), no induction of p53 and p21^{waf1/cip1} protein was observed after 2 Gy radiation treatment (figure not shown).

In certain cell types, the loss of p53 function caused enhanced resistance to ionizing radiation (35). By clonogenic and apoptotic

assays, our study demonstrated that the wild-type p53 containing SCC-61 cells was sensitive to radiation when compared with SQ-20B cells harboring mutant p53. BAX protein levels were elevated after radiation treatment in SCC-61 cells, which play a pivotal role in promoting cell death (36). Thus, sensitivity to radiation by SCC-61 cells may be attributable to the presence of functional p53 and its target genes. On the other hand, in SQ-20B cells, BCL-2 levels were elevated after treatment with Paclitaxel, radiation alone, and in combination. Ionizing radiation often decreases BCL-2 protein levels in p53 wild-type cell lines causing enhanced cell death (37). Radiation was found to up-regulate BCL-2 protein in cell lines lacking functional p53 (38). Similar findings were observed in SQ-20B cells, where radiation induced BCL-2 protein, and this may have contributed to the enhanced resistance to clonogenic inhibition and apoptosis.

Numerous studies have shown a correlation with p53 status and Paclitaxel sensitivity (7). A similar effect was observed in our study where Paclitaxel caused greater clonogenic inhibition and cell death in SCC-61 cells than in SQ-20B cells. Experimental conditions, such as Paclitaxel concentration, incubation time, radiation fractionation, radiation schedule, and sequence of Paclitaxel/radiation treatments also influence the effectiveness of a combined treatment. All this implies the involvement of other mechanisms in addition to G₂-M accumulation in the Paclitaxel-induced radio-sensitization (39). SCC-61 and SQ-20B cells showed a supra-additive effect with a mean radiation ER of 3.29 and 3.86, respectively, when treated with radiation in combination with 1 nM Paclitaxel. Particularly, these observations suggest that Paclitaxel in combination with radiation over-

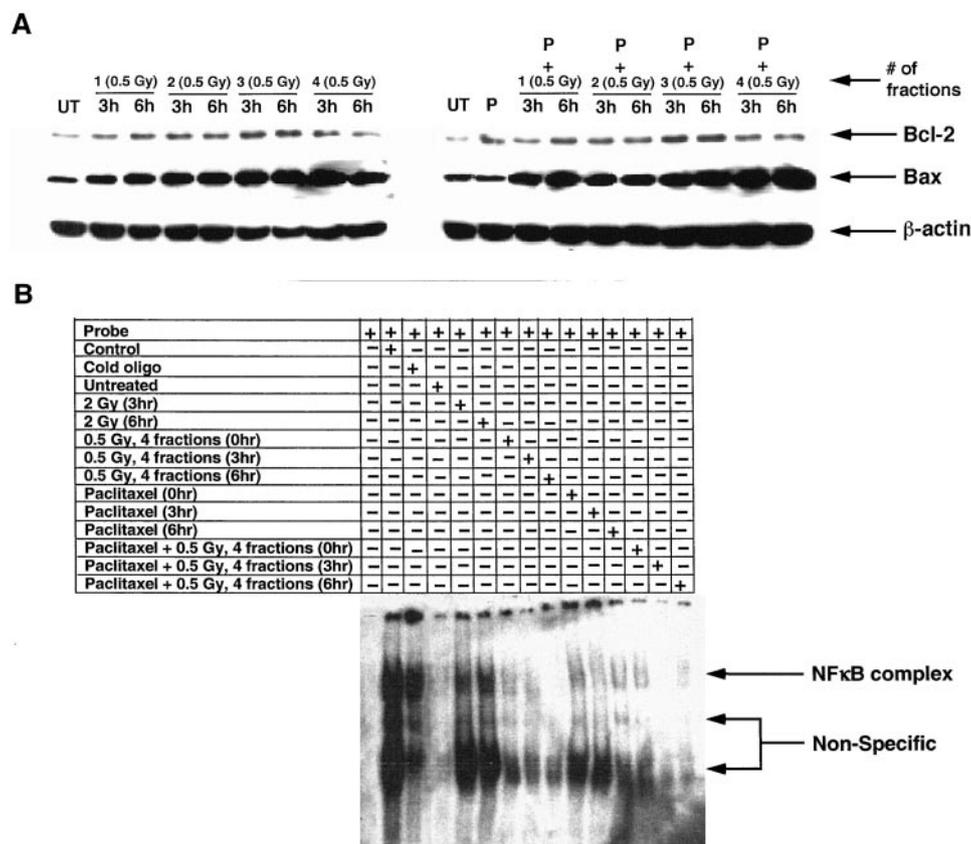


Fig. 5 Effect of LDFRT or LDFRT plus Paclitaxel on kinetics of pro-apoptotic and pro-survival factors in SQ-20B cells. A, Western blot analysis showing BCL-2 and BAX kinetics in SQ-20B cells. The cells were exposed to LDFRT alone or in combination with Paclitaxel plus LDFRT. The lysates isolated from Paclitaxel alone group is at the 24th h of continuous exposure. Whole cell protein extracts were subjected to Western blot analysis using anti-BCL-2 antibody and anti-BAX antibody. Anti-β-actin antibody was used as an internal loading control. B, the effect of single dose radiation (2 Gy), LDFRT, Paclitaxel (0.5 nm), and combination of Paclitaxel plus LDFRT on DNA-binding activity of NFκB activity in SQ-20B cells. EMSAs of NFκB-binding complexes from SCC-61 cells exposed to single radiation dose (2 Gy), LDFRT, Paclitaxel, and Paclitaxel (0.5 nm) in combination with LDFRT. Nuclear cell extracts (5 μg) from untreated or irradiated cells were incubated with ³²P-labeled NFκB DNA probe, followed by analysis of DNA-binding activities. Controls include probe only, positive control extract from HeLa cells treated with phorbol ester, and cold probe.

and overall response rate of 71%. Together, the data from this study and the clinical study strongly suggest that the use of such low doses of radiation in multiple fractions with a chemotherapeutic agent like Paclitaxel is a novel approach to achieve significant chemo-potential and also eliminate IRR.

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